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Official URL: https://doi.org/10.1111/nph.15883

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Sorroche, Fernando and Walch, Mathilda and Zou, Lan and Rengel, David and Maillet, Fabienne and Gibelin-Viala, Chrystel and Poinsot, Verena and Chervin, Christian and Masson-Boivin, Catherine and Gough, Clare and Batut, Jacques and Garnerone, Anne-Marie *Endosymbiotic Sinorhizobium meliloti modulate Medicago root susceptibility to secondary infection via ethylene.* (2019) New Phytologist, 223 (2). ISSN 0028-646X

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Endosymbiotic *Sinorhizobium meliloti* modulate *Medicago* root susceptibility to secondary infection via ethylene

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doi: 10.1111/nph.15883

Key words: ethylene, infection, legume, rhizobium, symbiosis.

Summary

- A complex network of pathways coordinates nodulation and epidermal root hair infection in the symbiotic interaction between rhizobia and legume plants. Whereas nodule formation was known to be autoregulated, it was so far unclear whether a similar control is exerted on the infection process.
- We assessed the capacity of *Medicago* plants nodulated by *Sinorhizobium meliloti* to modulate root susceptibility to secondary bacterial infection or to purified Nod factors in split-root and volatile assays using bacterial and plant mutant combinations. Ethylene implication in this process emerged from gas production measurements, use of a chemical inhibitor of ethylene biosynthesis and of a *Medicago* mutant affected in ethylene signal transduction.
- We identified a feedback mechanism that we named AOI (for Autoregulation Of Infection) by which endosymbiotic bacteria control secondary infection thread formation by their rhizospheric peers. AOI involves activation of a cyclic adenosine 3′,5′-monophosphate (cAMP) cascade in endosymbiotic bacteria, which decreases both root infectiveness and root susceptibility to bacterial Nod factors. These latter two effects are mediated by ethylene.
- AOI is a novel component of the complex regulatory network controlling the interaction between *Sinorhizobium meliloti* and its host plants that emphasizes the implication of endosymbiotic bacteria in fine-tuning the interaction.

Introduction

One of the most ecologically important and best-understood mutualistic interactions on Earth associates N_2-fixing soil bacteria, collectively known as rhizobia, and plants of the Leguminosae family. Thanks to this symbiotic association, wild and cultivated legume plants can get their nitrogen (N) supply from their N_2-fixing endosymbionts and can thus grow on N-depleted soils. This N_2-fixation takes place in specialized organs, called nodules, that rhizobia elicit on the roots of compatible legume plants. In the rhizosphere, most rhizobial bacteria synthesize specific lipo-chitooligosaccharide molecules, known as Nodulation Factors (NFs), that simultaneously trigger nodule organogenesis and the formation of dedicated infection structures called infection threads (ITs) that form in root hairs (eITs) before invading the root cortex (cITs) and the emerging nodule tissues (Jones *et al.*, 2007; Murray, 2011; Suzaki *et al.*, 2015; Dalla Via *et al.*, 2016; Miri *et al.*, 2016). Numerous plant genes required for IT formation and nodule organogenesis have been identified and tentatively organized in a complex genetic network (Murray, 2011; Oldroyd, 2013; Long, 2016; Kelly *et al.*, 2017). All through this process, plant innate immunity is challenged (Cao *et al.*, 2017).

Fine-tuning nodulation is central to the achievement of mutualism as it balances the N gains with the energy costs of nodule formation and functioning. Nodulation is regulated primarily via a systemic mechanism known as the autoregulation of nodulation (AON). During AON, CLE peptides synthesized in nodules travel to the shoot and meet specific LRR-Receptor-like-kinases (LRR-RLKs), called SUNN in *Medicago truncatula* and HAR-1 in *Lotus japonicus* (Penmetsa *et al.*, 2003; Magori & Kawaguchi, 2009; Reid *et al.*, 2011). Activation of these LRR-RLKS probably triggers cytokinin synthesis, thereby reducing root sensitivity to nodulation (Mortier *et al.*, 2012; Sasaki *et al.*, 2014). Local ethylene signaling also contributes to the negative control of nodulation (Penmetsa *et al.*, 2008; Gresshoff *et al.*, 2009). A *Medicago truncatula ETHYLENE INSENSITIVE2* mutant called *sickle* (*skl*) displays both a hypernodulation and a hyperinfection phenotype (Penmetsa & Cook, 1997; Penmetsa *et al.*, 2008), in agreement with former evidence that ethylene inhibits the NF signal transduction pathway (Oldroyd *et al.*, 2001). Recently, a long-distance regulatory mechanism positively controlling nodule formation was described in *M. truncatula* in which the MtCEP1 peptide modulates ethylene signaling upon interaction with the MtCRA2 receptor (Mohd-Radzman *et al.*, 2016).

Despite early cytological evidence for a control of IT formation and extension (Vasse *et al.*, 1993), the mechanism(s) regulating IT formation has(ve) remained elusive for a long time. Mutants of the model legumes *M. truncatula* and *L. japonicus* displaying a relaxed control of IT formation, resulting in a hyperinfection phenotype, have been described in the literature (Murray *et al.*, 2007; Vernie *et al.*, 2008; Mbengue *et al.*, 2010; Suzaki & Kawaguchi, 2014; Suzaki *et al.*, 2014; Yoro *et al.*, 2014; Reid *et al.*, 2018). However, these mutants either showed enhanced or decreased nodulation or N_2-fixation, thus raising the possibility that hyperinfection was a side effect of a reduced or altered control of nodulation or suboptimal N_2-fixation. Very recently, a mobile microRNA was shown to favor root susceptibility to infection on non-nodulated roots of Lotus (Tsikou *et al.*, 2018).

A few years ago, we identified in *Sinorhizobium meliloti,* a *Medicago* symbiont, a cyclic adenosine 3′,5′-monophosphate (cAMP) regulatory cascade consisting of three receptor-like adenylate cyclases (CyaD1, CyaD2, CyaK), a Crp-like cAMP-dependent transcriptional regulator called Clr, and a number of Clr-target genes among which *smc02178*, encoding a periplasmic protein of unknown function (Zou *et al.*, 2017). This cAMP cascade is expressed in nodules under the control of two unknown plant signals (Tian *et al.*, 2012; Garnerone *et al.*, 2018) sensed by the outer membrane receptor protein NsrA (Garnerone *et al.*, 2018). Null mutants in *clr*, *smc02178* or a triple *cyaD1cyaD2 cyaK* mutant displayed a hyperinfection phenotype on *Medicago sativa,* although their nodulation and N_2-fixation capacities were indistinguishable from wild-type (WT). The hyperinfection phenotype was best evidenced at 14 d post-inoculation (dpi), although it could be detected as early as 7 dpi (Tian *et al.*, 2012). Furthermore, the WT bacteria and the isogenic *clr* mutant were indistinguishable with respect to primary infection, as assessed by competitive inoculation assays (Tian *et al.*, 2012). Altogether these observations led us to hypothesize that this bacterial cAMP cascade was part of a pathway autoregulating plant secondary infection, that is IT formation on nodulated plants. (Tian *et al.*, 2012).

Here we provide conclusive evidence that cAMP cascade activation in endosymbiotic bacteria – that is, bacteria that have successfully elicited and invaded nodules – decreases the root sensitivity to bacterial NFs, thereby inhibiting secondary infection by rhizospheric bacteria. This Autoregulation Of Infection (AOI) pathway involves ethylene emission by nodulated roots.

Materials and Methods

Microbiological techniques

Bacterial strains and plasmids used in this study are described in Supporting Information Table S1. Bacteria were grown as described before (Tian *et al.*, 2012). Nodulation (Nod) factors were extracted from *Sinorhizobium meliloti* culture supernatants by butanol extraction, and purified by high-performance liquid chromatography (HPLC) on a semi-preparative C18 reverse phase column, as described before (Roche *et al.*, 1991). Nod factor structure was verified by mass spectrometry as described before (Poinsot *et al.*, 2016).

Plant material and plant seeds germination

Medicago sativa or *M. truncatula* seeds were sterilized by immersion in concentrated H_2SO_4 for 5–12 min (depending on seed batches), washed with sterile water, surface-sterilized with a diluted (25%) commercial bleach solution for 2 min, and thoroughly washed again with sterile water. Seeds were vernalized at 4°C on agar (1.5% w/v) plates for a week and then allowed to germinate overnight in the dark at 25°C. Germinated seedlings were transferred aseptically into 12 × 12-cm plates containing slanting Fåhraeus agar medium supplemented with 4 mM NH_4NO_3 and allowed to grow at 22°C in a 16 h : 8 h, light (day) : dark (night) photoperiod.

Plant preparation for split-root assays and volatile assays

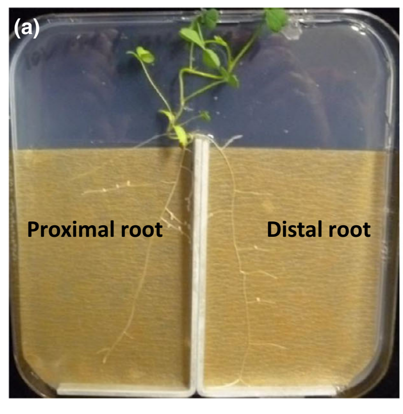
The primary roots of 2 d-old plantlets were cut at the hypocotyl to trigger formation of lateral roots. The root system was then covered with sterile paper to stick the emerging roots on the agar surface. After 5–7 d, plants with two lateral roots of a similar length were transferred on slanting nitrogen (N)-free Fåhraeus agar in compartmentalized squared plates containing two opposing L-shaped metal strings (Fig. 1a). To avoid root penetration into the Fåhraeus medium, the root systems were placed on a sterile paper and covered with a permeable transparent film (BioFolie 25; Sartorius AG, Vivascience, Hannover, Germany) to minimize desiccation and cross-contamination between compartments. The plates were closed with Parafilm to avoid desiccation and the root systems were shielded from light using black paper envelopes.

For so-called volatile assays (Fig. 4a), A17 (emitter) 2-d-old plantlets were grown in one compartment of the square plate and inoculated with wild-type (WT) or mutant *S. meliloti* strains. At 10 d post-inoculation(dpi), a 2-d-old seedling (either A17, proMtENOD11:GUS or MtSickleproENOD11:GUS) was introduced in the second (receiver) compartment of the square plate and allowed to grow for 4 d. Afterwards, the receiver root system was either monitored for ENOD11:GUS expression (Fig. 4b,c, see later) or infection (Fig. 4d, see later). When needed (Fig. 5c, see later), aminoethoxyvinylglycine (AVG; Sigma) was added to the Fåhraeus medium of the emitter plant compartment (0.5 μM final concentration), whereas the receiver plant compartment remained AVG-free.

Medicago sativa 'Europe' plants were alternatively cultivated on a sepiolite ('Oil Dri', UK) medium in open tubes as described before (Maillet *et al.*, 2011) and above. Plants inoculated by the WT and the *clr* strain were grown in separate (air-open) mini-glasshouses to prevent bacterial cross-contaminations.

proMtENOD11:GUS expression assays

The proximal roots of 3 d-old split-root plantlets prepared as described above or the emitter plants in the case of volatile assays were inoculated with 2×10^3 *S. meliloti* bacteria per plant (WT or



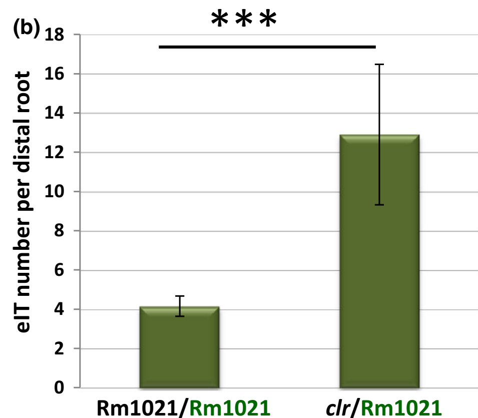


Fig. 1 Systemic control of epidermal infection thread (eIT_ formation in a split-root *Medicago sativa* assay. (a) Design of the split root assay. *Sinorhizobium meliloti* Rm1021 or isogenic *clr* mutant bacteria were inoculated on the proximal root. Fourteen days post-inoculation (dpi), wild-type (WT) green fluorescent protein (GFP)-labeled *S. meliloti* Rm1021 bacteria were inoculated on the distal root. (b) eIT formation by WT *S. meliloti* Rm1021 (green) on the distal root after inoculation of the proximal root with either Rm1021 (WT) or a *clr* mutant as indicated left of the slash. The bar features the ± SE values. ***, *P*-value 4.2 e^{-5} (Kruskal–Wallis test).

mutants). The distal roots or the roots of receiver plants were excised 14 d later and incubated overnight in 50 ml of purified *S. meliloti* Nod factors (NFs) (10^{-8} M in N-free Fåhraeus liquid medium). Roots were fixed and stained for GUS detection for 45 min to 4 h, as described previously (Journet *et al.*, 1994). When needed, roots mounted on slides were automatically scanned in a NanoZoomer Digital Scan System (Toulouse Imaging Platform, Toulouse, France). For quantification purposes, the level of NF-dependent proENOD11:GUS expression was scored into three classes as described before (Cerri *et al.*, 2012). Five to six experimenters scored roots independently in blind tests as follows: 'white' class, gathered roots showing no detectable blue color; 'blue' class, roots showing weak to poorly intense coloration; and 'dark blue' class, roots displaying a very intense blue color. Results were analyzed statistically using adapted models and tests, as described below.

Real-time quantitative RT-PCR analysis on *M. truncatula* seedlings

Medicago truncatula A17 seedlings were grown in the same volatile system as described above. Emitter plants were inoculated with either the WT Rm1021 strain or its isogenic *smc02178* derivative in at least three independent biological replicates. Receiver root systems (10 per assay) were treated with 10^{-8} M NFs for 16 h, and the regions close to root tips showing pMtENOD11::GUS expression in previous tests, were excised, ground in liquid N_2 using pestle and mortars, and stored on −80°C. A sample of the powder was further ground in 2-ml tubes with a 4 mm steel bead using an MM400 mill. The RNeasy Plant mini kit (Qiagen) and the RNase free DNase set (Qiagen) were used for extraction of total RNA. The quantity and the quality of RNA were evaluated, respectively, with the NanoDrop Lite Spectrophotometer (Thermo Scientific, Madison, WI, USA) and with the Agilent RNA 6000 Nano chips (Agilent Biotechnologies GmbH, Waldbronn, Germany). For the reverse transcription polymerase chain reaction (RT-PCR), the transcriptor reverse transcriptase (Roche) was mixed with polydT primers with RNAsin treatment. Samples were then diluted ×10 in water. For QPCR, the Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) was used. Primers and cDNA were mixed with the Light Cycler 480 SYBR Green master in a total volume of 8 µl. The PCR conditions used were: Denaturation: one cycle 95°C/5 min, Amplification: 45 cycles 95°C/10 s 60°C/20 s 72°C/20 s, Fusion: one cycle 95°C/10 s 65°C/15 s 95°C/continuous, Freeze 1 cycle 40°C/1 min. Two genes were used for normalization: a predicted ubiquitin gene (Medtr3g062450) and a predicted peptidase (Medtr3g065110), established previously as good reference genes displaying stable expression levels (Camps *et al.*, 2015; Larrainzar *et al.*, 2015). Primer sequences for these genes, as well as for *MtENOD11* and *MtNIN*, can be found in Table S1. The mean Cycle Threshold (Ct) of each sample was calculated with six technical replicates, and these values were used to calculate the $2^{-\Delta\Delta C_t}$ values.

Infection assays

For monitoring formation of epidermal infection threads (eITs) in root hairs, the distal roots (14 dpi of the proximal root) in split-root assays or 3-d-old receiver plantlets (volatile assays) were inoculated with 2×10^3 *S. meliloti* bacteria/plant carrying either green/red fluorescent protein (GFP/RFP) reporter fusions (Table S1; Fig. 1b) or a constitutive *hemA-lacZ* fusion (Table S1; Fig. 4d, see later). The number of abortive eITS was quantified 14 dpi under a Zeiss Axioplan microscope equipped with a AxioCam MRc camera (https://www.zeiss.com/microscopy/), as previously described (Tian *et al.*, 2012).

Measurements of ethylene production

A pool of three nodulated roots or shoots of 15 dpi plants grown on Fåhraeus medium were placed in autosampler crimp top vials

(46 × 22.5 mm) with 50 μl water to prevent desiccation, left opened for 30 min to allow stress-induced ethylene to escape and then hermetically closed with silicone/PTFE crimp caps. After incubation at 23°C for 3 h in the dark, 1 ml of gas was extracted from the headspace and injected into a gas chromatograph (Agilent GC7820, Agilent Biotechnologies) as described previously (Trapet *et al.*, 2016). The area of the ethylene peak was measured and compared to an ethylene standard of known concentration. The experiment was performed three times independently with 50–60 plants per bacterial strain.

Statistical methods

For infection assays (Figs 1b, 4d, see later), a Wilcoxon test was used. For proMtENOD11:GUS expression data analysis (Figs 4c, 5b,c, see later), adapted models and tests were used. Briefly, the distribution of gene expression into three classes was analyzed under the R environment (https://www.R-project.org/) using BASE, TIDYVERSE (https://CRAN.R-project.org/package=tidyverse) and ORDINAL (https://cran.r-project.org/web/packages/ordinal/) libraries. Experimental factor relevance was determined via the *P*-values obtained after performing ANOVA tests on Cumulative Link Mixed Models explaining the staining score. Detailed scripts can be obtained from the authors upon request. For ethylene measurements (Fig. 5a, see later), data were analyzed using a Student's *t*-test. In all figures we adopted the following rule to illustrate statistical significance: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; actual *P*-values are given in the text or in figure legends.

Results

Sinorhizobium meliloti modulates the infection sensitivity of distal roots in a split-root assay

We tested whether endosymbiotic bacteria altered the root sensitivity to secondary infection in a split-root assay (Larrainzar *et al.*, 2014). Split-root young plants of *M. sativa* (Fig. 1a) were inoculated on one side (called proximal) of the root system with a RFP-labeled *S. meliloti* WT Rm1021 strain (Table S1) or an isogenic *clr* mutant. After mature nodules had formed (14 dpi), the distal root was inoculated with a WT *S. meliloti* strain labeled with a GFP reporter gene fusion, and the extent of eIT formation on the distal root was quantified 14 dpi after the second inoculation. Strikingly, the number of eITs initiated on the distal root by the WT strain was significantly higher when the proximal root had been inoculated with the *clr* strain than with the WT strain (Fig. 1b). This result pointed to a long-distance modulation of the root infectiveness by WT *S. meliloti* that was not observed with the *clr* mutant.

Nodule counting confirmed that the *S. meliloti clr* mutant does not make more nodules than the WT, in either regular or split-root plant assays, even after 7 wk post-inoculation (wpi) (Fig. S1), thus extending previous observations (Tian *et al.*, 2012). Furthermore, the number of nodules on distal roots was lower than on the proximal roots, indicating that autoregulation of nodulation (AON) was not alleviated upon inoculation with the *clr* mutant (Fig. S1). N_2-fixation and plant growth also were unaffected by mutations inactivating this bacterial cyclic adenosine 3′,5′-monophosphate (cAMP) cascade (Fig. S1).

Endosymbiotic bacteria modulate the sensitivity of roots to NFs

Although the autoregulation of infection (AOI) phenotype was originally described in *M. sativa*, we have found that it also takes place in the model legume *M. truncatula* (Fig. S2). In order to get some molecular insight into the above-predicted change in root susceptibility to infection, we used a *M. truncatula* line carrying a stable proMtENOD11:GUS fusion (Journet *et al.*, 2001). MtENOD11 has for a long time been exploited as a specific, reliable and sensitive marker for NF-response and bacterial infection (Charron *et al.*, 2004; Ding *et al.*, 2008). MtENOD11 encodes a cell-wall associated protein expressed in root hair cells; its expression is under the regulation of two promoter elements triggered at distinct symbiotic stages (Cerri *et al.*, 2012). MtENOD11 expression first takes place at the pre-infection stage in response to NFs via the transcriptional regulators ERN1/ERN2 and, later on, during bacterial infection via NSP1/2 (Cerri *et al.*, 2012). We verified in a direct (i.e. non-split root) inoculation assay that there were more abundant discrete foci of proMtENOD11:GUS expression in plants inoculated with *S. meliloti clr* or *smc02178* mutants than in plants inoculated with WT bacteria, thus indicating that the proMtENOD11:GUS line was sensitive to AOI, as expected (Fig. S2). Furthermore, the AOI phenotype was observed with Rm1021 and RCR2011, two WT strains of *S. meliloti* (Fig. S2; Table S1).

In the split-root assay, the proximal root of proMtENOD11:GUS plants was either noninoculated (control) or inoculated with the WT, the isogenic *smc02178* mutant and the *clr* mutant, and 14 d later, proMtENOD11:GUS expression in the distal root was visualized after overnight incubation of excised roots with or without purified *S. meliloti* NFs (10^{-8} M). No proMtENOD11:GUS expression was detected in the absence of NFs.

Inoculation of the proximal root by WT *S. meliloti* had a global silencing effect on NF-induced pMtENOD11 expression in the distal root, as compared to noninoculated plants or plants inoculated with either the *clr* or *smc02178* bacterial mutant (Fig. 2). The proportion of distal roots that displayed detectable GUS expression following NF-treatment was lower upon inoculation of the proximal root with WT bacteria than with the bacterial mutants; it also was lower than in noninoculated roots (Fig. S3). Reduction of proMtENOD11:GUS expression was not observed upon inoculation of the proximal root with a non-nodulating *nodABC* bacterial mutant, thus indicating that nodulation of the proximal root was indeed required for the AOI phenotype (Fig. S4).

Altogether these data indicate that WT endosymbiotic bacteria decrease root infectiveness, most probably by decreasing the root sensitivity to NFs. This control does not operate in plants inoculated with *clr* or *smc02178 S. meliloti* mutants.

AOI is genetically distinct from AON

Systemic AON in *M. truncatula* is mediated by a LRR-RLK, called SUNN (Sagan *et al.*, 1995; Penmetsa *et al.*, 2003; Schnabel

Fig. 2 Autoregulation of infection (AOI) modulates Mt*ENOD11* expression in response to Nod factors (NFs). Split-root assay of proMtENOD11:GUS expression in response to NF addition (10^{-8} M). Distal roots were cut and incubated overnight in NFs before a 4-h coloration. proMtENOD11:GUS expression was monitored in the distal roots 14 d after the proximal root was noninoculated (ni) or inoculated with *Sinorhizobium meliloti* Rm1021 (WT), *smc02178* or *clr* mutant, as indicated. GUS, β-glucuronidase.

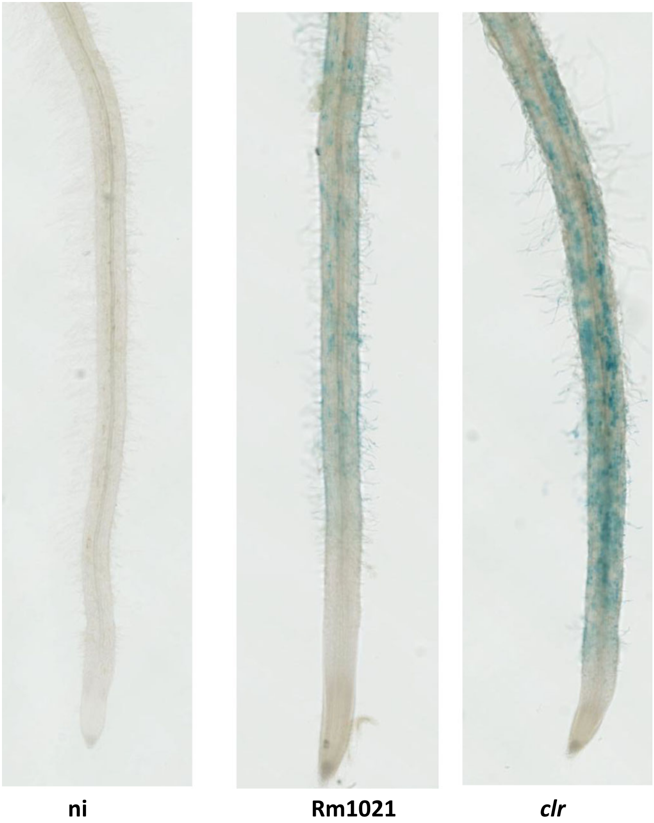


Fig. 3 *MtSUNN* does not mediate autoregulation of infection (AOI). Response of *Medicago truncatula sunn-2 proMt*ENOD11:GUS distal roots ($n = 5$) to purified Nod factors (NFs) (10^{-8} M) after the proximal roots were noninoculated (ni), or inoculated either with *Sinorhizobium meliloti* Rm1021 (wild-type, WT) or with a *clr* mutant. GUS, β-glucuronidase.

et al., 2005) which acts in the shoots as a receptor for mobile modified CLE peptides synthesized in nodules (Mortier *et al.*, 2012; Okamoto *et al.*, 2013; Suzaki *et al.*, 2015). The *Medicago* SUNN-2 *(TR122)* mutant is defective for AON, and displays a *c.* 10-fold higher number of nodules on roots (Sagan *et al.*, 1995; Penmetsa *et al.*, 2003; Schnabel *et al.*, 2005). In order to test whether systemic AOI required SUNN, we probed proMtENOD11:GUS activation by purified NFs in a *M. truncatula* SUNN-2 genetic background (Schnabel *et al.*, 2005). proMtENOD11:GUS activation by NFs in the distal root when the proximal root was noninoculated was significantly weaker in the *SUNN*-2 background than in the WT A17 background, suggesting a positive role of SUNN on MtENOD11 activation by pure NFs (Fig. 3). This observation is consistent with the recently described positive role of *sunn* in nodulation (Saha & DasGupta, 2015). Expression of MtENOD11 in response to NFs was consistently weaker after inoculation of the proximal root with the WT strain than after inoculation with the *clr* mutant. Hence, AOI operates in a *SUNN* background. Because nodulation appears to be unaffected in the *clr* and *smc02178* mutants (Fig. S1), this result indicates that AOI is genetically distinct of AON.

A volatile compound mediates AOI

The split-root assay results described above suggested that AOI was mediated by a mobile signal migrating from the proximal to the distal root either via the plant vascular system or as a volatile compound. We tested the second possibility by growing separate 'emitter' and 'receiver' plants inside the same square dish in such a way that their root systems were isolated from each other (Fig. 4a). In this set-up, we observed that the genotype of bacteria inoculated on the emitter plant influenced proMtENOD11:GUS expression in the receiver plant. Specifically, we observed that proMtENOD11:GUS expression in the receiver plant was significantly stronger when the emitter plant was inoculated with the *smc02178* bacterial mutant than when it was inoculated with WT bacteria (Fig. 4b,c). This was validated statistically by scoring the distribution of proMtENOD11:GUS expression intensity in three different classes and then testing significant differences in the score distribution among treatments, as described in the Materials and Methods section. MtENOD11:GUS expression data were complemented by quantitative (q) RT-PCR measurements on *MtENOD11* and the major, NF-dependent, symbiotic regulator *MtNIN*. Expression measurements on receiver roots following NF treatment (10^{-8} M, 16 h) showed a statistically significant higher gene expression of both genes when the emitter roots were inoculated with the *S. meliloti smc02178* mutant as compared to the Rm1021 WT strain (Fig. S5).

In order to ensure that the differential expression of MtENOD11 and MtNIN expression indeed reflected a difference

in root susceptibility to bacterial infection, we assessed using the same experimental design the impact of WT or mutant inoculation of the emitter plant on infection of the receiver plant by WT *S. meliloti*. We confirmed that inoculation of the emitter root with the WT or *clr S. meliloti* mutant differentially affected the distal root sensitivity to secondary infection by WT *S. meliloti* (Fig. 4d).

As, in these assay conditions, plants were unlikely to communicate via the culture agar medium but shared the same headspace, we hypothesized that a volatile compound emitted under the control of the bacterial symbiont affected root susceptibility to secondary infection events. It is noteworthy that the hyperinfection phenotype of the *clr* mutant was originally described on plants grown in open glass tubes on a slant of Fahreus solid medium (Tian *et al.*, 2012). Here we have confirmed the hyperinfection phenotype on plants grown in open tubes filled with a sepiolite substrate (Fig. S6), thus confirming that the AOI phenotype is not an artifact resulting from plants being grown in closed plates, and it is not dependent on a certain type of growth medium.

AOI involves ethylene

Ethylene gas is well-known for its negative effect on primary infection, making it an obvious candidate as the volatile compound mediating AOI (Guinel, 2015; Liu *et al.*, 2018).

We measured ethylene production by roots nodulated by WT bacteria or by the AOI-defective *smc02178* mutant. WT-nodulated roots produced significantly more ethylene (*c.* 30% more) than mutant-nodulated roots (Fig. 5a). In contrast, no statistically significant difference in ethylene production was observed for the corresponding shoots (Fig. 5a). Hence, there was a direct correlation between ethylene production and the AOI phenotype.

In order to further evaluate the implication of ethylene in AOI, we added aminoethoxyvinylglycine (AVG), a widely used inhibitor of ethylene biosynthesis, to the emitter plant culture medium and tested its impact on NF-dependent proMtENOD11:GUS expression in the distal root. We found that addition of AVG to the emitter plant compartment inoculated with the WT Rm1021 strain very significantly ($P=4.5\ e^{-5}$) enhanced MtENOD11 expression in the distal root, whereas AVG had no effect ($P=0.31$) when the emitter root was inoculated with the *smc02178* AOI-mutant (Fig. 5b). In other words, AVG selectively modified the impact of WT *S. meliloti* on MtENOD11:GUS expression, consistently with ethylene implication in AOI.

In *M. truncatula,* the *sickle* gene encodes the ortholog of the *Arabidopsis* EIN2 ethylene signaling protein. Accordingly a Mt*sickle* mutant is strongly affected in ethylene sensing (Penmetsa *et al.*, 2008). As direct AOI assessment (eIT counting) on Mt*sickle* roots was technically difficult, we used a

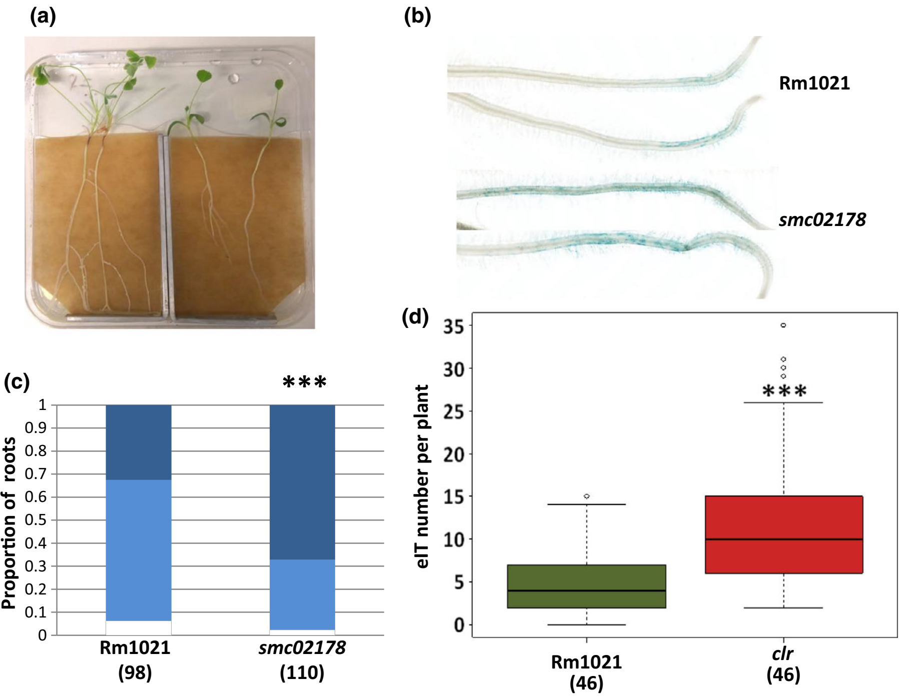


Fig. 4 A volatile compound mediates autoregulation of infection (AOI). (a) Design of the plant/plant assay. Emitter roots are in the left compartment. Receiver roots were incubated overnight in Nod factors (NFs) (10^{-8} M) before GUS coloration. (b) Most representative proMtENOD11:GUS expression in the receiver plant root in response to the inoculation status of the emitter plant. (c) GUS staining quantification. Roots were dispatched in three classes corresponding to no (white), weak (pale blue) and strong (dark blue) GUS staining (see the Materials and Methods section for details). The proportion of roots in each class is shown. ***, *P*-value $1.7\ e^{-11}$. (d) Epidermal infection thread (eIT) quantification on *Medicago sativa* receiver roots inoculated with wild-type (WT) *Sinorhizobium meliloti* (pXLGD4) after the emitter plant was inoculated with either *S. meliloti* WT or the *clr* mutant. The number of observed roots is indicated between brackets. The bar features the ± SE values. ***, $P > 1.0\ e^{-10}$ (Wilcoxon test). GUS, β-glucuronidase.

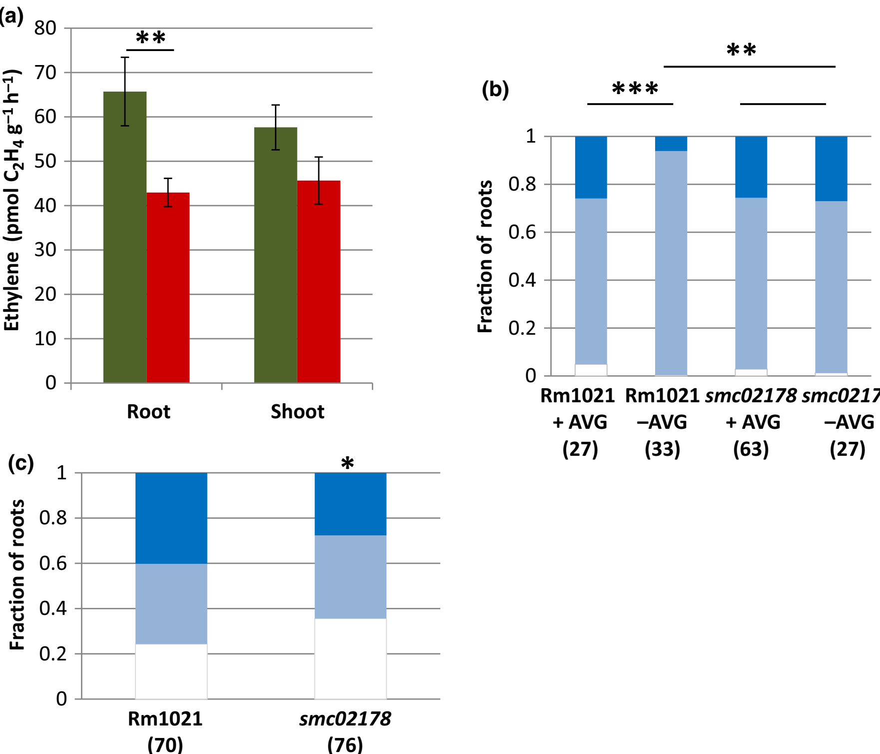


Fig. 5 Ethylene mediates autoregulation of infection (AOI). (a) Ethylene production by excised nodulated roots or shoots of plants inoculated by either the wild-type (WT) strain (green) or the *Sinorhizobium meliloti smc02178* mutant (red). **, $P > 4.0\,e^{-3}$ (Student's *t*-test). The bar features the ± SE values. (b) Effect of aminoethoxyvinylglycine (AVG) (0.5 μM) addition to the growth medium of emitter roots on Nod factor (NF)-dependent proMtENOD11:GUS expression in the distal root. ***, $P > 4.5\,e^{-5}$; **, $P > 4.0\,e^{-3}$. (c) NF-induced proMtENOD11:GUS expression in the *Mtsickle* mutant used as a receiver plant in the volatile assay (as in Fig. 4). *, $P > 0.02$. The number of tested roots is indicated between parentheses. GUS, β-glucuronidase.

Mt*sickle*proMtENOD11:GUS receiver plant (Ding *et al.*, 2008) in the volatile assay described above. We observed that the Mt*skl* mutation strongly attenuated the differential impact of the *S. meliloti* wt and *smc02178* mutant strain on NF-dependent proMtENOD11:GUS expression (Fig. 5c). However, a small difference may persist at a low significance level ($P = 0.02$), the origin of which is unknown.

Altogether these three lines of evidence indicate that ethylene is the likely volatile compound mediating AOI and that the Mt*ein2/skl* gene is involved in ethylene sensing in AOI.

Because ethylene could, in principle, originate from either the plant or the (WT) bacterial symbiont, we assessed ethylene production by bacteria carrying the pGMI50127 plasmid (Table S1) that constitutively expresses the cAMP signaling cascade *ex planta,* hence mimicking symbiotic conditions (Tian *et al.*, 2012). We observed a low level of ethylene production by WT *S. meliloti* that did not depend on the pGMI50127 plasmid (Fig. S7). Therefore, ethylene production in AOI is most likely of plant origin.

Discussion

Nodule and epidermal root hair infection thread (eIT) formation are tightly connected processes under positive and negative control by the plant. Nodulation is negatively controlled at early and late stages of the symbiotic interaction. The later process, known as autoregulation of nodulation (AON), involves plant CLE peptides synthesized in differentiated nodules. Formation of eITs is negatively regulated by ethylene at early stages (6–48 h) of the symbiotic interaction (Larrainzar *et al.*, 2015) under the control of nodulation (Nod) factors (NFs) synthesized by infecting bacteria. Here, we provide evidence for a later negative control of eIT formation, called secondary infection, taking place on nodulated plants between 1 and 2 wk post-inoculation (Fig. 6). In line with previous work (Tian *et al.*, 2012), we have shown here that endosymbiotic bacteria modulate the root susceptibility to secondary infection events by rhizospheric bacteria and we have shown the implication of ethylene in this process. We coined this new process autoregulation of infection (AOI) to underline its parallel with AON.

We have shown that AON and AOI are indeed independent processes. AOI does not require SUNN (the master regulator of AON in legumes), does not impact nodule formation, and is under both plant and bacterial genetic control. Furthermore, bacterial mutants defective for AOI are unaffected for primary infection thus indicating that AOI also is distinct from the long-known control of primary infection mediated by ethylene (Penmetsa & Cook, 1997). AOI is thus a new component of the complex regulatory network that controls the *Medicago*–*Sinorhizobium meliloti* symbiosis. The *clr* and *smc02178* genes are widespread in the *Sinorhizobium* genus, including *S. medicae* and *S. fredii* NGR234 and USDA257 that nodulate a large variety of legumes (e.g. soybeans, *Acacia* and *Vigna*) forming either indeterminate or determinate type of nodules. Conversely, AOI-marker genes such as *clr* and *smc02178* are not found in other rhizobium genera, for example *Rhizobium*, *Mesorhizobium* or *Bradyrhizobium* (J. Batut, unpublished). AOI is thus a genus-specific trait and is unrelated to the nodule ontogeny.

Initiation of eIT is specifically targeted by AOI in agreement with previous evidence that eIT and cIT formation are under

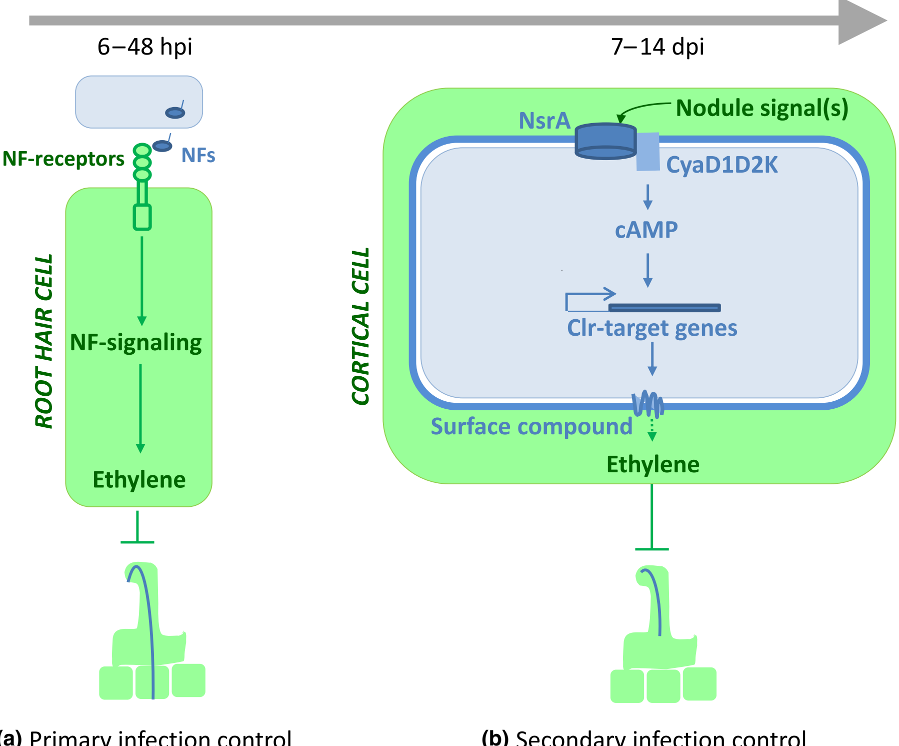


Fig. 6 Model for the control of primary and secondary infection by ethylene in the *Sinorhizobium meliloti*–*Medicago* symbiosis. (a) Hours after bacterial inoculation (hpi), Nod factor (NF) produced by rhizospheric/infecting bacteria trigger both symbiotic signaling mechanisms leading to nodulation and ethylene production that limits the number of successful (i.e. extended) infection events in root hairs (after (Penmetsa & Cook, 1997; Larrainzar *et al.*, 2015). (b) Autoregulation of infection (AOI) control of secondary infection. One to two weeks post-inoculation, endosymbiotic bacteria/bacteroids in nodules sense plant signals via the outer membrane receptor NsrA. NsrA triggers cyclic adenosine 3′,5′-monophosphate (cAMP) production by three inner membrane–associated adenylate cyclases (CyaD1, CyaD2, CyaK), thus allowing expression of Clr-target genes (including *smc02178*). A Clr-dependent compound, possibly a surface polysaccharide, then triggers ethylene production by the plant by a so-far unknown mechanism. Ethylene, in turn, limits epidermal infection thread (eIT) formation. Bacterial features are colored blue and plant features green. For clarity the peribacteroid membrane surrounding bacteroids was omitted.

different genetic control (Miri *et al.*, 2016) and are mediated by cell-autonomous mechanisms (Rival *et al.*, 2013). Altogether these findings indicate that IT formation is a highly controlled process to which different mechanisms contribute.

We have shown that AOI is mediated by a volatile compound that we identified as being ethylene, based on direct ethylene measurements of nodulated roots, the inhibitory effect of aminoethoxyvinylglycine on AOI and the substantial AOI suppression in a *Mtskl* mutant defective in ethylene signal transduction (Fig. 5). Available data suggest that AOI-associated ethylene is of plant origin although this remains to be confirmed. Altogether, this is evidence that ethylene negatively controls eIT formation at two different stages of the symbiotic interaction. Ethylene synthesis, induced in root hair cells under the control of NFs as early as 6 h after bacterial inoculation (Larrainzar *et al.*, 2015), negatively controls primary infection by controlling the number of successful infection events (Penmetsa & Cook, 1997). We have shown here evidence for a second burst in ethylene biosynthesis at a later stage of the symbiotic interaction, 1 or 2 wk after bacterial inoculation, once nodules are formed and infected. This ethylene burst is induced by endosymbiotic bacteria in a process involving a cyclic adenosine 3′,5′-monophosphate (cAMP) regulatory cascade and controls secondary infection at the level of eIT formation (Fig. 6). Our data thus provide further evidence that ethylene acts at different developmental stages during symbiosis (Larrainzar *et al.*, 2015), and suggest that primary and secondary infection can be controlled by ethylene at different stages, eIT persistence and eIT formation, respectively.

A role for endosymbiotic bacteria in fine-tuning the symbiotic interaction already has been proposed. It was recently shown that endosymbiotic *Mesorhizobium loti* control nodule number by synthesizing the hormone gibberellin in nodules (Tatsukami & Ueda, 2016). Together, the work on *M. loti* and the present work emphasize the thus-far overlooked role of endosymbiotic bacteria in fine-tuning the symbiotic interaction, controlling nodule number in *M. loti* and secondary eIT formation in *S. meliloti*. Interestingly, the gibberellin biosynthetic pathway does not exist in *S. meliloti* and in other rhizobia eliciting indeterminate-type nodules (Tatsukami & Ueda, 2016), whereas the *cyaD1D2K-clr-smc02178* cAMP-signaling cascade does not exist outside the *Sinorhizobium* genus (J. Batut, unpublished). This suggests that the capacity of rhizobial endosymbionts to modulate nodule formation or root infectiveness was selected by evolution and that different rhizobia have achieved different ways to do so.

Based on proMtENOD11:GUS expression, we have shown that endosymbiotic wild-type bacteria decrease the root response to bacterial NFs, which likely accounts for the decrease of root infectiveness to bacteria. This is fully consistent with the known role of ethylene in decreasing NF signaling and bacterial infection (Oldroyd *et al.*, 2001; Penmetsa *et al.*, 2008). Elucidation of the molecular mechanisms underlying AOI now requires two main issues to be addressed. First, the plant signals that trigger adenylate cyclase activity in the endosymbiotic bacteria (Tian *et al.*, 2012; Garnerone *et al.*, 2018) remain to be identified. A candidate receptor protein for these signals is NsrA, a recently characterized porin-like outer membrane protein (Garnerone *et al.*, 2018). Second, the pathway linking the activation of the bacterial cAMP-cascade in endosymbiotic bacteria to ethylene production in nodulated roots also needs to be deciphered. No cAMP-dependent production of ethylene could be evidenced in free-living bacteria (Fig. S7), suggesting the ethylene is produced by the plant. This would be consistent with the recent detection of *M. truncatula* ethylene biosynthesis gene expression in nodules (Larrainzar *et al.*, 2015). It has been established that ethylene production can be stimulated in plants by pathogen-associated molecular patterns (PAMPs) (Felix *et al.*, 1999; Lopez-Gomez *et al.*, 2012; Nascimento *et al.*, 2018). Along this line, two independent analyses of the Clr-dependent transcriptome of *S. meliloti* suggested the implication of Clr-target genes in the formation of an unknown bacterial surface polysaccharide (Krol *et al.*, 2016; Zou *et al.*, 2017). Our current working hypothesis is that the modification of the surface of the bacteroids – and possibly of the endosymbiotic bacteria before they are liberated from ITs – under Clr control may be perceived by a plant receptor in symbiosomes, ending up in a small burst of ethylene production in the nodule and/or the adjacent root by a so-far unknown mechanism.

Under standard laboratory conditions, inactivation of AOI had no detectable impact on nodulation or plant growth (Fig. S1). It is, however, possible that the amplitude and extent of AOI may vary according to abiotic or biotic conditions (e.g. different rhizobium strain/plant ecotype combinations). Given the implication of ethylene in AOI and the many roles of ethylene on plant health and growth (Gresshoff *et al.*, 2009; Nascimento *et al.*, 2018) it also is possible that AOI serves additional function(s) beyond controlling secondary eIT formation. Because ethylene is known to modify plant defence (Guinel, 2015; Cao *et al.*, 2017; Berrabah *et al.*, 2018; Nascimento *et al.*, 2018), AOI may contribute to shaping the microbiome of the nodulated plant.

Acknowledgements

We are grateful to our anonymous reviewers for their comments and suggestions and their help in editing the manuscript. We thank D. Capela (LIPM) for early assistance with ethylene measurements, E.P. Journet (LIPM, AGIR) for the gift of *sunn-2*proMtENOD11:GUS and sklproMtENOD11:GUS seeds, D. Barker (LIPM) for proMtENOD11:GUS seeds, and the Florimond-Desprez company (Cappelle en Pévèle, France) for the generous gift of *Medicago sativa* seeds, L. Kamel (LIPM) for the design of primers for the qRT-PCR experiments in the frame of the ANR 'Nice Crops' project (ANR-14-CE18-0008-02) and the FR AIB Imagery platform for help and advice. FS was supported by the Agreenskills Post-doctoral Fellowship Programme and the ANR AOI (ANR-15-CE20-0004-01). MW was supported financially by the ANR AOI (ANR-15-CE20-0004-01). LZ was supported by a PhD scholarship of the China Scholarship Council. This work was supported by the ANR AOI (ANR-15-CE20-0004-01) and the 'Laboratoire d'Excellence (LABEX)' TULIP (ANR-10-LABX-41). On behalf of the authors, the corresponding author indicates that no financial or nonfinancial competing interests exist.

Author contributions

FS, CM-B, CG, JB and AMG designed the experiments; FS, MW, LZ, FM, CG-V,VP, CC and AMG performed the experiments; FS, MW, DR, FM, CM-B, CG, JB and AMG analyzed the data; and FS, CG, JB wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 AOI has no impact on nodule number and AON.

Fig. S2 Evidence for AOI in the model legume *M. truncatula* A17 and the RCR21011 *S. meliloti* strain.

Fig. S3 AOI modulates Mt*ENOD11* expression in response to Nod factors in a split-root asssay.

Fig. S4 Nodulation is required for AOI.

Fig. S5 qRT-PCR measurements of NF-dependent *MtENOD11* and *MtNIN* gene expression in receiver roots.

Fig. S6 Hyperinfection phenotype of the *S. meliloti clr* mutant on *M. sativa* plants grown on sepiolite substrate in open tubes.

Fig. S7 Ethylene production by free-living *S. meliloti* bacteria.

Table S1 Bacterial strains, plasmids and primers used in this study.

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