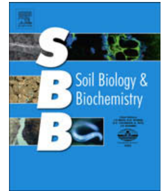
Soil Biology & Biochemistry 41 (2009) 2017–2020

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio



Short communication

Overproduction of the rhizobial adhesin RapA1 increases competitiveness for nodulation

Elías J. Mongiardini, Julieta Pérez-Giménez, M. Julia Althabegoiti, Julieta Covelli, J. Ignacio Quelas, Silvina L. López-García, Aníbal R. Lodeiro*

Instituto de Biotecnología y Biología Molecular, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata CCT La Plata-CONICET, Calles 47 y 115 (1900) La Plata, Argentina

ARTICLE INFO

Article history:
Received 9 January 2009
Received in revised form
3 July 2009
Accepted 13 July 2009
Available online 23 July 2009

Keywords:
Rhizobium
Clover
Adhesin
RapA1
Competition
Nodulation

ABSTRACT

Competition for nodulation is a complex problem where bacterial adhesins, which are required for root colonization, may play a role. However, the possible influence of adhesins on competitiveness was scarcely studied. In this work, the *Rhizobium leguminosarum* bv *trifolii* adhesion protein RapA1 was overproduced from a pHC60-derived plasmid and expressed in R200 strain. When an overproducing strain and a control-carrying empty vector were co-inoculated on clover plants, a positive effect of RapA1 on competition for nodule occupation was observed. Therefore, optimization of RapA1 expression may be considered for improvement of rhizobial competitiveness.

© 2009 Elsevier Ltd. All rights reserved.

The Rhizobia are a polyphyletic group of soil bacteria able to develop and occupy root nodules in legumes. After infecting and colonizing root nodules, rhizobia occupy an essentially protective environment where they fix N_2 and receive energy from the host plant. Thus, nodulation constitutes an advantageous trait for rhizobial proliferation and dispersal. Hence it is not surprising that soil-adapted rhizobial populations occupy most of the nodules in legume crops inoculated with non-indigenous, high quality N_2-fixer strains. Hence, competition for nodulation often impairs the performance of inoculated strains, which is a practical issue in relation to food production and of pertinence to understanding soil ecological interactions.

This problem has been studied for many years, and given its complexity, multiple approaches, such as bacterial genetics, optimization of plant variety-bacterial strain compatibility, enhancement of inoculant formulations, or inoculation technology, have been suggested to overcome it (López-García et al., 2009 and references therein). Since each root nodule often derives from a root hair infection carried out by a single clone, definition of which clone from the rhizosphere population succeeds in getting into the infection thread probably takes place at the onset of infection. Therefore, factors influencing rhizobial adhesion to roots and rhizosphere colonization such as motility, chemotaxis, signal perception, release of antibacterial substances and sensitivity, nutrient specificity, growth efficiency, and position in the soil profile have been identified as key factors in relation to competitiveness (Vande Broek and Vanderleyden, 1995; Toro, 1996; López-García et al., 2002; Okazaki et al., 2003; Brencic and Winans, 2005). Furthermore, studies of bacterial adhesion to plant surfaces identified several proteinaceous adhesins as required for these steps (Danhorn and Fuqua, 2007). However, in the case of rhizobia, any possible roles of the characterized adhesins (Ho et al., 1990; Smit et al., 1992; Wisniewski et al., 1994; Ausmees et al., 2001) for nodulation competition have not yet been demonstrated.

In *Rhizobium leguminosarum* bv *trifolii*, a family of adhesins, called Rap, was characterized (Ausmees et al., 2001). This family encompasses RapA, RapB, and RapC proteins, which are also present in the related species *R. leguminosarum* bv *viceae, R. leguminosarum* bv *phaseoli*, and *Rhizobium etli*. There are three copies of *rap*A gene in *R. leguminosarum* bv *trifolii*, named *rap*A1-3 (Ausmees et al., 2001). This redundancy in *rap* genes precludes obtaining knock-out *rap* mutants. Therefore, we compared the wild type with a RapA1-overproducer strain carrying the stable plasmid pHCRapAS, and observed that three-five times more RapA1 production–evaluated

* Corresponding author. Tel.: +54 221 425 0497; fax: +54 221 422 6947
E-mail address: lodeiro@biol.unlp.edu.ar (A.R. Lodeiro).

0038-0717/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2009.07.016

in culture medium–leaded to two to six times higher rhizobial adhesion specifically to plant roots, increased rhizobial adhesion to root hairs, and also enhanced rhizosphere colonization (Mongiardini et al., 2008). Since it exerted no influence on nodulation kinetics neither in *R. leguminosarum* bv *trifolii* nor in *R. etli*, we concluded that the positive effect of RapA1 on adhesion was not translated to nodulation (Mongiardini et al., 2008). Rhizobial adhesion to roots is not a simple phenomenon: a given rhizobial strain may adhere to different root surfaces in the same plant such as root hair tip, the epidermal surface or root apex. Furthermore, different modes may operate on the same surface, such as polar adhesion, lateral adhesion or cap-forming structures. Thus, the RapA1-promoted adhesion mode might be not symbiotically significant in the sense that rhizobia adhered through RapA1 might be non-infective or tend to colonize non-infectable root surfaces. Alternatively, nodulation kinetics experiments might be not sensitive enough to manifest any influence of RapA1-stimulated root adhesion and colonization on nodulation. Clarification of this aspect is important to consider or not RapA1 among the factors that influence rhizobial nodulation in field conditions, where the presence of competitor microbiota is common. To this end, we studied competition between RapA1-overproducers and wild type strains.

By introducing the plasmid pHCRapAS into *R. leguminosarum* bv *trifolii* R200 we obtained a RapA1-overproducer strain (Mongiardini et al., 2008). This plasmid derives from pHC60, a stable replicative plasmid vector that carries the *par*AB toxin-antitoxin system and the *gfp*P64L/S65T gene (Cheng and Walker, 1998). The pHC60 vector derives from RK2, which has 5–10 copies per bacterial genome, and was reported to maintain this property in different bacterial host species (Chen and Winans, 1991; Cheng and Walker, 1998). To see whether maintenance of pHCRapAS or empty pHC60 vector affected *R. leguminosarum* bv *trifolii* R200 growth in liquid culture, we compared growth kinetics of this strain with or without plasmids in yeast extract-mannitol (YM) medium (Vincent, 1970) in agitated flasks at 120 rev min^{-1} and 28 °C. From growth curve slopes at exponential phase we calculated minimum generation times, which were 2.34 h for wild type R200, 4.01 h for R200 carrying pHC60, and 3.65 h for R200 carrying pHCRapAS. Thus, the presence of these plasmids slightly reduced growth rate.

Since both pHCRapAS and pHC60 encode the green fluorescent protein (GFP), we evaluated nodule occupancy in a series of competition experiments co-inoculating clover plantlets with mixtures of the GFP-tagged strains and non-fluorescent ones. In all experiments clover seeds were surface-sterilized and grown as described (Mongiardini et al., 2008). Then, germinated seeds were transferred, under laminar flow, to sterile vermiculite pots irrigated with plant nutrient Fåhraeus modified solution (FMS) (Lodeiro et al., 2000) and sets of 25 plants were inoculated with each rhizobial strain mixture of interest at the indicated cell densities. After planting and inoculation pots were carried to the greenhouse for 25 days with watering as required. Then, nodules were observed at 10× magnification with a Leica MZ8 stereomicroscope equipped with a GFP Plus fluorescence module with an excitation filter of 480 nm, an emission filter of 505 nm, and a blocking filter of 510 nm. The proportion of fluorescent nodules was registered for each individual plant, and transformed to the arc sin root square. Statistical analysis was done with the transformed values by analysis of variance employing a significance level of $p < 0.05$. Significant differences from the null hypothesis of 50%:50% nodule occupancy were detected with the calculated F parameter, which was compared with the corresponding tabulated F value for the same degrees of freedom at the desired significance level. When calculated F value was higher than tabulated one, we compared all mean values with Tukey test to evaluate mean differences through Student's t. Any possible rhizobial contamination was assessed with uninoculated controls, and experiments were discarded if at least one nodule was observed in such negative controls. In addition, we prepared positive controls by inoculating separate sets of clover plants with each single strain to assess for stability of green fluorescent label in nodules. As before, any experiment was discarded if in a positive control inoculated with a GFP-tagged strain alone, less than 100% nodules were fluorescent. As an additional contamination control, we also discarded experiments if a fluorescent nodule appeared in positive controls inoculated with non-fluorescent strains alone. All experiments were repeated at least twice to assess for reproducibility.

In a preliminary experimental series we prepared mixtures of each GFP-tagged strain – carrying either pHCrapAS or pHC60 – with the R200 wild type, to measure the competitiveness of each plasmid-carrying strain against the wild type used as reference. When we employed 1:1 mixtures, we observed that, in all cases, less than 1% of nodules were fluorescent. After increasing GFP-tagged:wild type ratio by 500:1, fluorescent nodules percentage reached only 20–30%. Since cell concentration by centrifugation or filtration is impractical here because this would lead to RapA1 loss, wild type cells had to be employed at very low numbers to reach this ratio. This leaded to stochastic effects that increased variance, preventing detection of any significant influence of RapA1 overproduction. These results cannot be explained by plasmid loss or GFP being below the threshold required for scoring as positive fluorescence, since in all cases both controls with single inoculation gave 100% nodules with green fluorescence.

As an alternative approach, we prepared isogenic strains to perform direct competitiveness tests. To this end, we constructed another pair of pHC60-derived plasmids without GFP encoded, one of them overproducing RapA1. This plasmid, named pHC60*rapA1*, was previously described (Mongiardini et al., 2008) and carries the *rap*A1 gene in the same way as pHCrapAS, but does not contain *gfp*P64L/S65T. The other plasmid was generated by *gfp*P64L/S65T gene removal from pHC60 through digestion with *EcoRI* and religation to create the plasmid pHCempty, which encodes neither GFP nor RapA1. After mobilizing these plasmids into *R. leguminosarum* bv *trifolii* R200 by conjugation (Mongiardini et al., 2008) we examined RapA1 production from YM broths in western blots of total protein extracts with anti-RapA1 antibodies (Ausmees et al., 2001; Mongiardini et al., 2008). In Fig. 1 we confirmed that pHC60*rapA1* leaded to RapA1 overproduction at similar levels as pHCrapAS, while pHCempty did not, as pHC60. Therefore, we can expect similar behaviors in *R. leguminosarum* bv *trifolii* R200 carrying these new plasmids as previously observed with the same methodology for pHCrapAS and pHC60 (Mongiardini et al., 2008).

With these plasmid-carrying R200 strains we inoculated clover plants with 1:1 mixtures as follows: a) pHCRapAS:pHCempty (i.e. $GFP^+/RapA1^+$ vs $GFP^-/RapA1^-$); b) pHC60: pHC60*rapA1* (i.e. $GFP^+/RapA1^-$ vs $GFP^-/RapA1^+$). If RapA1 overproduction has a positive influence on competitiveness, we would see a higher percentage of fluorescent nodules in plants inoculated with mixture *a*, since here RapA1 overproduction and GFP are encoded in the same strain, and at the same time, we would see a lower percentage of fluorescent nodules in plants inoculated with mixture *b*, because here RapA1 overproduction is encoded in a strain that does not encode GFP. In addition, these combinations would be mutually confirmatory about the role of RapA1 on competition for nodulation, and will allow us to evaluate the proportion of nodules occupied simultaneously by both competitor strains.

The experiment was repeated twice with essentially the same results, as shown in Table 1. There it can be seen that RapA1 overproduction significantly ($p < 0.05$) enhanced *R. leguminosarum* bv *trifolii* competitiveness for nodulation against an isogenic strain producing regular levels of this adhesin. Through the difference in

Download English Version:

https://daneshyari.com/en/article/2025595

Download Persian Version:

https://daneshyari.com/article/2025595

Daneshyari.com