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RESEARCH PAPER

Multiple steps control immunity during the intracellular accommodation of rhizobia

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Abstract

Medicago truncatula belongs to the legume family and forms symbiotic associations with nitrogen fixing bacteria, the rhizobia. During these interactions, the plants develop root nodules in which bacteria invade the plant cells and fix nitrogen for the benefit of the plant. Despite massive infection, legume nodules do not develop visible defence reactions, suggesting a special immune status of these organs. Some factors influencing rhizobium maintenance within the plant cells have been previously identified, such as the M. truncatula NCR peptides whose toxic effects are reduced by the bacterial protein BacA. In addition, DNF2, SymCRK, and RSD are M. truncatula genes required to avoid rhizobial death within the symbiotic cells. DNF2 and SymCRK are essential to prevent defence-like reactions in nodules after bacteria internalization into the symbiotic cells. Herein, we used a combination of genetics, histology and molecular biology approaches to investigate the relationship between the factors preventing bacterial death in the nodule cells. We show that the RSD gene is also required to repress plant defences in nodules. Upon inoculation with the bacA mutant, defence responses are observed only in the dnf2 mutant and not in the symCRK and rsd mutants. In addition, our data suggest that lack of nitrogen fixation by the bacterial partner triggers bacterial death in nodule cells after bacteroid differentiation. Together our data indicate that, after internalization, at least four independent mechanisms prevent bacterial death in the plant cell. These mechanisms involve successively: DNF2, BacA, SymCRK/RSD and bacterial ability to fix nitrogen.

Key words: bacA, CRK, DNF2, innate immunity, nifA, nifH, nitrogen fixation.

Introduction

Legume plants can be cultivated without application of nitrogen fertilizers on soil poor in nitrogen. This capacity is the result of the associations with soil bacteria, referred to as rhizobia, that fix nitrogen for the benefit of the legume (for recent review, see Udvardi and Poole, 2013). These symbioses are efficient thanks to the spectacular bacterial density within the symbiotic organ, the nodule. Despite the massive and chronic colonization by bacteria, legume nodules do not develop defences. As an illustration of this, expression of defence-related genes is drastically reduced in nodule infected cells as compared to non-infected cells (Limpens et al., 2013).

A growing body of recent publications indicates that (the control of) plant innate immunity plays a major role during the early steps of rhizobium/legume interactions (Jones et al., 2008; Yang et al., 2010; Gough and Jacquet, 2013; Liang et al., 2013; Lopez-Gomez et al., 2012; Okazaki et al., 2013).

However, the control of legume immunity is not only critical during the first steps of the symbiotic process but is also required once the nodule is formed when bacteria are released into the plant cells. In Medicago truncatula Gaertn., one of the favourite model legumes, two genes required to repress defence-like reactions into the nodules have been identified.
Defensin-like peptides (Mergaert et al., 2003; Alunni et al., 2007). NCR peptides are specifically produced in infected cells and have a toxic effect on bacteria and prevent their division. As a consequence, in M. truncatula, intracellular rhizobia (called bacteroids) are elongated bacteria, up to 10 µm long, that contain multiple genome copies, up to 24C (Mergaert et al., 2006). NCR toxicity is partially countered by the bacterial BacA protein (Haag et al., 2011), which is believed to act as a peptide importer. The bacterial bacA mutants are more susceptible to NCR peptides in vitro as well as in planta (Van de Velde et al., 2010; Haag et al., 2011). Indeed, once released into the plant cells the bacA mutant does not elongate as the WT bacteria and is rapidly killed (Haag et al., 2011). Interestingly, the ability of the bacA mutant to survive within the plant cells is restored in the M. truncatula dnf1 mutant (Haag et al., 2011). DNF1 is required for bacteroid differentiation and encodes a nodule-specific subunit of a signal peptidase necessary to address plant proteins (including NCR peptides) to the cellular compartments containing the bacteroids (Wang et al., 2010). In addition to DNF1, three other genes (RSD, DNF2 and SymCRK) are also required for bacteroid elongation in M. truncatula (Bourcy et al., 2013; Sinharoy et al., 2013; Berrabah et al., 2014a). These three genes are specifically expressed in nodules and the symbiotic organs of the rsd, dnf2 and symCRK mutants accumulate brown material (Bourcy et al., 2013; Sinharoy et al., 2013; Berrabah et al., 2014a), reminiscent of defence reactions.

Defence-like reactions are not developed in bacA-triggered nodules indicating that the lack of bacteroid elongation and the lack of nitrogen fixation do not elicite dnf2- and symCRK-like defences (Berrabah et al., 2014b). However, the bacA mutant is unable to colonize massively the plant cells and the question remains as to whether the lack of nitrogen fixation associated with a massive bacterial intracellular colonization could elicit the plant defences.

We aim to better understand the repression of defence-like reactions in nodules by identifying the factors involved in this process and by determining precisely the sequence of events that conduct defence development in the mutants. Herein we show that early bacterial death is associated with bacterial inability to fix nitrogen and that RSD is also required to prevent defence-like reactions in nodules. In addition, by combining the use of the dnf2, symCRK and rsd mutants together with various bacterial mutants, we define two steps in the symbiotic control of immunity after rhizobia internalization.
then stained with methylene blue 0.01% for 10 to 15 mins and nodule sections were washed in 6.1% chlorine solution prior observation.

**Acetylene reduction assay**

An acetylene reduction assay was conducted as previously described (Berrabah et al., 2014a, b). The method is derived from the one described in Koch and Evans (1966). Briefly, entire plants were individually incubated in 10 ml vials containing air supplemented with 250 μl acetylene. After at least 1 h at room temperature, 200 μl of the gas phase were analysed by gas chromatography coupled with flame ionization detection.

**In situ hybridization**

*In situ* hybridizations followed the protocol described in Boualem et al. (2008). Twenty-one dpi nodules of *M. truncatula* cultivated *in vitro* on BNM-agar were analysed using RNA probes labelled with dioxygenin and revealed using antibodies coupled to alkaline phosphatase. The anti-sense probe was designed on extracellular cysteine-rich SymCRK domain to specifically target the SymCRK transcript. The sense probe was used as a negative control; probes were synthethized using T7/SP6 primers after target sequence cloning in the pGEMT easy vector. The primers used to amplify the target region are listed in Supplementary Table S1.

**Results**

The nifA fix mutant undergoes bacterial death that is not associated with symCRK/dnf2-like defence reactions

Using the bacterial bacA mutant that is unable to differentiate elongated bacteroids, we previously showed that lack of bacteroid differentiation does not elicit expression of defence genes or phenolics accumulation that are observed in the dnf2 and the symCRK nodules (Berrabah et al., 2014b). Nevertheless, the bacA mutant is hypersensitive to NCR peptides and this sensitivity does not allow massive colonization of the plant cells (Haag et al., 2011). In order to determine if a massive colonization by ineffective bacteria triggers defence reactions, *M. truncatula* WT plants were inoculated with the *S. melliloti* nifA mutant. NifA is a key regulator of the nitrogenase synthesis and the corresponding mutant does not fix nitrogen (Zimmerman et al., 1983; Dixon and Kahn, 2004). Nodules induced by nifA are infected and bacteroids clearly undergo terminal differentiation (Fig. 1). However, in contrast to the WT, the mutant bacteria prematurely died as revealed by the live/dead assay that stains viable bacteria green and dead bacteria red (Fig. 1). The bacterial death occurs only after bacteroid elongation in the nifA mutant (Supplementary Fig. S1). Beyond genes responsible for nitrogenase synthesis, NifA regulates additional genes (Bobik et al., 2006) and the nifA mutant displays pleiotropic phenotypes (Gong et al., 2007). To determine whether nifA bacteroid death is caused by the lack of nitrogen fixation or to another nifA-regulated process, nifH bacteroid viability was also studied; nifH encodes a subunit of the nitrogenase. Like for the nifA mutant, bacteroid death was observed for elongated nifH bacteroids (Supplementary Fig. S1) suggesting that lack of nitrogen fixation is responsible for the death of nifA and nifH elongated bacteroids. In order to determine if defence reactions such as those observed in the nodules of the symCRK and the dnf2 mutants could be responsible for nifA bacterical death, the induction of these defences

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**Fig. 1.** Mutation in nifA elicits early bacterial death that is not mediated by symCRK/dnf2-dependant defence reactions. (A): a–d: On these sections of 26dpi nodules imaged using a confocal microscope after live/dead staining assay, the early death of the nifA mutant within the plant cell is observable. Green and red stain alive and dead bacteria respectively. a and b represent WT bacteria induced nodules, c and d: nifA induced nodules, note that like the WT bacteria, the nifA mutant undergoes terminal differentiation, scale bars correspond to, 20μm (a, c) and 10μm (b, d). (B): nifA mutant does not elicit the accumulation of phenolics in nodules. Sections of WT plant nodules induced by WT bacteria or by nifA mutant as well as dnf2 and symCRK nodules induced by WT bacteria were stained with methylene blue to reveal phenolics in blue. Scale bars correspond to 500 μm. (C): PR10, VSP, BGL and NDR1 defence-related genes are not induced in nifA-triggered nodules. In contrast these defence-related genes are induced in dnf2 and symCRK nodules as revealed by RT-qPCR analysis. Results are expressed as induction fold vs R108 nodules induced by WT bacteria after normalization using Mtactin2 constitutive gene as an internal standard.
was evaluated by RT-qPCR and histological approaches. In contrast to the \textit{dnf2} and \textit{symCRK} mutant nodules, the expression of the defence-related genes \textit{PR10}, \textit{VSP}, \textit{BGL}, and \textit{NDR1} was not induced in the \textit{nifA}-triggered nodules, which also do not accumulate phenolics (Fig. 1). In agreement of a role of \textit{nifA} after \textit{DNF2} and \textit{SymCRK}, mutation in the \textit{nifA} gene does not prevent the development of defence-like reactions in \textit{dnf2} and \textit{symCRK} nodules (Supplementary Fig. S2). Together these results suggest that lack of nitrogen fixation induces early death of the bacteria. Furthermore this bacterial death is not associated with defence-like reactions observed in the \textit{dnf2} and \textit{symCRK} mutants (Fig. 1).

\textit{DNF2}, \textit{bacA} and \textit{SymCRK} act successively during the symbiotic process

In order to get an insight into the development of the symbiotic process and the timing of immunity suppression during symbiosis, nodulation tests were conducted using \textit{dnf2} and \textit{symCRK} mutants in combination with bacterial mutants altered in surface components or in the symbiotic process. Defence-like reactions were evaluated 14 days after inoculation in the nodules induced by \textit{S. meliloti} Sm1021 \textit{bacA}, and \textit{nifH} mutants. Defence reactions were monitored by following the expression of the \textit{NDR1} and \textit{PR10} genes using RT-qPCR. In addition, nodule sections were observed under bright field illumination to reveal the presence of necrotic zones accumulating phenolics. The \textit{eps} and \textit{kps} mutants elicit a moderate induction of the expression of \textit{NDR1} and \textit{PR10} genes in nodules of the WT plants (Fig. 2A). These inductions were not associated with the development of necrotic zones in the WT plant nodules (Fig. 2B). In contrast, the expression of defence-related genes was observed when these bacterial mutants were nodulating \textit{dnf2} and \textit{symCRK} plant mutants and this was associated to the presence of necrotic zones (Fig. 2B). In nodules developed on WT plants inoculated by Sm1021 or its \textit{nifH} or \textit{bacA} mutant derivatives, the \textit{NDR1} and \textit{PR10} genes are not induced (Fig. 2A) and these nodules do not accumulate phenolics. In contrast to the WT plants, \textit{dnf2} and \textit{symCRK} nodules display necrosis and induction of the defence markers with these strains, except for \textit{symCRK} inoculated with the \textit{bacA} mutant; in these nodules do not differentiate and are not maintained viable in both the \textit{dnf2} and \textit{bacA} nodules. In contrast, in the \textit{dnf1} \textit{bacA} mutant, bacteria do not undergo terminal differentiation but bacteroids remain viable during colonization (Haag \textit{et al.}, 2011). Thus, this mutant constitutes a good tool to discriminate whether the colonization defect and/or the differentiation defect is responsible for incorrect \textit{symCRK} expression. We have used two \textit{Tnt1} insertion mutant lines for \textit{DNF1} (NF8776 and NF17452) in the \textit{M. truncatula} R108 background (in which the \textit{dnf2} and \textit{symCRK} mutants used in this study were generated). In these lines the \textit{Tnt1} retrotransposon is inserted in the first intron 131 bp and 324 bp after the predicted start codon. However, we could not observe any symbiotic phenotypes in the progeny, possibly as a result of the insertion in the intron or of the absence of homozygous mutant (Supplementary Fig. S7). The expression of

The double mutant \textit{dnf2}\textit{symCRK} was analysed with respect to defence development in nodules elicited with WT bacteria or \textit{bacA} mutant strain. We did not observe any additive effect of the mutation in the double mutant nodules upon inoculation with the WT bacteria (Fig. 2C, Supplementary Figs S3 and S6). When inoculated with the \textit{bacA} mutant, defence reactions were observed in the \textit{dnf2}\textit{symCRK} nodules but not on the \textit{symCRK} mutant (Fig. 2C, Supplementary Fig. S3). These results thus indicate that the double mutant behaves as the \textit{dnf2} mutant in terms of defence reactions and that \textit{DNF2} is epistatic to \textit{SymCRK} in the \textit{M. truncatula} R108 background. Together, these results suggest that \textit{DNF2}, \textit{bacA} and \textit{SymCRK} act successively during the symbiotic process.

\textbf{SymCRK correct expression requires initiation of bacteroids differentiation}

In order to improve our understanding of the \textit{SymCRK} gene function, its expression pattern inside nodules was studied using \textit{in situ} hybridization in WT nodules. This experiment (Fig. 3A) suggests that \textit{SymCRK} is specifically expressed in infected cells in agreement with its timing of expression (Berrabah \textit{et al.}, 2014a; see also below). In order to define more precisely the role of \textit{SymCRK} and \textit{DNF2} during the symbiotic process, we investigated the expression of the two genes in \textit{M. truncatula} nodules induced by the \textit{S. meliloti} \textit{nifA} and \textit{nifH} mutants altered in the production of nitrogenase (for review, see Dixon and Kahn, 2004) as well as by the \textit{bacA} mutant. In nodules induced by the \textit{nifA} and \textit{nifH} bacterial mutants (Fig. 3B), expression of the two symbiotic genes \textit{SymCRK} and \textit{DNF2} was not altered, indicating that nitrogen fixation is not necessary for their expression. \textit{DNF2} transcript accumulation is not modified in nodules induced by the \textit{bacA} mutant or in \textit{symCRK} mutant nodules induced by WT bacteria placing \textit{DNF2} upstream or in a parallel pathway to \textit{BacA} and \textit{SymCRK}. In contrast, the level of \textit{SymCRK} transcripts was reduced in WT nodules induced by the \textit{bacA} mutant or in \textit{dnf2} mutant nodules induced by WT bacteria, in agreement with the data shown above, indicating that \textit{SymCRK} act downstream of \textit{DNF2} and \textit{bacA} during the symbiotic process. As mentioned previously, bacteroids do not differentiate and are not maintained viable in both the \textit{dnf2} and the \textit{bacA} nodules. In contrast, in the \textit{dnf1} \textit{bacA} mutant, bacteria do not undergo terminal differentiation but bacteroids remain viable during colonization (Haag \textit{et al.}, 2011). This, mutant constitutes a good tool to discriminate whether the colonization defect and/or the differentiation defect is responsible for incorrect \textit{symCRK} expression. We have used two \textit{Tnt1} insertion mutant lines for \textit{DNF1} (NF8776 and NF17452) in the \textit{M. truncatula} R108 background (in which the \textit{dnf2} and \textit{symCRK} mutants used in this study were generated). In these lines the \textit{Tnt1} retrotransposon is inserted in the first intron 131 bp and 324 bp after the predicted start codon. However, we could not observe any symbiotic phenotypes in the progeny, possibly as a result of the insertion in the intron or of the absence of homzygous mutant (Supplementary Fig. S7). The expression of
SymCRK was thus studied in the only dnf1 available mutant (generated in the A17 background). This experiment shows that SymCRK expression was reduced in the dnf1 mutant, suggesting that not only bacterial colonization but also bacteroid differentiation is required for SymCRK expression (Fig. 3C).
RSD represses defence-like reactions and act downstream of bacA and DNF2

Like dnf2 and symCRK, rsd displays necrotic nodules in which bacteroids do not undergo terminal differentiation (Sinharoy et al., 2013). In order to determine if rsd also shares the defence-like reactions observed in the dnf2 and symCRK mutants, induction of defence-related genes was evaluated in rsd-1 nodules. Expression of the PR10 and NDR1 genes was evaluated by RT-qPCR and was found to be induced in rsd-1 as compared to WT nodules (Fig. 4). In addition, presence of phenolic compounds was detected in rsd-1 nodules (Fig. 4) indicating that rsd develops defence-like reactions similar to those of symCRK and dnf2. In order to position RSD with respect to SymCRK and DNF2 during the development of the symbiosis, we analysed the expression of RSD in the dnf2 and in the symCRK backgrounds. RSD expression is strongly reduced in both mutants (Fig. 4C) suggesting a role for RSD downstream of DNF2 and SymCRK.

In order to determine whether the development of defence-like reactions in the rsd-1 mutant is dependent on bacterial invasion and differentiation, we evaluated defences in this mutant upon nodulation with the bacA mutant. In contrast to dnf2 and, similarly to symCRK, the rsd mutant does not accumulate phenolics upon infection with bacA (Fig. 4A). In agreement with these results, defence-related gene expression was strongly reduced in rsd/bacA nodules as compared to rsd nodules triggered by WT bacteria (Fig. 4B). Thus, similar to what is observed in the symCRK mutant, development of defence-like reactions in rsd relies on the presence of a functional bacA.

Discussion

Herein we have investigated the sequence leading to the symbiotic suppression of immunity in legume nodules after rhizobia internalization within the plant cells (chronic infection). We showed that the bacterial mutants nifA and nifH died prematurely within the plant cells despite that defence reactions similar to those observed in symCRK and dnf2 nodules were not detected in these nodules. In addition, we showed that DNF2 and SymCRK act successively during the symbiotic process and that bacA is required after DNF2 and before SymCRK. Furthermore, our data indicate that rsd-1 behave like the symCRK mutant and acts after bacA. These results are in agreement with the observation that SymCRK and RSD expression is reduced in WT plants nodulated with bacA, a condition in which expression of DNF2 is not modified (Fig. 3B; Sinharoy et al., 2013). In addition, results obtained with the dnf1 mutant suggest that the initiation of bacteroid differentiation rather than the massive intracellular colonization is important for SymCRK activity. Interestingly, in the distant phylogenetic system, soybean/Bradyrhizobium japonicum, the nifA mutant triggers nodules displaying dark brown zones of necrotic appearance (Fischer et al., 1986) reminiscent of symCRK/dnf2-like defences. Altogether our results indicate that multiple steps are required for the symbiotic suppression of immunity after bacterial internalization. Furthermore the present study indicates that at least two types of defence can be the cause of bacterial death in the plant cells, one associated with nodule necrosis and induction of PR10 and NDR1 defence genes and another observed upon nifA- or nifH-triggered nodulation.

Based on our results, we propose a model focused on the intracellular stage of symbiosis, which describes the actors preventing bacteroid death (Fig. 5). In this model, DNF2 is...
the earliest actor identified as required for symbiotic suppression of immunity. Its requirement is determined by environmental conditions that influence the development of defence-like reactions in nodules (Berrabah et al., 2014).

After DNF2, the bacA bacterial gene prevents the killing activity of NCR peptides before SymCRK and RSD prevent defence-like reactions possibly triggered by massive intracellular invasion or initiation of bacteroid differentiation. Finally, the production of nitrogenase prevents bacteroid death. In order to add more actors to this model, necrotic mutants (such as those described in Pislariu et al., 2012; Domonkos et al., 2013) will probably represent good tools. Such mutants could in future studies be classified according to their behaviour upon inoculation in combination with bacterial symbiotic mutants (amongst which is bacA) but also based on their symbiotic properties using dnf2 permissive media (Berrabah et al., 2014).

Immunity suppression is not only required for the intracellular stage of the symbiosis; various actors have been identified or proposed to be important to prevent plant defences during the infection process. For example the nodulation (Nod) factors might participate in the suppression of plant immunity triggered by microbial motifs (Liang et al., 2013).

In agreement with this novel Nod factor's role, the type three secretion system (often used by phytopathogenic bacteria to suppress plant immunity) of Bradyrhizobium elkanii can bypass the requirement of Nod factors for the nodulation of soybean (Okazaki et al., 2013). Also, two soybean resistance genes modify the rhizobial host range of the plant (Yang et al., 2010) and exopolysaccharides were shown to be important to reduce the induction of defence-related genes at the early stages of the symbiosis (Jones et al., 2008). Finally, defence-related phytohormones also interfere with the nodulation process (Penmetsa and Cook, 1997; Stacey et al., 2000).
et al., 2006; Sun et al., 2006). These data indicate that suppression of immunity during the early steps of the symbiotic process is required for nodulation. In agreement with this, the expression of a PRI0 gene is drastically reduced in bumps (young nodules) and mature nodules as compared to roots. We showed that DNF2 prevents defense-like reactions in nodules only after bacterial internalization (Bourcy et al., 2013) and that in bumps, the expression of the PRI0 gene is similar in the dnf2 and WT nodules (Bourcy et al., 2013). These results indicate that a mechanism distinct from DNF2 and SymCRK suppresses immunity in early developing nodules and reinforce the hypothesis of the existence of multiple steps in the suppression of plant immunity during the symbiotic process. It is now a challenge to gather the identified actors of the symbiotic suppression of immunity within an integrated model.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Lack of nitrogen fixation triggers bacterial death after bacteroid elongation.
Supplementary Fig. S2. dnf2 and symCRK act before nifA.
Supplementary Fig. S3. Defence reactions are abolished in the symCRK bacA nodules.
Supplementary Fig. S4. Nitrogen fixation is not restored in symCRK/bacA nodules.
Supplementary Fig. S5. NCR99 expression is reduced in bacA-triggered nodules of the dnf2 and symCRK mutants.
Supplementary Fig. S6. bacA viability is not restored in the symCRK mutant.
Supplementary Fig. S7. DNF1 gene structure (based on MTR_3go27890 sequence) and position of the Tnt1 insertions present in mutant lines NF8776 and NF17452.
Supplementary Table S1. List of primers used during this study.

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