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ORIGINAL PAPER

Functional analysis of the two cyclophilin isoforms of *Sinorhizobium meliloti*

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Abstract The nitrogen fixing *Sinorhizobium meliloti* possesses two genes, *ppiA* and *ppiB*, encoding two cyclophilin isoforms which belong to the superfamily of peptidyl prolyl *cis/trans* isomerases (PPIase, EC: 5.2.1.8). Here, we functionally characterize the two proteins and we demonstrate that both recombinant cyclophilins are able to isomerise the Suc-AAPF-pNA synthetic peptide but neither of them displays chaperone function in the citrate synthase thermal aggregation assay. Furthermore, we observe that the expression of both enzymes increases the viability of *E. coli* BL21 in the presence of abiotic stress conditions such as increased heat and salt concentration. Our results support and strengthen previous high-throughput studies implicating *S. meliloti* cyclophilins in various stress conditions.

Keywords Abiotic stress · Cyclophilin · Peptidyl-prolyl *cis/trans* isomerase · *Sinorhizobium meliloti*

Introduction

Peptidyl-prolyl *cis/trans* isomerases are ubiquitous enzymes found in all kingdoms of life. They are classified into three protein families: the cyclophilins, the FK506 binding proteins (FKBPs), and the parvulins (Fanghänel and Fischer 2004). Prolyl *cis/trans* isomerizations are intrinsically slow reactions since there is a large energy barrier between the *cis* and *trans* states of the prolyl peptide bond and often they represent a rate limiting step in protein folding (Brandts et al. 1975). Therefore, PPIases, by catalyzing this isomerization reaction, play a critical role in protein homeostasis (Fischer and Aumüller 2003).

Among the many physiological roles identified for cyclophilins their implication in cell cycle control, signal transduction and transcriptional regulation is well documented (Göthel and Marahiel 1999). Moreover they contribute to the virulence of pathogens (Bell et al. 2006; Viaud et al. 2002; Hacker and Fischer 1993) and to stress tolerance and pathogenicity of bacteria (Söderberg and Cianciotto 2008; Reffuveille et al. 2012; Hermans et al. 2006). Cyclophilins are also essential for adaptation under stress conditions (Andreeva et al. 1999) and their ability to provide tolerance to various stresses has been identified in many species (Sykes et al. 1993; Lee et al. 2015; Trivedi et al. 2013). In *E. coli* they negatively modulate motility and biofilm formation ability (Skagia et al. 2016).

The nitrogen fixing bacterium *Sinorhizobium (Ensifer) meliloti* is able to form symbiotic relationships with certain legumes including the model legume *Medicago truncatula* (Galibert et al. 2001). Many molecular studies have provided insight into the mechanisms regulating symbiosis and the response of *S. meliloti* to various stimuli (Barnett et al. 2004; Barra-Bily et al. 2010; Teplitski et al. 2004; Tiricz et al. 2013). Mining these resources we observed the involvement of the bacterial cyclophilins to these processes so here we further studied the functional properties of the enzymes regarding their catalytic and chaperone activity and their ability to confer protection from various stresses when overexpressed in *Escherichia coli*.

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Methods and materials

Bacterial strains and growth conditions

Escherichia coli XL-Blue1 strain (Invitrogen) was used for the propagation of recombinant forms of the plasmid pCDFDuet-1 (Novagen). *E. coli* strain BL21 (DE3) (Novagen) was used for the expression of recombinant proteins. All *E. coli* strains were grown in LB medium supplemented with streptomycin when necessary.

Heterologous expression of PpiA and PpiB in E. coli and purification of recombinant proteins

The coding sequence of PpiB (SMc01208) and the coding sequence of PpiA (SMc01700) without the first 23 amino acids comprising the probable leader peptide were PCR amplified with *S. meliloti* genomic DNA as template. The primers used were PpiB-F: 5′-AAACTGCAGGCCGCGATCAAGGATCCGGA-3′ with PpiB-R: 5′-TTTAAGCTTTCAGGCGTCGGCGGCAACCC-3′ and PpiA-F: 5′-GGGCTGCAGCAGTCCGGCGAAAATATCCTG-3′ with PpiA-R: 5′-GGGAAGCTTTTACCTGCCGACCTTAACGCTG-3′ carrying restriction sites for ligation to the pCDFDuet1 expression vector. The underlined nucleotides at each primer represent PstI and HindIII, respectively. The absence of undesired alterations was confirmed by nucleotide sequencing. Synthesis and purification of recombinant proteins in *E. coli* BL21 (DE3) cells was performed as previously described (Dimou et al. 2011).

Peptidyl-prolyl *cis/trans* isomerase enzymatic assay

PPIase activity was measured as previously described (Kofron et al. 1991; Dimou et al. 2011). The assay buffer (50 mM Hepes buffer pH: 8.0 and 100 mM NaCl) was mixed with 50 μg α-chymotrypsin (dissolved in 1 mM HCl) (Fluka) and subsequently with the appropriate amount of enzyme. The reaction was rapidly initiated inside the cuvette with the addition of 25 μM Suc-AAPF-pNA (dissolved in trifluoroethanol with 0.45 M LiCl) (Bachem) and the increase in absorbance at 390 nm was monitored at 4 °C using a HITACHI U-2800 spectrophotometer equipped with a thermostated cell holder.

Citrate synthase thermal aggregation assay

Citrate synthase (Sigma) was denatured by incubation at 45 °C, in 40 mM Hepes pH: 7.5, for 15–20 min, in the absence or in the presence of additional proteins, as previously described (Buchner et al. 1998; Dimou et al. 2011). Protein aggregation was measured by monitoring the increase in absorbance at 500 nm using a HITACHI U-2800 spectrophotometer equipped with a thermostated cell holder. Protein disulfide isomerase (Sigma) and albumin (Research Organics) were used in positive and negative control reactions respectively.

Survival of *E. coli* strains during various stresses

Wild type BL21 (DE3) *E. coli* strain and BL21 (DE3) *E. coli* expressing PpiA or PpiB were grown in LB medium at 30 °C. Cells were collected during stationary growth phase and 5 μl from serial dilutions were spotted on LB agar medium supplemented with 0.1 mM IPTG. For each treatment the plates were incubated for 12 h. To test survival during temperature stress, the plates were incubated at 40 °C or at 25 °C. To test survival during salt stress, the LB medium was supplemented with 2% NaCl and to test survival during H_2O_2 stress, the LB medium was supplemented with 0.4 mM H_2O_2. To test survival associated with defects in outer membrane and cell envelope assembly LB was supplemented with 2% SDS and to test survival during water deficit LB was supplemented with 1% PEG_{3000}. Furthermore, LB medium was buffered to different pH values using Hepes and Mes.

Results

PpiA and PpiB encode putative cyclophilins

In *S. meliloti* genome there are two genes coding for putative cyclophilins. PpiA (SMc01700) is predicted to have a periplasmic localization (http://www.psort.org/psortb/) due to a signal peptide consisting of the first 23 amino acid residues (http://www.cbs.dtu.dk/services/SignalP/) while PpiB (SMc01208) is most likely a cytoplasmic protein (http://www.psort.org/psortb/). PpiA and PpiB share 30 and 32% identity with the cyclophilin prototype PPIA (Kallen and Walkinshaw 1992) and are characterized by a well conserved catalytic site (Fig. 1).

Recombinant PpiA and PpiB have peptidyl-prolyl *cis/trans* isomerase activity

To study the physiological role of PpiA and PpiB, we initially investigated whether they are active as peptidyl-prolyl *cis/trans* isomerases using a chymotrypsin coupled assay. Both enzymes were expressed as recombinant proteins with a $(His)_6$-tag added at their N-terminus using the pCDFDuet1 vector and purified under native conditions by Ni–NTA chromatography (Fig. 2a, d).

The standard PPIase activity assay utilizes the conformational specificity of chymotrypsin which cleaves 4-nitroanilide from succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide only

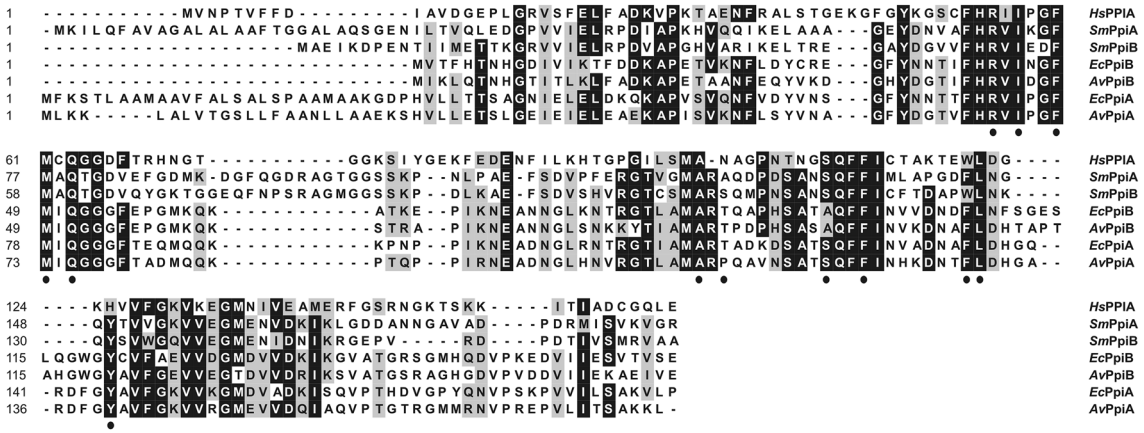

Fig. 1 Protein sequence alignment between *S. meliloti* cyclophilins and related proteins. The sequences included are *Homo sapiens* PPIA (NP_066953), *Escherichia coli* PpiA (NP_417822) and *Escherichia coli* PpiB (NP_415058), *Azotobacter vinelandii* PpiA (ACO78121) and *Azotobacter vinelandii* PpiB (ACO78539) and *S. meliloti* PpiA (NP_385689) and *S. meliloti* PpiB (NP_385690). Multiple sequence alignment was performed using ClustalO (http://www.ebi.ac.uk/Tools/msa/clustalo/). *Black boxes* indicate identical amino acids while *grey boxes* indicate similar. *Black dots* indicate amino acid residues involved in substrate binding of *Homo sapiens* PPIA (Kallen and Walkinshaw 1992)

when the Xaa-Pro peptide bond retains the *trans* conformation. The remaining substrate with the Xaa-Pro bond retaining the *cis* conformation is rapidly converted to the *trans* conformation in the presence of PPIases and the *trans* form is subsequently cleaved by chymotrypsin leading to the formation of the colored product 4-nitroaniline (Kofron et al. 1991). In the presence of PpiA or PpiB we observed an accelerated interconversion of the peptide substrate from *cis* to *trans* form compared to the uncatalyzed reaction (Fig. 2b, e), indicating that both enzymes are active PPIases.

We also calculated the specificity constant k_{cat}/K_M of each enzyme using kinetic data obtained in the presence of increasing concentrations of PpiA or PpiB (Table 1), which were comparable to other cyclophilins from various organisms ranging between 10^6 and 10^7 $M^{-1}s^{-1}$ (Schönbrunner et al. 1991; Dimou et al. 2011).

Recombinant PpiA and PpiB have no chaperone activity

Since many single domain cyclophilins have also been characterized by chaperone activity (Chakraborty et al. 2002; Zhang et al. 2013; Dimou et al. 2011), we further investigated the possible chaperone activity of PpiA and PpiB using the citrate synthase thermal aggregation assay (Buchner et al. 1998). At temperatures above 37 °C, citrate synthase loses its native conformation and undergoes spontaneous aggregation which can be prevented by the presence of a putative chaperone. At control reactions addition of 50 μM of protein disulfide isomerase efficiently prevents citrate synthase aggregation while addition of up to 50 μM of albumin has no effect (data not shown). However, neither recombinant PpiA or PpiB were able to suppress thermal aggregation of citrate synthase when added in 10× molar concentration to the assay mixture (Fig. 2c, f), indicating that at least under our experimental conditions, these two PPIases are not characterized by additional chaperone activity.

Overexpression of recombinant PpiA or PpiB confers *E. coli* resistance to heat and salt stresses

To further elucidate the physiological role of cyclophilins, we analyzed the survival of the wild type *E. coli* BL21 strain in comparison to the *E. coli* BL21 strain overexpressing PpiA or PpiB using the pCDFDuet1 vector, during growth under different stress conditions including thermal, acidic, osmotic, oxidative and detergent stresses (Fig. 3).

BL21 cells overexpressing PpiA or PpiB, grown on LB medium at 30 °C, resulted in 10 to 100 fold reduction in viability compared to BL21 cells. We assume that the observed growth reduction under these typical growth conditions is a physiological consequence of the cyclophilins expression which although requires further investigation regarding the molecular mechanism involved, it can be considered as the standard growth pattern. We observed no difference from this growth pattern during growth under most of the stress conditions tested (Fig. 3). However, growth at 40 °C as well as at 2% NaCl resulted in 10 to 100 fold increase in growth compared to BL21 cells indicating that PpiA or PpiB expression enhances the viability of BL21 strain under these conditions.

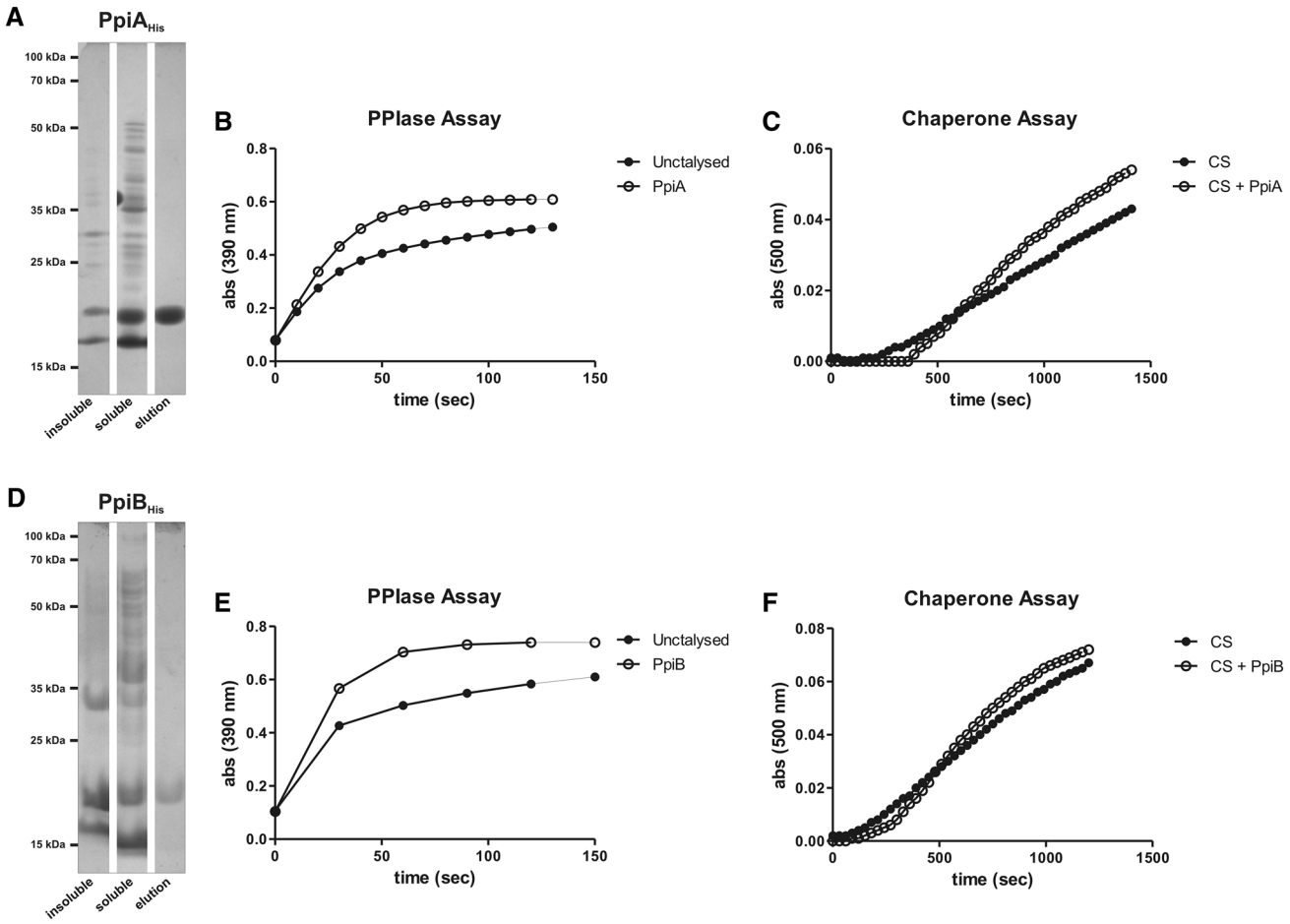

Fig. 2 PpiA and PpiB exhibit peptidyl prolyl *cis*/*trans* isomerase activity but have no influence on the thermal aggregation of citrate synthase. **a, d** SDS–PAGE analysis of the insoluble fraction from *E. coli* BL21 (DE3) cells overexpressing PpiA or PpiB, the soluble fraction and the elution fraction after the Ni–NTA purification of the recombinant proteins. **b, e** Hydrolysis of the peptide Suc-AAPF-pNA by α-chymotrypsin in the absence (*dot*) and presence of 0.17 μM PpiA or PpiB (*open circle*). The background absorbance of p-nitroaniline release is due to the initial presence of the *trans* form of Suc-AAPF-pNA. **c, f** Aggregation of 0.5 μM citrate synthase in the absence in the absence (*dot*) and presence of 5 μM PpiA or PpiB (*open circle*). Citrate synthase was incubated at 45 °C. Aggregation was monitored by measuring the turbidity of the solution at 500 nm in the absence and the presence of additional components. The results are representative of three series of measurements performed with different preparations of enzymes

Table 1 Specificity Constance of PpiA and PpiB for the synthetic tetrapeptide Suc-AAPF-pNA

Substrate	PpiA k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	PpiB
Suc-Ala-Ala-Pro-Phe-pNA	0.02	0.01

PPIase activity was determined at 390 nm with the isomer specific proteolytic assay at 4 °C in 50 mM Hepes pH: 8.0, 100 mM NaCl. k_{cat}/K_M is given in $\mu M^{-1}s^{-1}$

The above results indicate that during thermal and osmotic stresses, which both result in increased protein aggregation (Mogk et al. 2011), the possible prolyl isomerization catalysis by PpiA and PpiB improves cell viability due to enhanced refolding of unfolded proteins. However, since both enzymes were expressed as cytoplasmic recombinant proteins, we cannot fully realize the physiological role of the periplasmic PpiA. While PpiB probably confers resistance via prolyl isomerization of its own specific protein substrates, PpiA could act non specifically on PpiB's substrates or other protein substrates.

Discussion

In the present study, a functional analysis of the purified recombinant PpiA and PpiB has demonstrated that both enzymes are active PPIases with no additional chaperone activity as other single domain cyclophilins (Chakraborty et al. 2002; Zhang et al. 2013; Dimou et al. 2011),

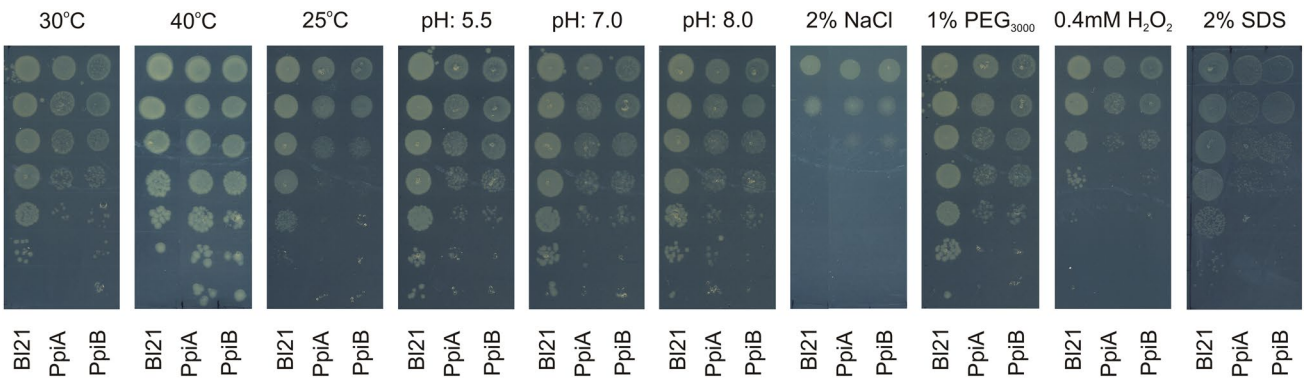

Fig. 3 PpiA or PpiB overexpression confers increased resistance of *E. coli* to heat and salt stresses. Cell viability of BL21 (DE3) wild type cells (BL21 label) and BL21 (DE3) cells expressing PpiA or PpiB (PpiA or PpiB label) was determined by spotting 5 µl of serial dilutions of stationary grown cultures on LB medium supplemented with 0.1 mM IPTG and additional compounds as indicated. Bacterial cultures were incubated at 30 °C or at the indicated temperatures for 12 h

indicating possible variations in their mechanism of action. Furthermore, the cytoplasmic expression of both proteins improved cell viability during heat and salt stresses, environmental conditions frequently encountered by rhizobia, indicating that the possible catalysis of prolyl isomerization reactions on protein substrates enhances the refolding of specific unfolded proteins conferring resistance to these stresses.

There is a close interaction between a host legume and free-living *S. meliloti* throughout the course of symbiosis. After initial signal exchange and bacterial attachment at the root surface, root inner cortical cells dedifferentiate and begin to divide in order to form the nodule meristem (Suzaki et al. 2015). Invasion involves the delivery and release of the rhizobia via an infection thread into the plant cell cytoplasm (Oke and Long 1999), where differentiated bacteroids reduce inert dinitrogen into biologically usable ammonium. Both cyclophilins show decreased expression in differentiated bacteroids from wild type nitrogen fixing or *fixJ*$^{-}$ nodules compared to free living bacteria (Barnett et al. 2004), indicating that their physiological function is not correlated with the ability of *S. meliloti* to fix nitrogen during symbiosis. Similarly, genes whose expression decreases in bacteroids are mostly involved in "housekeeping" and cell surface functions or belong to the *nod* genes or the flagellar/chemotaxis regulon (Barnett et al. 2004). However, a Tn5 insertion into the *ppiB* gene resulted in alterations in lipopolysaccharide banding pattern and in lower proficiency at forming a symbiosis with alfalfa than the wild type strain since nitrogen fixation occurred at reduced levels (Campbell et al. 2003).

During the transition from soil to the invasion and colonization of the developing root nodules, *S. meliloti* encounters numerous abiotic stimuli and plant signal molecules such as flavonoids, reactive oxygen species, nodule-specific cysteine-rich peptides or microaerobiosis (Djordjevic et al. 2003; Naya et al. 2007; Gibson et al. 2008). Oxidative stress is the most intensively investigated stress given that a prolonged oxidative burst with both superoxide and hydrogen peroxide is detected in nodules several weeks after the initial infection (Santos et al. 2001). We observed that both PpiA and PpiB cyclophilins, along with important known determinants of oxidative stress resistance, are downregulated in a *S. meliloti* mutant lacking the RNA chaperone Hfq (Barra-Bily et al. 2010). The *hfq* mutant is affected for H_2O_2, menadione and heat stress resistance (Barra-Bily et al. 2010), and downregulation of the expression of cyclophilins could be to some extent responsible for the observed phenotypes. Interestingly, a recent study on the RNA-binding features of *S. meliloti* Hfq (Torres-Quesada et al. 2014) identifies PpiB among the mRNAs targeted by Hfq. Furthermore, PpiA is downregulated in a deletion mutant of SMc01113, which possibly helps in recognizing the sRNAs during their Hfq mediated interaction with the target mRNAs, and in bacteria overexpressing the sRNA sra35 (Pandey et al. 2011). In agreement to these results, we have observed a better resistance of *E. coli* to heat stress when we overexpressed PpiA and PpiB. However, overexpression of each cyclophilin did not improve the resistance of *E. coli* to H_2O_2, indicating a diverse functional role of the two cyclophilins during different stress conditions. Collectively, these results signify the impact of possible post-transcriptional regulation on the function of cyclophilins during different growth stages and stress conditions.

In further support of the regulated expression of cyclophilins during symbiosis and stress conditions, the expression of *S. meliloti ppiA* is generally downregulated by the antimicrobial nodule-specific cystein-rich peptides NCR247 and NCR335 similarly to many genes involved in basic cellular functions such as transcription/translation

and energy production (Tiricz et al. 2013). Furthermore, *ppiA* from *Bradyrhizobium japonicum* is downregulated by exposure to hydrogen peroxide which causes effects similar to those caused by antimicrobial peptides (Tiricz et al. 2013; Majchrzykiewicz et al. 2010; Jeon et al. 2011). Expression of the legume NCR genes requires the presence of *S. meliloti* and is activated only in the symbiotic cells (Kereszt et al. 2011). *In planta* the activity of such antimicrobial peptides contributes to the loss of rhizobium cell division capacity directing it into irreversible terminal differentiation (Kereszt et al. 2011). Although the general mode of action of these peptides is the disruption of cell membranes or the formation of pores which eventually will lead to cell lysis, they might have intracellular targets as well (Maróti et al. 2011). It would be interesting to further clarify the mechanism of cyclophilins action during this bactericidal process by means of identifying their protein substrates and their effects on them.

The production and exchange of specific signals like *N*-Acyl homoserine lactones (AHLs) between individual cells enables the coordination of bacterial gene expression in a population density-dependent manner (Miller and Bassler 2001). Quorum sensing regulates diverse rhizobial genes mainly associated with rhizosphere adaptation while some interactions between rhizobia and legumes are also influenced (Sanchez-Contreras et al. 2007). Treatment of *S. meliloti* with a partially purified AHL mimic substance from the unicellular green alga *Chlamydomonas reinhardtii* affected the accumulation of PpiB in the opposite way to the addition of the bacterium's own AHL signals, which increased the expression of PpiB (Teplitski et al. 2004). Consequently, the quorum sensing-regulated expression of PpiB implies a significant effect on its functions during free living or symbiotic growth.

Concluding, our study demonstrates the prolyl isomerase activity of *S. meliloti* cyclophilins and illustrates the importance of these enzymes as foldases that improve *E. coli* response during certain stress conditions. These results strengthen the previously published high-throughput studies pointing towards a possible involvement of cyclophilins in stress adaptation or susceptibility depending on the stress and in effective nodulation as well. Further studies are, however, necessary in order to identify the protein substrates of the two cyclophilins and to clarify the mechanism of action of these enzymes during the free living state of *S. meliloti* as well as in symbiosis with legumes.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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