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Full Length Research Paper

The cell division gene *ftsZ2* of *Sinorhizobium meliloti* is expressed at high levels in host plant *Medicago truncatula* nodules in the absence of *sinI*

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In this study, we investigated the role of quorum sensing (QS) in expression of the *Sinorhizobium meliloti* cell division gene *ftsZ2* in free-living cells and within nodules of its host plant, *Medicago truncatula*. Using a *ftsZ2* promoter reporter fusion, we were able to track the expression of this gene in wild-type *S. meliloti* cells and QS mutant backgrounds *in planta*. Our findings revealed that expression of *ftsZ2* in free-living cells was changed or reduced in *sinI* mutant cells compared with wild-type cells while expression *in planta* was substantially higher in the absence of *sinI*. The results further clarify the involvement of quorum sensing in the control of bacterial cell division during the symbiosis *in planta*.

Key words: *Sinorhizobium meliloti, Medicago truncatula,* quorum sensing.

INTRODUCTION

The symbiosis between the Gram-negative bacterium *Sinorhizobium meliloti* and its legume host *Medicago truncatula* is one of the best characterized host–bacterial interactions (Jones et al., 2007). Quorum sensing (QS), a cell-cell signaling and gene regulatory mechanism, plays an important role in this symbiosis (Gurich and González, 2009; Miller and Bassler, 2001; Sanchez-Cantreras et al., 2007). *Sinorhizobium meliloti* (1021) has a single *N*-acyl homoserine lactone (AHL) synthase, SinI, that is responsible for the production of several long-chain AHLs, ranging from C12-C18 in length (Marketon et al., 2002; Marketon and Gonzalez, 2002; Teplitski et al., 2003). However, the *S. meliloti* strain Rm41 has two AHL synthases (Marketon et al., 2002; Marketon and Gonzalez, 2002).

This bacterium has multiple Lux-R type receptors: SinR, ExpR, and six other proteins with homology to LuxR-like regulators that has not yet been completely characterized or proven to be functional AHL receptors (Galibert et al., 2001; Hoang et al., 2004; Pellock et al., 2002; Sanchez-Cantreras et al., 2007). Expression of *sinI* is dependent on *sinR.* However, ExpR has been shown to enhance the expression of *sinI* via binding to the *sinI* promoter in the presence of the SinI-AHL oxo-C14-HSL (McIntosh et al., 2008). Gurich and Gonzalez (2009) observed that ExpR is a highly versatile regulator with a unique ability to show different regulatory capabilities in the presence or absence of auto inducers. In addition, it is worth to mention that *S. meliloti* has an additional QS system, the *mel* system, which is responsible of short chain AHLs and whose genetic basis is not yet elucidated or identified completely (Marketon et al., 2002; Marketon and Gonzalez, 2002).

Mutant *sinI S. meliloti* cells are considerably delayed the ability to initiate nodules, which indicates that QS regulation plays an important role in the rate or efficiency of nodule initiation (Gao et al., 2005). Thus, cell division may be central for the initiation and maintenance of a successful symbiosis and the regulation of symbiotic cell division in plant and bacterial partners. However, little is currently known regarding the molecular players that regulate rhizobial cell division during symbiosis and the contribution of QS in this process. In this study, we investigated the role of quorum sensing (QS) in expression of the *Sinorhizobium meliloti* cell division gene

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Table 1. Bacterial strains and plasmids used in this study[a].

Strain or plasmid	Relevant characteristic(s)	References
Sinorhizobium meliloti		
wild-type	8530 (*expR*$^+$), Sm^r	Pellock et al. (2002)
sinI mutant	8530 with in-frame *sinI* deletion), Sm^r	Gao et al. (2005)
expR mutant	1021, *expR102*:: ISRm2011-1 *expR*, Sm^r	Galibert et al. (2001)
sinR mutant	Δ170 (8530 *sinR* $^-$), Sm^r	This study
wild-type (pSRf, pDG71)	Sm^r, Gm^r, Tet^r	This study
sinI mutant (pSRf, pDG71)	Sm^r, Gm^r, Tet^r	This study
expR mutant (pSRf, pDG71)	Sm^r, Gm^r, Tet^r	This study
sinR mutant (pSRf, pDG71)	Sm^r, Gm^r, Tet^r	This study
Escherichia coli		
DH5α (pSRf)	Gm^r	This study
HB101(pRK2013)	pRK2013 helper strain, Kan^r	Ditta et al. (1980)
Plasmids		
pBBR1MCS5	Gm^r	Kovach et al. (1995)
pSRf	pBBR1MCS5(p*ftsZ2*:: tdRfp),Gm^r	This study
pDG71	(p*trp*::Gfpmut3), Tc^r, Km^r	Bringhurst et al. (2001)

[a]Sm^r, Tet^r, Nm^r, Gm^r, Kan^r= resistance to streptomycin, tetracycline, neomycin, gentamycin and kanamycin, respectively.

ftsZ2 in free-living cells and within nodules of its host plant, *Medicago truncatula*. FtsZ controls initiation of bacterial cell division and is the best-known cell division protein among prokaryotes. *S. meliloti* is the only bacterium we are aware of with two different ftsZ copies, *ftsZ1* and *ftsZ2* (Margolin and Long, 1994). *ftsZ1* is essential for free-living growth in *S. meliloti* bacteria. On the other hand, *ftsZ2* is mostly required for differentiation *in planta* and is nonessential for viability of *S. meliloti* (Margolin and Long, 1994). In the current study, we explored the expression of *ftsZ2* gene of *S. meliloti* in wild-type (Rm8530) and in different QS mutant backgrounds which are unable to synthesize the quorum sensing signals (AHLs) and the AHL receptors SinR or ExpR. We found that expression of *ftsZ2* in free-living cells was changed or reduced in *sinI* mutant cells compared with wild-type cells while expression *in planta* was substantially higher in the absence of *sinI*. This finding enhanced our knowledge regarding the contribution of the QS in regulating *ftsZ2* expression in *S. meliloti.*

MATERIALS AND METHODS

While *S. meliloti* has two *ftsZ* copies, only expression of *ftsZ2* was examined in this study while *ftsZ1* expression, was not investigated. To determine the effect of QS on *ftsZ2* gene expression in free-living cells, we measured *ftsZ2* expression in wild-type, *sinI*, *expR* and *sinR* mutant *S. meliloti* backgrounds. The *ftsZ2* promoter reporter fusion was created by cloning tdRfp (Tomato tandem dimer red fluorescent protein) (Shaner et al., 2004) into the pBBR1MCS5 plasmid (Kovach et al., 1995) generating the promoterless plasmid derivative pSRf (pBBR1MCS5-tdRfp) as listed in Table 1. The predicted promoter region of the *S. meliloti* gene *ftsZ2* was amplified by PCR and cloned into the pSRf plasmid. It has been shown that the td-Tomato Rfp of Shaner et al. (2004) fluoresces intensely and is easily detected in single cells of *S. meliloti* with high relative brightness and expressing efficiently. The resulting promoter fusion plasmid was mobilized into *S. meliloti* wild-type and QS mutant cells by triparental mating as described previously (Ditta et al., 1980; Ruvkun and Ausubel, 1981). To differentiate between *ftsZ2* expressing and non-expressing cells, we placed a second plasmid into each strain (pDG71) that contains Gfpmut3 under control of the constitutive *trp* promoter from *Salmonella typhimurium* (Bringhurst et al., 2001; Rosado and Gage, 2003). Cultures of *S. meliloti* strains were incubated at 30°C with shaking in TA media with appropriate antibiotics (Table 1) then diluted in defined M9 glycerol medium containing appropriate antibiotics. Diluted cultures were added to glass culture tubes containing a mixture of C14- and C16:1 HSLs at final concentration of 1 µM each. Parallel control cultures were prepared in the same manner without added AHLs. Rfp expression per cell in wild-type cells at time zero was set at 100%. Reported values are the averages of three separate trials, 10 replicate wells each. *S. meliloti* cultures were sampled at 0, 1, 24 and 48 h to measure the fluorescent levels in free-living cells at different time points. These time points represent early and late growth phases. Under the conditions tested and throughout the course of the experiment, there was no considerable difference from 0 to 1 h as the cells are dividing while at 24 and 48 h there is difference comparing to 0 h. This may be due to stationary phase as bacteria divide less with slower growth rate. The mutants seem to have different starting points which may be due to the experimental setup or to the mutation.

RESULTS AND DISCUSSION

As shown in Figure 1, in the absence of added cognate

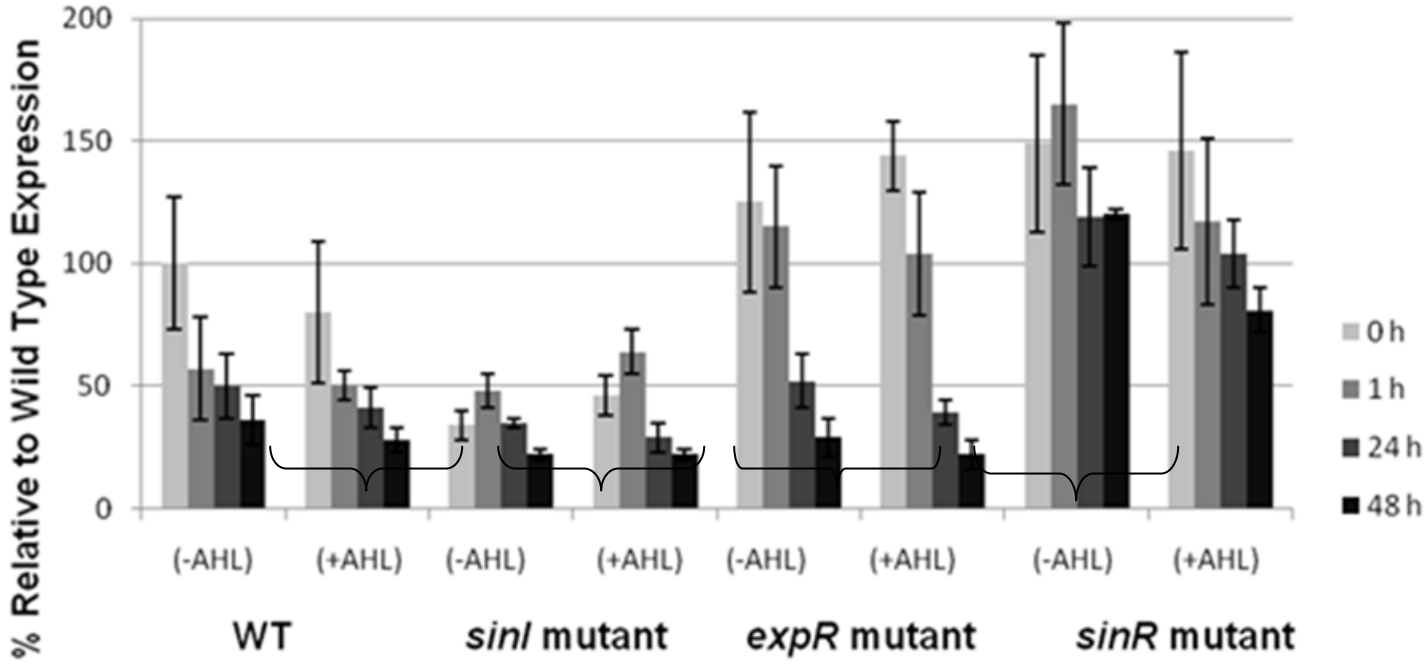

Figure 1. *In vitro ftsZ2* expression in wild- type and QS mutant strains of *Sinorhizobium meliloti* containing both the *ftsZ2*::tdRfp promoter fusion plasmid and the p*trp*- Gfpmut3 plasmid which were grown with or without added AHLs (1 µM each C14-HSL and C16:1-HSL). All samples 0, 1, 24 and 48 h were measured for OD at 600 nm and Rfp. Rfp/OD values were calculated and normalized to wild-type cell expression levels in the absence of added AHLs. Error bars represent S.D. Aliquots of these cultures were transferred to wells of black 96-well microtiter plates and absorbance was measured using a Victor2 plate reader. Fluorescence of tdRfp was measured using 550 and 580 nm excitation and emission filters respectively. For mut3Gfp measurements, 485 and 535 nm excitation and emission filters were used.

AHLs in the wild-type background expression of *ftsZ2* declined over time: it was 50% less than initial levels at 24 h and 64% less at 48 h. At time zero, expression of *ftsZ2* in the *sinI* mutant background was 34% of that in the wild-type background indicating 66% reduction. However, at 1 h levels were almost equivalent. At 24 and 48 h, levels of *ftsZ2* in *sinI* mutant cells were slightly reduced in contrast to wild-type cells. Additionally, while there was a decline in *ftsZ2* expression overtime in the *sinI* mutant background, it was less substantial than in the wild-type background. In the *expR* mutant background, *ftsZ2* expression started out slightly higher than in the wild-type cells but declined over time in similar pattern to that observed in wild-type cells. In *sinR* mutant cells, *ftsZ2* expression was also initially higher than in wild-type cells; however, there was little reduction in expression overtime.

The addition of *S. meliloti* cognate AHLs had a little effect on *ftsZ2* expression in either wild-type or QS mutant free-living cells under the conditions tested. Our results demonstrate that expression of *ftsZ2* in free-living cells was reduced in the absence of *sinI* and the addition of neither C14-HSL nor C16:1-HSL altered expression in this mutant background signifying that *ftsZ2* expression in free-living *S. meliloti* cells require *sinI* but is not rescued by added AHLs. This suggests that *ftsZ2* expression may be QS regulated in free-living cells. Further, expression in *expR* and *sinR* mutant cells was higher than that of wild-type cells which suggests that *ftsZ2* expression in free-living cells does not require ExpR or SinR, or, either or both repress *ftsZ2* expression in free-living *S. meliloti* cells under the conditions tested in this study. Thus, *ftsZ2* expression in a double mutant *sin*R *exp*R *S. meliloti* cells may be needed and this will be the subject of a future research.

To study *ftsZ2* gene expression *in planta* we visually tracked the expression of *ftsZ2* by the appearance of Rfp in the nodules of *Medicago truncatula* infected with wild-type or QS mutant cells using Confocal Laser Scanning Microscopy (CLSM). All of the nodules were harvested and sampled at the same time point 21 days post-infection. Growth of plants and infection with *S. meliloti* strains were as described previously (Gao et al., 2005) with some modifications. *M. truncatula* seeds were surface sterilized by ethanol followed by 30% bleach. Surface-sterilized and imbibed seeds were germinated in the dark in sterile petri dishes. Two-day-old seedlings were transferred to growth pouches wetted with sterile two fold diluted N-free Hoagland's solution and inoculated along the length of the root with 200 µl of inoculum containing approximately 10^7 CFU/ml of *S. meliloti* cells. Nodulation assays revealed no statistically significant

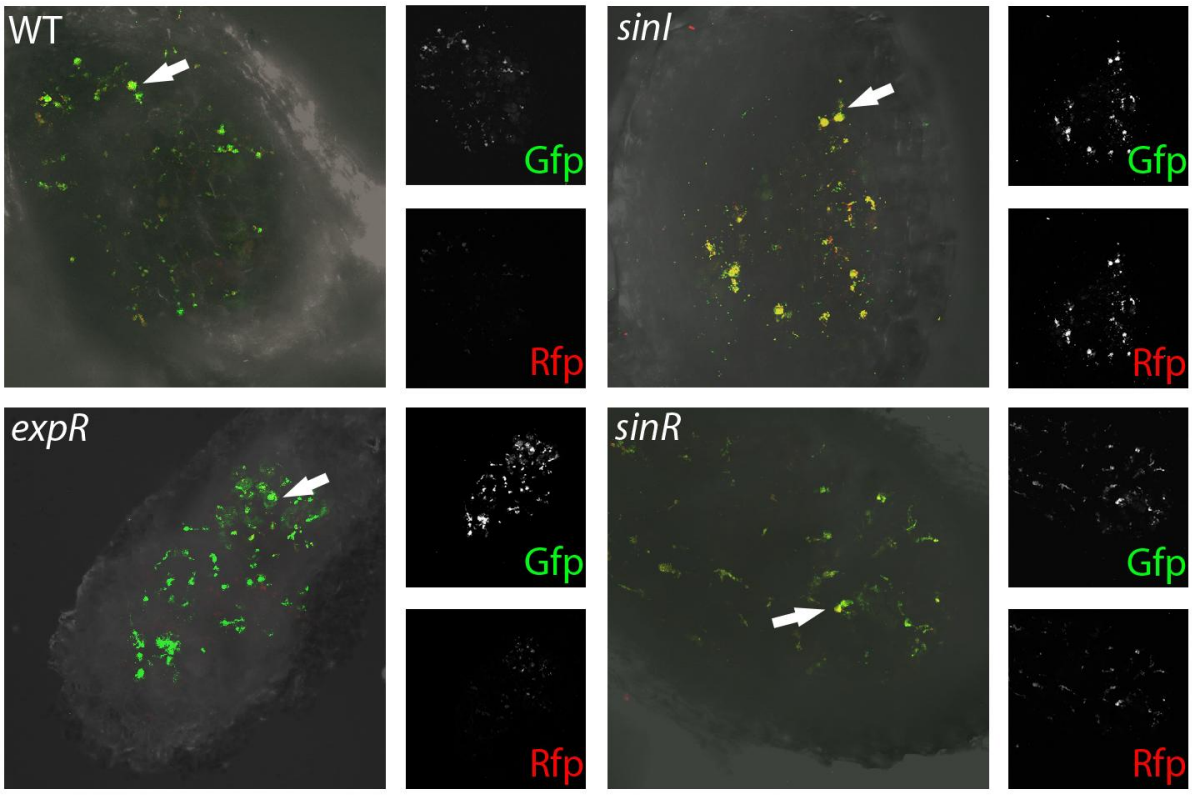

Figure 2. Confocal Laser Scanning Microscopy (CLSM) images of *Medicago truncatula* nodules harvested at 21 days post-infection with wild-type or QS mutant strains of *S. meliloti*. All strains contained both the p*ftsZ2*::tdRfp promoter fusion and constitutively expressed p*trp*::Gfp mut3 fusion on separate plasmids. Nodules were immersed in PIPES buffer (pH 7) and bisected longitudinally using a double-edged razor blade. 488 and 543 nm excitation lasers were used for Gfp and Rfp respectively; 515 – 565 nm emission band width for Gfp and 555-605 nm emission bandwidth for Rfp were used. Smaller images show Gfp and Rfp in separate channels. The larger image for each train are the combined overlay of Gfp, Rfp and differential interference contrast (DIC) channels. All images are Z-stacks of 2.75 µm slices of the bisected nodules. Arrows indicate representative areas where Gfp and Rfp expression co-localized; areas of co-location that are yellow indicate higher levels of Rfp.

difference in the number of nodules on plants infected with cells harboring both plasmids compared with plasmidless cells (data not shown). As illustrated in Figure 2 there was little or no Rfp signal in nodules infected with wild-type cells indicating low *ftsZ2* expression. In contrast, Rfp levels were markedly higher in nodules infected with *sinI* mutant cells indicative of higher *ftsZ2* expression. The images in Figure 2 are representative of dozens of nodules examined with Z stacks of the entire half of each nodule thus represent expression throughout the nodule in order to give enough evidences for making accurate conclusions regarding the increase or the decrease in expression of *ftsZ2* in the whole nodule, although this way of examining the expression of *ftsZ2* is not quantitative. Expression of *ftsZ2* in nodules infected with either *expR* or *sinR* mutant cells was somewhat elevated compared to wild-type cells, but not as high as in the *sinI* mutant background (Figure 2).

The results suggest that QS does play a role in controlling *ftsZ2* expression at some point during nodule development. The elevated levels of *ftsZ2* expression in the *sinI* mutant background *in planta* could be the result of plant AHL mimics that, in the absence of bacterial AHLs result in the activation of *ftsZ2.* This stimulation of *ftsZ2* in the nodules that lack AHL synthase *sinI* raises the important possibility that there exist additional symbiotic signals that stimulate the expression of this gene other than AHLs.

Moreover, AHLs could reach levels in nodules infected with wild-type cells results in repression of *ftsZ2*. This possibility suggests that *ftsZ2* repression is SinI AHL dependent and tied to QS via SinI. AHLs absence. Finally, *ftsZ2* repression in nodules infected with wild-type cells also may suggest a role in regulating bacterial cell division during symbiosis. The moderate increase in *ftsZ2* expression in *expR* and *sinR* mutant backgrounds *in planta* parallels that in free-living cells suggesting the same lack of dependence upon these two receptors and the possibility of repression by them. Furthermore, as six

other potential receptors exist it is possible that one or more of these are required for optimal *ftsZ2* expression *in planta.* Many studies have described *ftsZ2* expression, but expression in nodules is inconsistent between these studies and its expression hasn't been studied in different QS mutants as we did in this study. A transcriptome study has shown that expression of both *ftsZ1* and *ftsZ2* genes are reduced in *S. meliloti* bacteroids (Becker et al., 2004). RT-PCR expression analysis by Gurich and Gonzalez (2009) compared *ftsZ2* expression in free-living cells and bacteroids and found it was substantially reduced in nodules at 8 days and 4 weeks post infection. Using RIVET Gao and Teplitski (2008) found that *ftsZ2* expression was activated in nodules; however, the timing and duration of expression was not pinpointed. RIVET only allows for detection in culturable cells such that this study only detected expression in free-living, undifferentiated saprophytic rhizobia within the nodule (Gao and Teplitski, 2008). None of these studies investigated the role of QS on *ftsZ2* expression *in planta* or in free-living cells.

The results presented in this study extend the findings by Gao and Teplitski (2008) who showed, using RIVET, that *ftsZ2* was activated in nodules infected with wild-type cells at some point during the four week post-infection period. However, since RIVET cannot be used to measure transcriptional repression, it cannot be used to determine if *ftsZ2* is repressed at some point in wild-type cells. Unlike previous studies in wild-type cells of *S. meliloti*, we showed increased *ftsZ2* expression in the absence of *sinI* indicating that cell division may continue for longer time more than in wild-type cells during nodulation in the absence of bacterial AHLs. This may contribute to the delay in nodulation in a *sinI* mutant *S. meliloti* (Gao et al., 2005) and a role in the continued production of bacterial AHLs in nodulation.

In summary, the results further clarify the involvement of QS in the control of bacterial cell division during the symbiosis *in planta.* Our results are added to understand the role of QS in controlling bacterial cell division during the symbiosis *in planta.*

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