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Determining the Site of Action of Strigolactones during Nodulation¹

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Strigolactones (SLs) influence the ability of legumes to associate with nitrogen-fixing bacteria. In this study, we determine the precise stage at which SLs influence nodulation. We show that SLs promote infection thread formation, as a null SL-deficient pea (*Pisum sativum*) mutant forms significantly fewer infection threads than wild-type plants, and this reduction can be overcome by the application of the synthetic SL GR24. We found no evidence that SLs influence physical events in the plant before or after infection thread formation, since SL-deficient plants displayed a similar ability to induce root hair curling in response to rhizobia or Nod lipochitooligosaccharides (LCOs) and SL-deficient nodules appear to fix nitrogen at a similar rate to those of wild-type plants. In contrast, an SL receptor mutant displayed no decrease in infection thread formation or nodule number, suggesting that SL deficiency may influence the bacterial partner. We found that this influence of SL deficiency was not due to altered flavonoid exudation or the ability of root exudates to stimulate bacterial growth. The influence of SL deficiency on infection thread formation was accompanied by reduced expression of some early nodulation genes. Importantly, SL synthesis is down-regulated by mutations in genes of the Nod LCO signaling pathway, and this requires the downstream transcription factor *NSP2* but not *NIN*. This, together with the fact that the expression of certain SL biosynthesis genes can be elevated in response to rhizobia/Nod LCOs, suggests that Nod LCOs may induce SL biosynthesis. SLs appear to influence nodulation independently of ethylene action, as SL-deficient and ethylene-insensitive double mutant plants display essentially additive phenotypes, and we found no evidence that SLs influence ethylene synthesis or vice versa.

Nodulation results from the intimate relationship of nitrogen (N)-fixing rhizobacteria and leguminous plants. The uptake of rhizobia and the organogenesis of the nodule in which the bacteria are hosted only occur after the exchange of specific chemical signals through the rhizosphere between the rhizobia and plant host. The exudate from roots of the host includes flavonoids that induce the rhizobia to produce signals including specific Nod lipochitooligosaccharides (LCOs; Peters et al., 1986). Following the recognition of these Nod LCOs by receptor-

like kinases, including the Nod LCO receptors NFP and LYK3, there is an induction of oscillations in nucleus-associated calcium levels via the action of DMI2 and DMI1. This is sensed by DMI3, which, along with parallel pathways, influence a range of transcription factors (e.g. *NIN*, *NSP1*, *NSP2*, and *IPD3*) that, in turn, coordinate the expression of nodulation-associated genes such as *ENODs* (for early nodulation; Yano et al., 2008; Venkateshwaran et al., 2013; Singh et al., 2014; Genre and Russo, 2016; Fig. 1). Corresponding physical changes induced by this perception pathway include root hair curling, infection thread formation, and concomitant cell division in the inner cortical and pericycle cells that leads to the formation of the nodule meristem and, ultimately, colonized nodules (Fig. 1). In some species, the meristem is maintained in the mature nodule (indeterminant nodulators), while in other species, the nodule meristem is lost at maturity (determinant nodulators; Ferguson et al., 2010).

Plant hormones play critical roles in a number of these processes. One of these is strigolactones (SLs), a group of plant hormones synthesized from a carotenoid precursor via the sequential action of enzymes including *D27*, *CCD7*, *CCD8*, *MAX1*, and *LBO* and perceived via a complex of the *D14* receptor, *MAX2* F-box protein, and *D53* (Smith and Li, 2014; Brewer et al., 2016). SLs

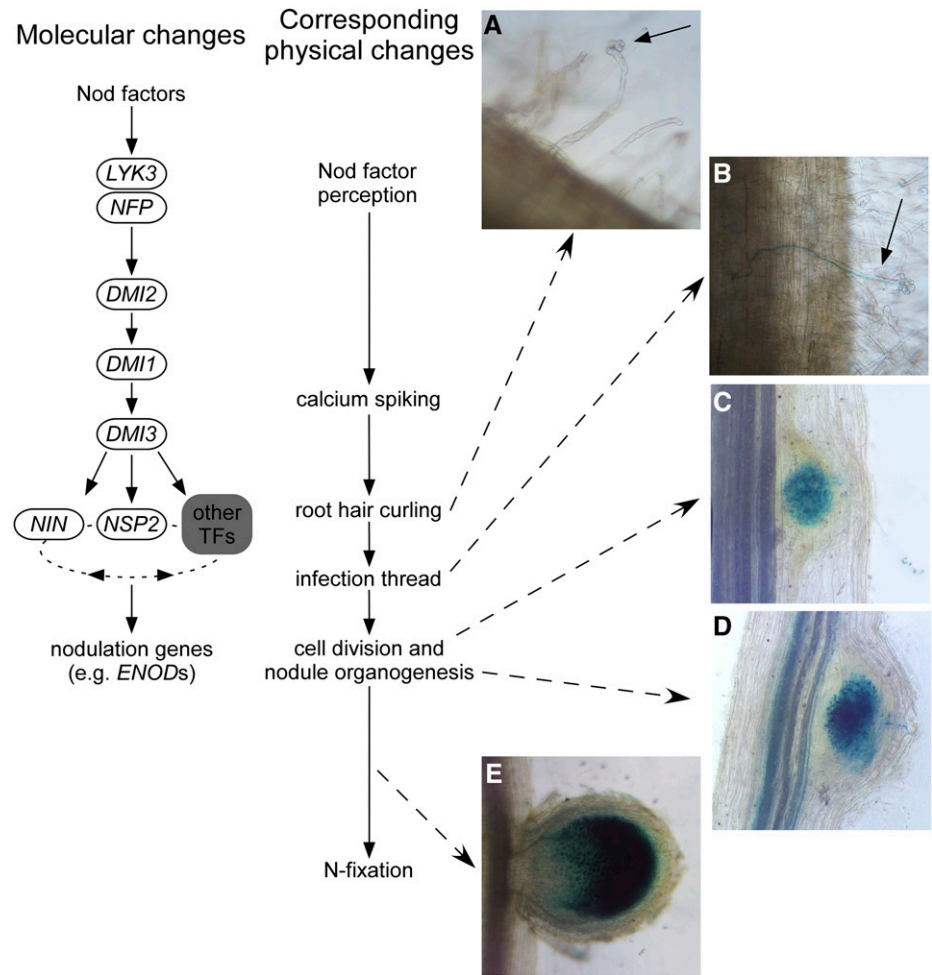
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E.L.M., C.H., and E.F. performed the experiments; N.W.D. devised the ethylene/acetylene and flavonoid analyses; S.F., E.S., and S.C. synthesized the Nod LCO; E.L.M. and E.F. analyzed the data; E.F. conceived the project and wrote the article with contributions from J.B.R. and E.L.M.

Figure 1. Scheme of the physical and molecular changes during nodule development in pea, including the proposed positions of genes in the Nod LCO signaling pathway that influence a complex nonlinear network of transcription factors (TFs). A, Root hair curling. B, Infection thread formation. C, Developing nodule not visible to naked eye. D, Maturing nodules. E, Young mature nodule. All photographs show pea roots colonized with blue-stained *lacZ*-labeled rhizobia.



play important roles in shoot and root development and during the interaction of plants with microorganisms (Foo and Reid, 2013; Smith and Li, 2014; López-Ráez et al., 2017). For example, SLs are exuded from plant roots and act as a rhizosphere signal to promote the symbioses of plants with phosphorus-acquiring arbuscular mycorrhizal (AM) fungi by promoting spore germination and hyphal branching (Akiyama et al., 2005). This AM signaling pathway shares functional elements with nodulation, including elements of the Nod LCO perception pathway outlined above (Venkateshwaran et al., 2013). SLs appear to be a common signal in AM and nodulation, as reports in several species, using both genetic and application approaches, indicate that SLs also exert a primarily positive role during nodulation (Soto et al., 2010; Foo and Davies, 2011; Liu et al., 2013; De Cuyper et al., 2015).

The specific stage at which SLs influence the interaction with rhizobia is not yet clear. A significant reduction in SL biosynthesis, due to lesions in the *CCD7* or *CCD8* gene, leads to significant reductions in the number of nodules in species that form both indeterminate (pea [*Pisum sativum*]) and determinate (*Lotus japonicus*) nodules (Foo and

Davies, 2011; Liu et al., 2013). Consistent with this positive role of SLs in nodulation is the observation that nodule number is elevated by the application of the synthetic SL, GR24, in pea and *Medicago sativa* (Soto et al., 2010; Foo and Davies, 2011). In contrast, GR24 application has been reported to lead to a small decrease in nodule number in *Medicago truncatula* (De Cuyper et al., 2015), and *D27 RNAi* lines in this species did not have altered nodulation (van Zeijl et al., 2015). However, gene expression studies in *M. truncatula* from two independent groups show that the expression of several key SL biosynthesis genes (*D27*, *CCD7*, and *CCD8*) is elevated following challenge with rhizobia (Breakspear et al., 2014; van Zeijl et al., 2015). In the case of *CCD8*, promoter fusion studies revealed that this expression occurred specifically in infected root hairs and developing nodule primordia (Breakspear et al., 2014), and in mature nodules, the expression of *D27*, *CCD7*, and *CCD8* was restricted to the meristem and distal infection zone (van Zeijl et al., 2015). Overall, these mutant and gene expression studies suggest that SLs may act at several stages to promote rhizobial infection and nodule formation.

As outlined above, one of the first steps in the interaction of legumes and N-fixing rhizobia is signal exchange through the rhizosphere. However, although they are well known for their role as a rhizosphere signal in AM symbioses, there is no direct evidence that SLs influence rhizobia in an analogous way. Application of the synthetic SL GR24 directly to rhizobia does not promote bacterial growth or signaling (Moscatiello et al., 2010; Soto et al., 2010). The recent report that GR24 induces increased swarming behavior in some rhizobia is intriguing (Peláez-Vico et al., 2016). However, there is currently no known connection between swarming and nodulation, and indeed, plant root exudates from nonlegumes and some, but not all, legume hosts were shown to induce swarming behavior (Tambalo et al., 2014). One possibility yet to be explored is that SL status may influence other root exudates, including flavonoids.

Outputs of the Nod LCO signaling cascade include changes to hormone biosynthesis and/or perception. There is some indication that the Nod LCO signaling pathway may influence SL biosynthesis. *M. truncatula* mutants with lesions in *NSP1* and/or *NSP2* (transcription factors downstream of Nod LCO signaling) have reduced levels of SLs in the absence of rhizobia, and this is reflected in reduced expression of the SL biosynthesis gene *D27* (Liu et al., 2011). The gene expression studies outlined above by van Zeijl et al. (2015) found that application of Nod LCOs induced the expression of several SL biosynthesis genes, and this was disrupted in plant mutants with lesions in the Nod LCO response pathway (*dmi1*, *dmi2*, *dmi3*, *nsp1*, and *nsp2*), although SL levels were not quantified in that study. Like many developmental processes, hormones often interact to control nodulation. Ethylene is known to be a negative regulator of nodulation (Penmetsa and Cook, 1997; Guinel, 2015; Foo et al., 2016c). Recent work in *M. truncatula* investigated the potential interaction between ethylene and SL during nodulation and found that ethylene-insensitive *skl/ein2* mutants did not display altered nodulation when treated with GR24 (De Cuyper et al., 2015). This suggests that SLs may act upstream of ethylene action during nodulation, but further studies are required to fully test this hypothesis.

In this study, we pinpoint the stage at which SLs appear to influence nodulation and examine interactions between SLs, the Nod LCO signaling pathway, and ethylene during nodulation in pea. Studies with severe SL biosynthesis *ccd8* mutants and application studies with the synthetic SL GR24 suggest that SLs act specifically to promote infection thread formation. We found no evidence that SL deficiency influences physical events before or after this crucial stage, including flavonoid production, root hair curling, or nitrogen fixation in mature nodules. We also report studies with an SL receptor mutant, *d14*, which suggest that CCD8 products may promote infection thread formation by influencing the bacterial partner rather than via the SL response system in the plant. This influence of SL deficiency on infection thread formation is accompanied

by a reduction in the expression of certain ENOD genes that are well-known markers of the early events during the formation of symbioses. SL levels are up-regulated by elements of the Nod LCO signaling pathway, and SLs appear to influence nodulation largely independently of ethylene action.

RESULTS

Stages of Nodulation in an SL-Deficient Mutant

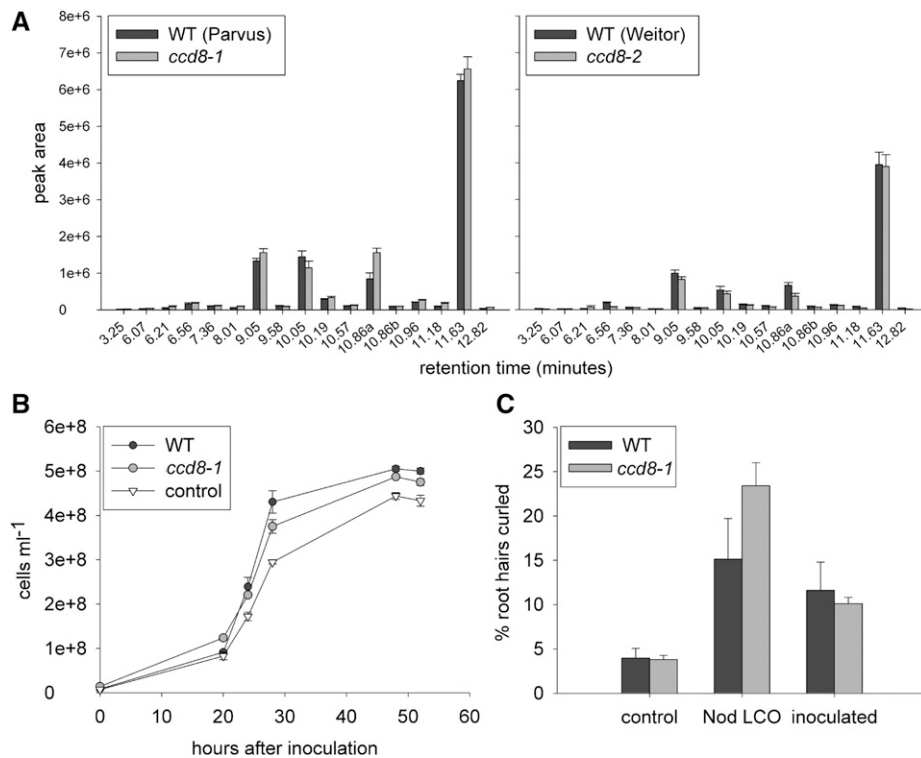
SLs have been reported to have a generally positive role in promoting nodule formation (Foo and Davies, 2011), although it is not clear at what stage SLs influence the interaction between legumes and rhizobia. We used null SL-deficient *ccd8* mutants, disrupted in a key enzyme of the SL biosynthesis pathway (Sorefan et al., 2003; Gomez-Roldan et al., 2008), in a series of studies to pinpoint the stage at which nodulation was disrupted in this mutant.

One of the earliest events in the interaction between a legume host and rhizobia is flavonoid exudation by the host root. This, in turn, induces the production of Nod LCOs from rhizobia. We examined the flavonoid profile of several alleles of *ccd8* SL-deficient mutants and compared them with their wild-type progenitor lines. We found no difference in the identity or significant reduction in the levels of flavonoids between two null SL-deficient mutants, *ccd8-1* and *ccd8-2*, and their respective progenitor lines when analyzed by ANOVA (Fig. 2A). We also examined the influence of root exudates from wild-type and SL-deficient *ccd8-1* plants on bacterial growth in vitro and found that both had a positive influence on bacterial growth and that there was no significant difference overall between the genotypes (Fig. 2B). These studies suggest that SLs do not influence nodulation by influencing the production of flavonoids or rhizobial population growth.

In many legume species, including pea, root hairs are the site of rhizobial infection, and one of the first physical changes during nodulation is root hair curling. Previous studies have reported that pea *ccd8* mutants do not display altered root hair number or length (Foo and Davies, 2011). We found that there also was no significant difference in the number of curled root hairs produced by the SL-deficient mutant *ccd8-1* compared with wild-type plants in response to pure Nod LCO or application of the compatible rhizobia (Fig. 2C).

Following root hair curling, uptake of the rhizobial bacteria occurs through infection thread structures that channel rhizobia into dividing cells that will form the nodule. Using *lacZ*-labeled rhizobia, we were able to investigate the formation of infection threads and developing nodules (nodules not yet visible to the naked eye but visible and stained blue under magnification) in an SL-deficient *ccd8-1* line (Fig. 3, A–D). We found that *ccd8-1* mutants formed significantly fewer infection threads compared with wild-type plants in two independent experiments (Fig. 3A; $P < 0.05$), and a two-way

Figure 2. Influence of the wild type (WT) and SL-deficient *ccd8* mutants on early events in nodulation. A, Flavonoid profile in root exudate of *ccd8-1* and *ccd8-2* mutants and their respective wild types, cv Parvus and cv Weitor ($n = 4$). B, Growth of *Rhizobium leguminosarum* bv *viciae* (RLV248) over time after treatment with wild-type cv Parvus or *ccd8-1* root exudates compared with a solvent control ($n = 4$). C, Percentage of root hairs curled in wild-type cv Parvus and *ccd8-1* mutant plants 5 d after treatment with 0.1 μM Nod LCO or 10% solution of *R. leguminosarum* bv *viciae* culture (inoculated) compared with a solvent control ($n = 4$ –5). Values are means \pm SE, and analysis of each experiment by ANOVA indicated no significant differences between *ccd8* and its wild-type progenitor.



ANOVA across experiments found a significant genotype effect ($P < 0.01$). The infection threads that did form in *ccd8-1* mutants were similar in morphology to those observed in the wild type (Fig. 3B), ranging from immature infection threads to mature infection threads connected to developing nodules. There did not appear to be a reduction in the frequency of infection threads leading to nodules (i.e. a higher abortion rate) in SL-deficient mutants, as the approximate 30% to 40% reduction in infection thread formation in *ccd8-1* mutants was mirrored by a similar significant reduction in the number of developing nodules compared with the wild type (Fig. 3, C and D; $P < 0.05$). Both reductions in infection thread formation and developing nodules in *ccd8-1* are consistent with the 30% to 40% decrease in mature nodules reported previously in *ccd8-1* mutant plants (Foo and Davies, 2011). No other changes were observed in the roots (or root hairs) of *ccd8-1* plants as a consequence of the reduced infection thread formation.

To examine whether the influence of CCD8 products (presumably SLs) on infection thread formation is via the SL receptor D14, we compared infection thread formation in SL-deficient *ccd8* mutant plants with that in *d14* mutant plants (de Saint Germain et al., 2016). As observed above, *ccd8-2* mutants formed significantly fewer infection threads and nodules than wild-type plants (Supplemental Fig. S1, A and B). In contrast, we found no decrease in the number of infection threads or nodules formed on *d14* mutants compared with the wild type, indicating that the D14 SL receptor is not required for the effect of the CCD8 product on nodule development. As

observed previously (Urquhart et al., 2015), both *ccd8-2* and *d14* mutants have somewhat shorter lateral roots than the wild type, consistent with the reported SL deficiency and insensitivity of these mutants (Supplemental Fig. S1C).

To check whether the reduced infection thread formation in *ccd8* plants was due to the SL deficiency in this mutant, the response to applied GR24 was examined in wild-type and *ccd8* plants (Fig. 4). A two-way ANOVA showed a significant effect of GR24 application ($P < 0.01$) and also a significant interaction between genotype and GR24 treatment ($P < 0.01$). As observed previously (Fig. 2A; Supplemental Fig. S1A), infection thread formation was reduced significantly in *ccd8* mutants compared with the wild type. The fact that GR24 significantly increased infection thread formation in *ccd8* mutants (Fig. 4A) provides strong support for the view that the action of CCD8 on infection thread formation is via its known effect on SL biosynthesis. Similarly, a two-way ANOVA also showed a strong treatment effect of GR24 on the total number of nodules ($P < 0.001$) and a significant interaction between treatment and genotype ($P < 0.05$). The alterations seen in infection thread formation across both control and GR24-treated genotypes were reflected in similar changes in total nodule number (Fig. 4B). The approximately 50% reduction in the total number of nodules seen in *ccd8* mutants compared with wild-type plants was fully restored by GR24 treatment, consistent with the previously reported positive effects of SLs on the number of visible nodules seen in pea (Foo and Davies, 2011).

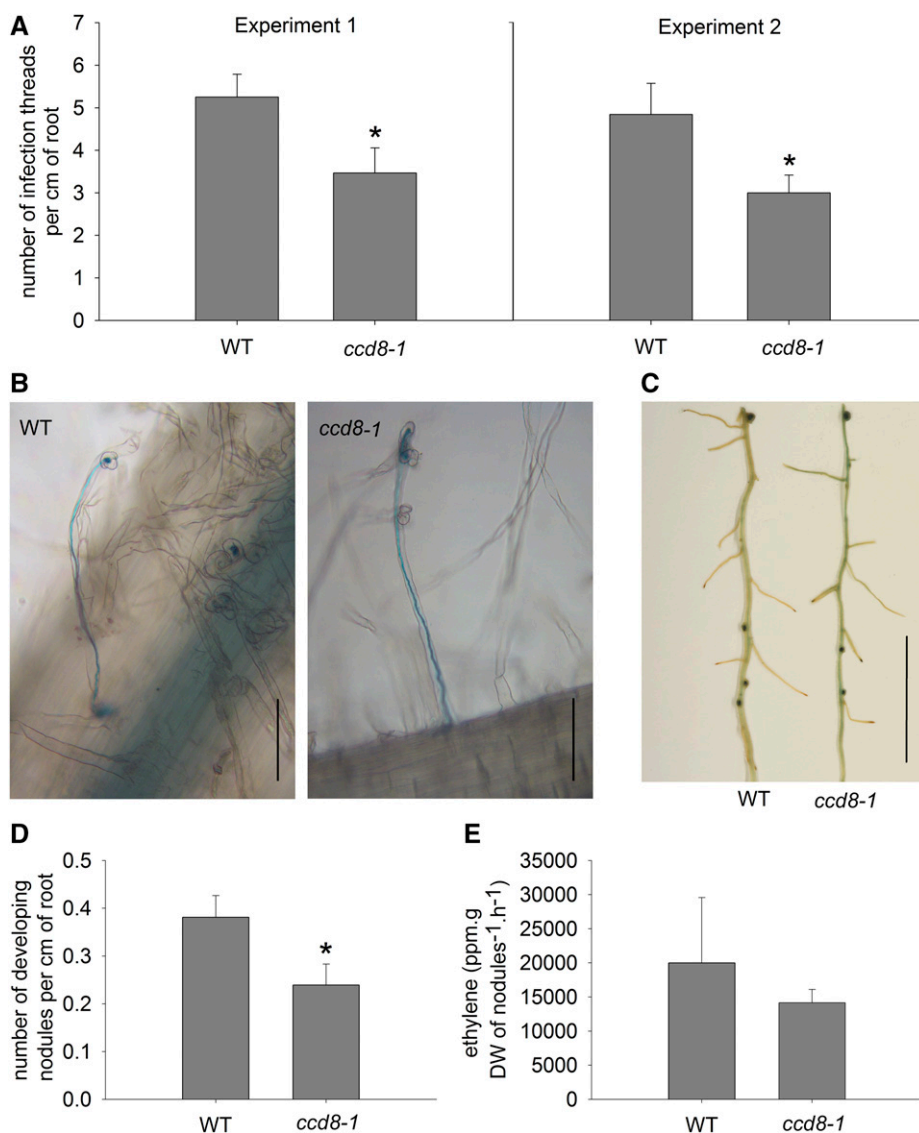


Figure 3. Nodule development in wild-type cv Parvus (WT) and SL-deficient *ccd8-1* plants infected with *lacZ*-labeled *R. leguminosarum* bv *viciae*. A, Number of infection threads per cm of root (four to 10 root segments from $n = 6-11$ plants) in two independent experiments. B, Photograph of stained roots showing infection threads in wild-type and *ccd8-1* plants. Bars = 0.1 mm. C, Photograph of stained root showing nodules in wild-type and *ccd8-1* plants. Bar = 5 mm. D, Number of developing nodules per cm of root (10 root segments from $n = 15$ plants). E, Ethylene evolution per gram nodule dry weight (DW) from the acetylene reductase assay ($n = 4$). Values are means \pm SE, and values statistically different from the wild type are indicated by asterisks: *, $P < 0.05$. In B and C, *R. leguminosarum* is stained blue.

Some legumes, such as *Lupinus* spp., undergo infection not through root hairs but via cracks in the epidermis (Tang et al., 1993; González-Sama et al., 2004). Interestingly, we found that application of the synthetic SL (+)-GR24 did not enhance nodule formation in blue lupin (*Lupinus angustifolius*; Supplemental Fig. S2), while similar doses of GR24 have been shown to alter nodulation in pea, *M. truncatula*, and *M. sativa* (Soto et al., 2010; Foo and Davies, 2011; De Cuyper et al., 2015). The fact that SLs only appear to influence nodule formation in species that use root hair infection is consistent with an important role for SLs during the infection thread stage of infection.

Maturation of the nodule ultimately results in a functional nodule in which rhizobia are able to fix nitrogen. We found that, like other root tissue, mature nodules also contain SLs, although the levels were approximately 4 times lower than in surrounding mature root (the level of fabacyl acetate in nodule tissue was

0.05 ng g⁻¹ fresh weight, compared with 0.19 ng g⁻¹ fresh weight in root tissue). To determine whether SLs influence nodule function, we examined the ability of nodules that do form on SL-deficient mutants to fix nitrogen using the acetylene reductase assay (Fig. 3E). The SL-deficient *ccd8-1* nodules were clearly functional and, for a given mass of nodules, did not have a significantly different acetylene reductase rate from comparable wild-type nodules, indicating that SL-deficient nodules can fix nitrogen.

Gene Expression during Early Nodulation in an SL-Deficient Mutant

During early interactions with rhizobia, the expression of a suite of *ENODs* is induced via the action of the Nod LCO signal transduction pathway. In pea, this includes *ENOD12a*, *ENOD12b*, and *ENOD40*

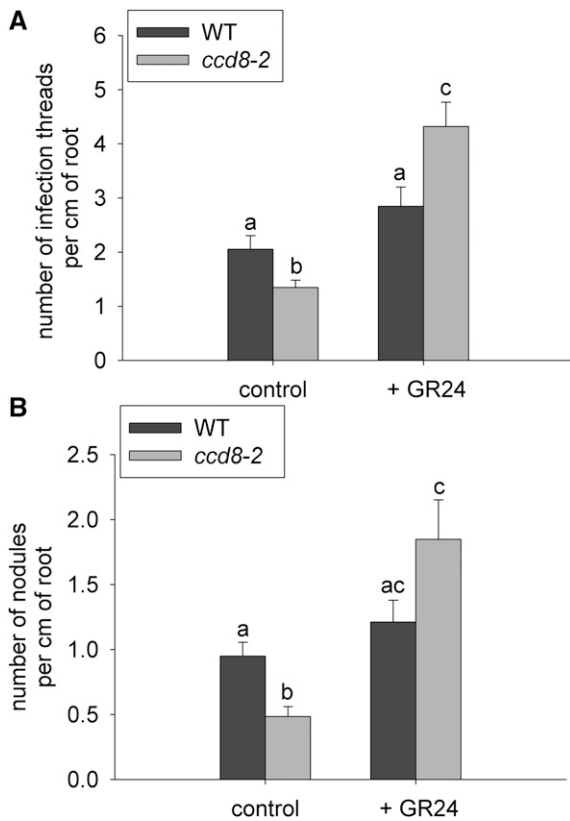


Figure 4. Nodule development in wild type cv Torsdag (WT) and SL-deficient *ccd8-2* plants infected with *lacZ*-labeled *R. leguminosarum* bv *viciae* treated with synthetic SL (+)-GR24 or solvent control. A, Number of infection threads per cm of root. B, Total number of nodules per cm of root. Values are means \pm SE (one to two root segments from $n = 12-13$ plants), and values with different letters are significantly different ($P < 0.05$).

(Govers et al., 1991; Schneider et al., 1999). Induction of these genes by compatible rhizobia is severely impaired, at least at some time points, in mutants disrupted in elements of the Nod LCO signal transduction pathway, such as *dmi2* (Schneider et al., 1999). We found that, in the days following inoculation with rhizobia, the induction of some of these genes was reduced significantly in SL-deficient *ccd8-1* mutant plants compared with wild-type cv Parvus plants (Fig. 5). For example, 4 d after inoculation, the expression of *ENOD12b* was significantly lower in roots of *ccd8-1* plants compared with the wild type (Fig. 5; $P < 0.01$). We also found that, in wild-type plants, the expression of the SL biosynthesis gene *D27* was elevated significantly following challenge with the rhizobia (Supplemental Fig. S3; $P < 0.05$), consistent with similar reports in *M. truncatula* (van Zeijl et al., 2015). Recent reports suggest that SLs may also influence disease development in some systems (López-Ráez et al., 2017), although studies with a pea SL-deficient *ccd8* mutant have not revealed any influence of this mutation on pea disease caused by *Pythium irregulare* or *Fusarium oxysporum* (Blake et al., 2016; Foo et al.,

2016a). To examine if a *ccd8* mutation may influence disease response after challenge with rhizobium, the expression of three key disease marker genes (Supplemental Fig. S4) as well as callose and lignin deposition were examined in *ccd8* and wild-type plants exposed to rhizobia. No trends of consistently elevated or reduced gene expression (Supplemental Fig. S4) or callose or lignin deposition were observed between *ccd8* and wild-type roots.

In addition to inducing nodulation-specific molecular and physical changes, rhizobia (via Nod LCO signaling) also induce changes in root architecture (Oláh et al., 2005). Given that SLs have been implicated in the

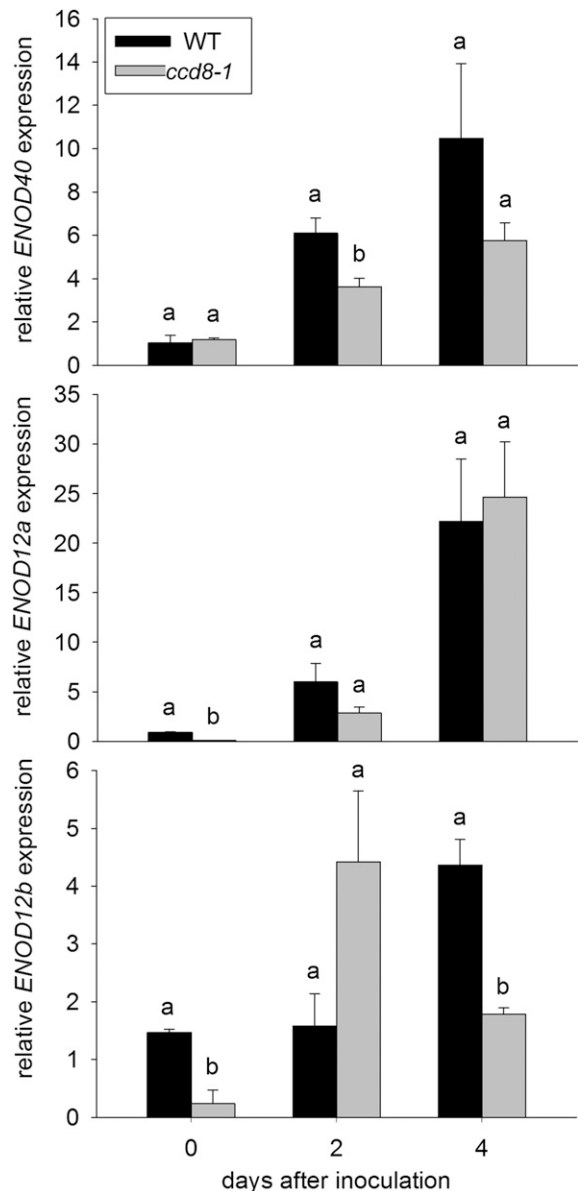


Figure 5. Expression of *ENOD* genes 0, 2, and 4 d following inoculation with *R. leguminosarum* bv *viciae* in wild type (WT) cv Parvus and the SL-deficient *ccd8-1* mutant. Values are means \pm SE ($n = 3$). For days 0, 2 and 4, values with different letters are significantly different ($P < 0.05$).

control of root architecture (Rasmussen et al., 2013), it was interesting to examine whether CCD8 plays a role in modifying root architecture in response to Nod LCO. Two-way ANOVAs showed that Nod LCO treatment significantly affected lateral root number and length (Supplemental Fig. S5; $P < 0.01$). However, there was no significant interaction between genotype and treatment, indicating that *ccd8-1* plants responded to Nod LCO in a similar way to wild-type plants.

Common SYM Mutants Have Impaired SL Biosynthesis

Previous studies in *M. truncatula* have identified SL biosynthesis and/or the expression of SL biosynthesis genes as dependent in part on some elements of the LCO signaling pathway (Liu et al., 2011; Breakspear et al., 2014; van Zeijl et al., 2015), but it is not clear from these studies if this is due to indirect effects of individual genes in this pathway or if the LCO signaling pathway as a whole influences SL biosynthesis. To address this question, we systematically examined SL levels in nonnodulated roots of pea mutants disrupted in Nod LCO receptors (*nfp* and *lyk3*), LCO signaling elements (*dmi1*, *dmi2*, and *dmi3*), and downstream transcription factors (*nsp2* and *nin*). We found that the major canonical SL present in pea tissue, fabacyl acetate, was reduced significantly in mutants disrupted in one Nod LCO receptor (*lyk3*), signaling elements (*dmi1*, *dmi2*, and *dmi3*), and one downstream transcription factor (*nsp2*) compared with their respective wild-type progenitor lines (Fig. 6A; $P < 0.05$ – 0.001). The SLs orobanchol and orobanchyl acetate also were detected in some experiments, and these also were reduced in *dmi3* (Supplemental Fig. S6). In contrast, there was no significant reduction in fabacyl acetate levels in mutants disrupted in the NIN transcription factor (Fig. 6A). This indicates that SL levels are influenced by the Nod LCO signaling pathway but not via the transcription factor encoded by the *NIN* gene. This relatively small

(approximately 2-fold) reduction in SL levels in the roots is not sufficient to significantly promote shoot branching in these lines (data not shown), a phenotype observed in severely SL-deficient lines (Beveridge et al., 1997).

It is interesting that, in some cases, this reduction in SL levels was accompanied by significant reductions in the expression of the SL biosynthesis genes *D27* and *CCD8*, but in many cases, the expression of these genes did not reflect SL level, and for *CCD7*, the expression was elevated in several cases (Fig. 6B). This may be due to the well-established feedback regulation of the SL biosynthesis pathway (Foo et al., 2005; Umehara et al., 2008) and is consistent with other hormones, where the expression of hormone biosynthesis genes often is not an accurate proxy for hormone levels (Symons and Reid, 2008).

SLs Act Largely Independently of Ethylene to Influence Nodulation

It was suggested recently that ethylene may act downstream of SLs during nodulation (De Cuyper et al., 2015), and we used several approaches to examine ethylene-SL interactions during nodulation. We examined whether endogenous SLs influence endogenous ethylene and *visa versa* in pea using the ethylene-insensitive *ein2* mutant (Weller et al., 2015; Foo et al., 2016c) and the SL-deficient *ccd8-1* mutant. We found no indication that the *ein2* mutant significantly altered the expression of the SL biosynthesis genes *CCD7*, *CCD8*, and *D27* or the level of the major SLs produced by pea when compared with wild-type cv Torsdag plants (Fig. 7A). Similarly, we found no significant difference in ethylene production or in the expression of the key ethylene metabolism genes *ACC synthase (ACS1)* or *ACC oxidase (ACOX)* in *ccd8-1* mutants compared with wild-type cv Parvus plants (Fig. 7B). An independent experiment with both SL-deficient *ccd8-1* and *ccd7-3* mutants confirmed that SL

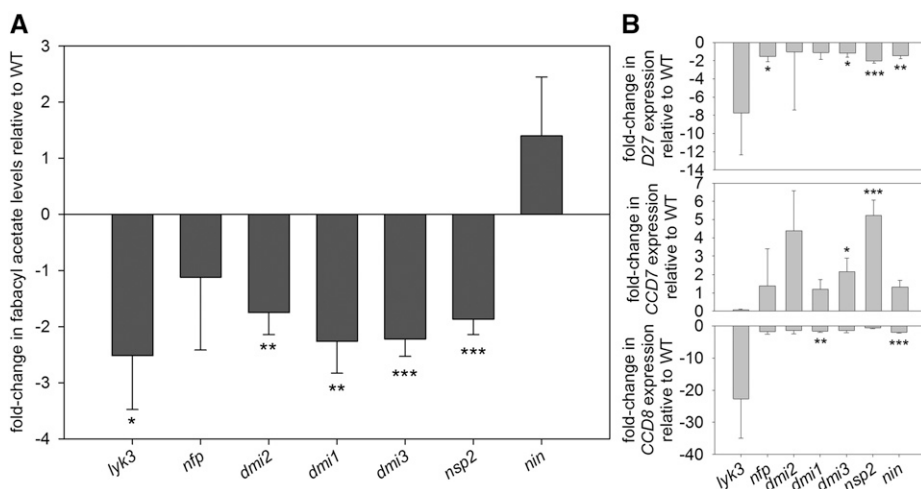
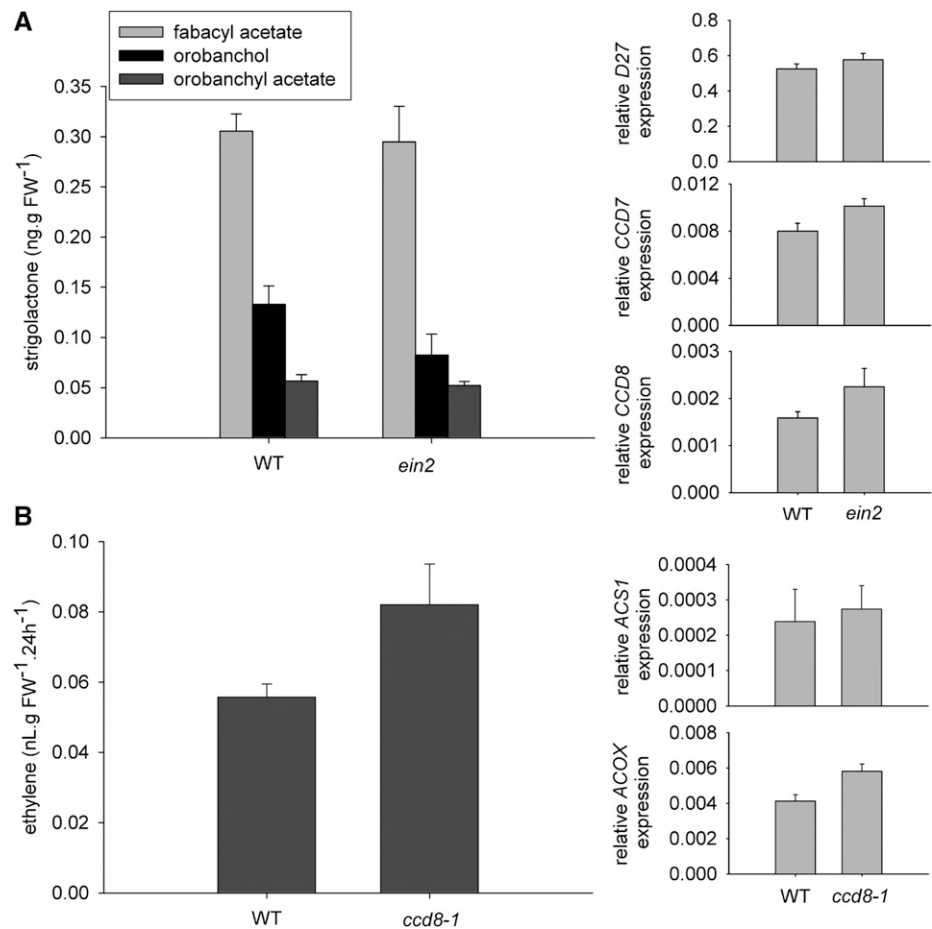


Figure 6. SL levels and expression of SL biosynthesis genes in root tissue of various symbiosis mutants. A, Fold change relative to respective wild types (WT) of the major canonical SL in pea, fabacyl acetate, in root tissue of various pea symbiosis mutants. B, Fold change relative to the respective wild type of the expression of SL biosynthesis genes *CCD7*, *CCD8*, and *D27* in root tissue of pea symbiosis mutants. Values statistically different from the wild type are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Values are means \pm 95% confidence interval ($n = 4$).

Figure 7. SL and ethylene levels and expression of biosynthesis genes in SL-deficient *ccd8-1* or ethylene-insensitive *ein2* plants compared with their respective wild types (WT). A, Levels of three SLs, fabacyl acetate, orobanchol, and orobanchyl acetate, and relative expression of the SL biosynthesis genes *CCD7*, *CCD8*, and *D27* in root tissue of wild-type (cv Torsdag) and *ein2* mutant plants. B, Ethylene evolution from whole plants of the wild type (cv Parvus) and *ccd8-1* and expression of the ethylene metabolism genes *ACS1* and *ACOX* in roots of these plants. Values are means \pm SE ($n = 4$), and analysis of each parameter by Student's *t* test indicated no significant differences between the mutant and the wild type. FW, Fresh weight.



deficiency does not substantially alter ethylene production (data not shown).

During nodulation, SLs do not appear to act downstream of ethylene, as, like wild-type plants, the SL-deficient *ccd8-2* mutant could significantly elevate visible nodule number in response to the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG; Fig. 8A). However, GR24 could not elevate nodulation in the ethylene-insensitive *ein2* mutant even though a small but significant increase occurred in wild-type cv Torsdag plants (Fig. 8A) and *ccd8* mutants (Foo and Davies, 2011). We further investigated the relationship between SL and ethylene during nodulation by examining the nodulation phenotype of a double mutant disrupted in the SL biosynthesis gene *CCD7* and also the ethylene signaling component *EIN2* and comparing these double mutant plants with wild-type, *ein2*, and *ccd7* segregants from the cross between *ein2* and *ccd7* (Fig. 8, B and C). We found that, like *ein2* single mutant plants, the *ein2 ccd7* double mutants formed significantly more visible nodules than the wild type ($P < 0.01$). However, visible nodule number in the double mutant plants was significantly lower than in the *ein2* single mutant plants ($P < 0.05$). Indeed, the phenotype of *ein2 ccd7* double mutants appears to be essentially additive, as the approximate 40% reduction in nodule number in *ein2 ccd7* compared with

the *ein2* single mutant is consistent with an approximate 40% significant reduction in nodule number of the *ccd7* single mutant compared with the wild type ($P < 0.05$). Taken together with the application studies, this finding suggests that SL and ethylene are likely to act largely independently to control nodule number.

DISCUSSION

Over the last decade, SLs have been implicated in a wide range of developmental processes (Foo and Reid, 2013; Smith and Li, 2014). However, the exact mechanism by which SLs influence each process is still emerging. This has been the case for the role of SLs in the interaction between legumes and N-fixing rhizobia, with a number of studies indicating that SLs influence the ultimate number of mature nodules in several species, but the specific point during nodulation at which this occurs has not been established (for review, see López-Ráez et al., 2017). In this study, we show that infection thread formation is reduced specifically in SL-deficient *ccd8* mutants during nodulation and that this effect may occur due to the action of the *CCD8* product on the bacterial partner. This is presumably due to the reduced SL levels in this mutant, as exogenous GR24 can overcome this reduction

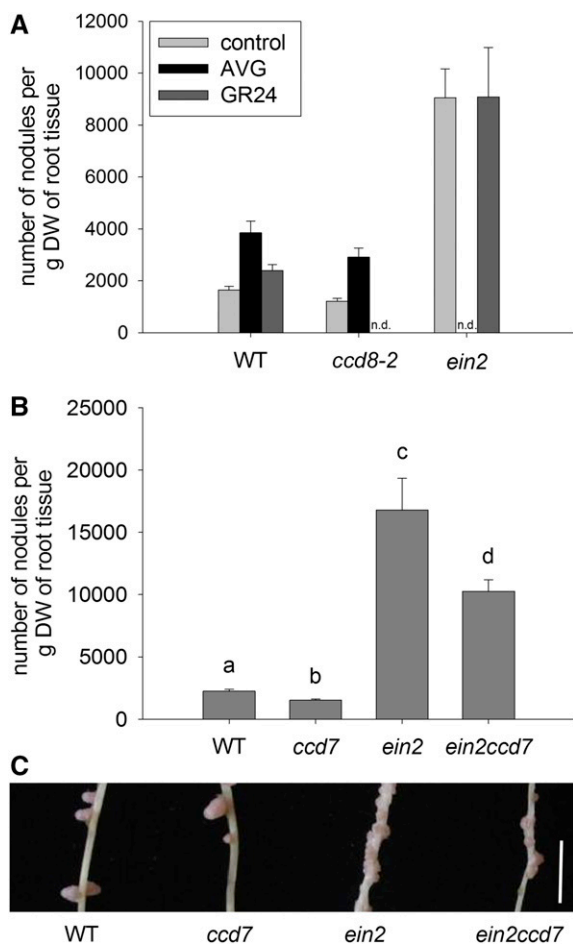


Figure 8. Interactions between ethylene and SL during nodulation. A, Nodule number in wild-type cv Torsdag (WT), SL-deficient *ccd8-2*, or ethylene-insensitive *ein2* pea mutants treated with the synthetic SL (+)-GR24, the ethylene synthesis inhibitor AVG, or solvent control ($n = 6-11$). n.d., Not done. B, Nodule number in wild-type, SL-deficient *ccd7*, *ein2*, and double mutant *ccd7 ein2* segregants ($n = 5-7$). Values are means \pm SE. For A, separate two-way ANOVAs were performed for the *ccd8* and *ein2* comparisons, and for B, a one-way ANOVA was performed, and values with different letters are significantly different ($P < 0.05$). DW, Dry weight. C, Photograph of nodules on secondary roots (tertiary roots have been removed). Bar = 5 mm.

in infection thread formation. Application and genetic studies indicate that this influence of SLs early in nodulation appears to be independent of ethylene signaling. The disruption of infection thread formation in an SL-deficient mutant is accompanied by reduced expression of some early nodulation genes, indicating that SLs result in a modification of the Nod LCO signaling pathway. Furthermore, we show that SL levels are down-regulated by mutations in the Nod LCO signaling pathway, and this appears to be dependent on NSP2 but independent of the NIN transcription factor.

In species that undergo root hair infection, root hair curling is one of the first physical events observed following challenge with rhizobia or specific Nod LCOs. We found that SLs do not appear to influence this very

early event, as SL-deficient plants had a similar number of curled root hairs when challenged with compatible rhizobia or Nod LCOs as wild-type plants (Fig. 2C). However, SL-deficient *ccd8* mutants did have reduced formation of infection threads (Figs. 3A and 4A; Supplemental Fig. S1), the next physical event in nodulation that allows the uptake of rhizobia from the soil and the ultimate delivery of the rhizobia into the dividing cells that will form the nodule. This reduction could be overcome by the application of GR24 (Fig. 4). These results suggest a positive influence of SLs on infection thread formation in pea, which is consistent with studies in *M. truncatula* that found that the expression of the SL biosynthesis gene *CCD8* was specifically up-regulated in infected root hairs and developing nodule primordia (Breakspear et al., 2014). Other studies in *M. truncatula* also found that, later in development, the SL biosynthesis genes *D27*, *CCD7*, and *CCD8* were expressed in the meristem and distal infection zone of mature nodules, leading to speculation that SLs may play a role during the later stages of nodule organogenesis or function (van Zeijl et al., 2015). However, although we found that nodules do contain low levels of SLs, the nodules that do develop on SL-deficient mutants of pea are of a similar size to those on wild-type plants (Foo and Davies, 2011) and also can fix atmospheric nitrogen (Fig. 3E).

In addition to infection via root hairs, many other legume-rhizobia interactions involve the uptake of rhizobia through cracks in the epidermis. Interestingly, when we examined the influence of SLs on the nodulation of lupin, a legume that uses crack-entry uptake of rhizobia, we found that the synthetic SL (+)-GR24 had no influence on nodule number (Supplemental Fig. S2). This is in contrast to reports of the effects of GR24 on nodule number in a range of root hair-entry species (pea, *M. truncatula*, *M. sativa*, and *L. japonicus*; Soto et al., 2010; Foo and Davies, 2011; Liu et al., 2013; De Cuyper et al., 2015). This observed lack of effect of SL on a species that does not form infection threads is consistent with our hypothesis that SLs appear to influence infection thread formation specifically.

While SL-deficient *ccd8* mutants form fewer infection threads and ultimately nodules than wild-type plants, this is not observed in *d14* SL receptor mutants (Supplemental Fig. S1). This is consistent with previous studies that showed that pea *max2* mutants, disrupted in another key SL signaling component, also did not display reduced nodulation (Foo et al., 2013). This suggests that, like its role in AM development, SLs may influence nodulation by affecting the microbial partner rather than the plant partner. One possibility is that SL-deficient *ccd8* plants produce altered levels of compounds known to be important in nodulation, such as flavonoids. However, we found no major difference in the profile or level of flavonoids in root exudates from SL-deficient *ccd8* and wild-type roots (Fig. 2A). We also found that SL-deficient *ccd8* root exudates had a similar positive influence on bacterial growth to wild-type root exudates (Fig. 2B), suggesting that products resulting

from CCD8 action do not influence nodulation by directly affecting bacterial growth. This is consistent with previous studies showing that application of the synthetic SL GR24 does not influence the growth or Nod LCO production of rhizobia, including the pea-compatible *R. leguminosarum* (Moscatiello et al., 2010; Soto et al., 2010). Recent reports suggest that SLs such as GR24 may influence rhizobial motility by affecting swarming behavior, although the specific function of swarming in nodulation per se is still unclear (Tambalo et al., 2014; Peláez-Vico et al., 2016). Given the discrete window in which we have shown that CCD8 influences nodulation, after root hair curling but before infection thread formation, future work could examine the role of products of CCD8 action, presumably SLs, in the dialogue between the plant and its bacterial partner during this crucial stage of nodule development.

The physical and molecular events that occur during nodulation are regulated in part via the Nod LCO signaling pathway (Fig. 1). Indeed, previous studies have indicated that the Nod LCO signaling pathway may influence SL biosynthesis, with some mutants in this pathway reported to have lower SL levels and/or expression of SL biosynthesis genes (Liu et al., 2011; van Zeijl et al., 2015). We examined this systematically, quantifying the level of SLs produced in pea mutants with lesions in each step of the signaling pathway (Fig. 6; Supplemental Fig. S6). We found that SL levels are reduced in mutants in core elements of the Nod LCO pathway, including a Nod LCO receptor (LYK3), and signaling elements (DMI1, DMI2, and DMI3). This positive influence on SL levels in wild-type plants requires the downstream transcription factor NSP2 but not NIN. Nonmycorrhizal species such as *Arabidopsis* (*Arabidopsis thaliana*) have lost several of these key SYM genes (DMI2 and DMI3; Delaux et al., 2014) but retained others. It would be interesting to examine the influence of these genes on noncanonical SL levels, since recent reports indicate that *Arabidopsis* does not appear to produce canonical SLs (Xie et al., 2015; Brewer et al., 2016). Given the role of CCD8 in influencing infection thread number, it is interesting that the *nsp2* mutation blocks bacterial colonization of the curled root hairs while the *nin* mutation (also known as *sym35*) blocks the next stage, which is the initiation of infection threads (Tsyganov et al., 2002; Borisov et al., 2003). Recent progress has been made in our understanding of infection thread formation (Fournier et al., 2015), and it will be interesting to explore specific nonplant roles for SLs during this process in the future.

The promotion of SL levels by the Nod LCO signaling pathway, together with the fact that the Nod LCO pathway is required for rhizobia and/or Nod LCOs to elevate the expression of some SL biosynthesis genes (van Zeijl et al., 2015), suggest that Nod LCOs may induce SL biosynthesis. However, when the SL levels in the root infection zone (1–2 cm behind the root tip) of wild-type pea were measured 1, 2, 3, and 7 d after inoculation, we did not find a consistent increase in SL levels (data not shown). If SL levels are elevated specifically in root hairs, as suggested by the root hair

expression of CCD8 in *M. truncatula* (Breakspear et al., 2014), such localized increases in levels may be difficult to detect in extracts from root sections.

Overall, we found that SLs act largely independently of ethylene in nodulation. We found no evidence that SL deficiency influences ethylene levels, as ethylene levels and the expression of ethylene metabolism genes were not altered significantly in *ccd8-1* compared with wild-type plants (Fig. 7B). Importantly, we found a 7- to 8-fold increase in nodule number of *ein2* mutants on either a wild-type or SL-deficient *ccd7* background (Fig. 8B). This additive phenotype suggests that ethylene and SLs control nodule number largely independently. The fact that the nodulation of ethylene-insensitive *ein2* pea mutants was not responsive to GR24 (Fig. 8A) but that nodule number could be elevated in SL-deficient pea mutants treated with the ethylene synthesis inhibitor AVG (Fig. 8A) suggests that the ethylene response of the plant has a much greater influence on nodulation than SL exudation. SL-ethylene interactions also have been investigated in root hair elongation and leaf senescence in *Arabidopsis*, and there is evidence for somewhat different interactions and independent effects of each hormone (Kapulnik et al., 2011; Ueda and Kusaba, 2015), reflecting the common observation that plant-hormone interactions are often specific to a given developmental process (Vanstraelen and Benková, 2012).

In conclusion, we have highlighted a specific role for SLs during nodule development. SLs appear to promote infection thread formation and may do so by influencing the bacterial partner. We have revealed that many elements of the Nod LCO signaling pathway are required to up-regulate SL levels, and this and other studies support the idea that Nod LCOs may elevate SL biosynthesis during nodulation. Although important roles for SLs during the nutrient regulation of development have been established (Brewer et al., 2013), it is important to note that SLs are not required to modulate nodulation in response to nitrogen or phosphorus (Foo et al., 2013). It appears that SLs may play a small but significant role during nodulation, and future studies may explore the role of SL action, either directly or indirectly, on the rhizobial partner, including in other plant-rhizobium symbioses with different developmental features, such as actinorhizal interactions that form via intracellular infection and root hair penetration.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The pea (*Pisum sativum*) lines used were the SL-deficient line *ccd8-1* (also known as *rms1-1*; Beveridge et al., 1997) derived from wild-type cv Parvus; the SL receptor mutant *d14* (de Saint Germain et al., 2016) derived from cv Torsdag (also known as *rms3-1* and K487; Arumingtyas et al., 1992); the ethylene-insensitive *ein2* mutant (Weller et al., 2015) derived from cv Torsdag; and the symbiosis mutants *nfp* (*sym10*; Madsen et al., 2003) and *dmi2* (*sym19*; Stracke et al., 2002) derived from cv Frisson, *dmi1* (*sym8*; Edwards et al., 2007) and *nin* (*sym35*; Borisov et al., 2003) derived from cv Finale, and *dmi3* (*sym9*; Lévy et al.,

2004) and *nsp2* (*sym7*; Kaló et al., 2005) derived from cv Sparkle. The *ccd8-2* line (also known as *rms1-2*) mutant was derived from cv Weitor (Beveridge et al., 1997), and this line was used in Figure 2. The *ccd8-2* line also has been backcrossed to cv Torsdag (also known as *rms1-2T*; Foo et al., 2013), and this line was employed in Figures 4 and 8 to allow direct comparison with *ein2* and in Supplemental Figure S1 to allow direct comparison with *d14*. The double mutant *ein2 ccd7* was derived from a cross between *ccd7-3* (also known as *rms5-3T*; Foo et al., 2013) and *ein2*. In all cases, comparisons were made between the mutant line and its wild-type progenitor line. Seeds were surface sterilized with 70% (v/v) ethanol and grown in growth cabinets (18-h photoperiod, 20°C day, 15°C night, under cool-white fluorescent tubes [100 $\mu\text{mol m}^{-2} \text{s}^{-1}$]), two per pot in vermiculite, under conditions to exclude rhizobial bacteria, unless stated otherwise.

Flavonoid Analysis and Bacterial Growth Assays

Plants were grown for 21 d as described by Foo et al. (2016c), removed from pots, placed on damp paper, and five 10- × 10-mm filter papers were placed 1 cm from root tips. After 2 h, the papers were pooled from 20 plants per genotype, and flavonoids were extracted in 1:1 ethyl acetate:methanol overnight at 4°C. The sample was dried and resuspended in 100 μL of 0.4% acetic acid. A total of 10 μL was injected onto an Acquity UPLC BEH C_{18} column using an Acquity H-series UPLC device coupled to an Acquity Photodiode Array detector (PDA) (Waters) in series with a Xevo triple quadrupole mass spectrometer. The column was held at 35°C, flow rate was 0.35 mL min^{-1} , with 100% A (1% acetic acid):0% B (acetonitrile) increasing to 90% A:10% B (v/v) at 0.5 min and 70% A:30% B (v/v) at 20 min, and the PDA was monitored continuously over the range 230 to 500 nm. The initial selection of targeted flavonoids was based on peaks with UV λ_{max} between 325 and 380 nm, supported by subsequent positive and negative ion tandem mass spectrometry spectra and, where possible, by characteristic flavonoid full UV spectra.

The mass spectrometer was operated in several different modes in separate injections. Initially, negative ion full-scan survey scans were acquired over the mass-to-charge ratio (*m/z*) range 200 to 1,700 every 0.3 s, with a cone voltage of 40 V, after which ScanWave daughter scans at 32 and 45 V collision energy at 2,000 *m/z* per second were acquired automatically from the strongest ions. Subsequent runs targeted some of the stronger putative flavonoids (e.g. *m/z* 771, 787, and 1,023) to acquire higher quality ScanWave daughters with a collision energy of 40 V. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L h^{-1} , the desolvation temperature was 450°C, and the capillary voltage was 2.7 kV in all cases. Positive ion tandem mass spectrometry data also were acquired in precursor scan mode using *m/z* 287 and 303 as precursor ions (targeting kaempferol- and quercetin-containing flavonoids, respectively). This indicated that the majority of flavonoids present were highly glycosylated and acylated, consistent with the finding of kaempferol and quercetin sophorotriosides and several acylated versions of these in pea shoots (Ferreeres et al., 1995). While the molecular masses of five of the compounds found in pea roots through this process (772, 788, 934, 950, and 964 D) were the same as those reported for pea shoot flavonoid sophorotriosides, there was insufficient sample to determine their structures. Peaks identified as probable flavonoids through this method were then targeted by selected ion monitoring, using a dwell time of 25 ms per ion (Supplemental Table S1).

For bacterial growth assays, root exudates were collected and extracted as described above and resuspended in 1 mL of water. Four replicate cultures of *Rhizobium leguminosarum* bv *viciae* (RLV248) were grown in yeast-mannitol broth with 250 μL of the extracted root exudate (or 250 μL of water in control samples) at 25°C and 120 rpm. Bacterial growth was measured 0, 20, 24, 28, 48, and 52 h after inoculation by measuring the A_{600} of 1 mL of culture on an Spectrostar Nano spectrophotometer (BMG Labtech).

Root Hair Curling, Infection Thread, and Developing Nodule Studies

Plants grown for root hair curling studies were grown for 7 d and inoculated with 75 mL of sterile water (control), 0.1 × 10⁻⁶ M Nod LCO (CO-IV [C18:1 Δ 11Z, Ac]), or a 10% solution (v/v) of a 3-d-old culture of *R. leguminosarum* bv *viciae* (RLV248) grown in yeast-mannitol broth (an equal volume of sterile yeast-mannitol broth and/or solvent [50% acetonitrile (v/v)] also was included in all treatments/controls). Nod LCO was prepared by a chemoenzymatic approach combining biotechnological synthesis of the saccharidic backbone in transgenic *Escherichia coli* followed by chemical acylation with cis-vaccenic acid (Samain et al., 1999; Ohsten Rasmussen et al., 2004; Chambon et al., 2015). Five

days after treatment, six to 10 roots per plant were stained briefly with Toluidine Blue and examined with a light microscope, and the percentage of curled root hairs was recorded.

For infection thread and developing nodule studies, seedlings were inoculated 10 d after planting with 75 mL of a 10% solution (v/v) of a 3-d-old culture of *R. leguminosarum* bv *viciae* (RLV3841) carrying pXLGD4 (carrying the *lacZ* reporter gene; supplied by John Innes Centre) grown in Tryptone Yeast (TY) medium with streptomycin (200 $\mu\text{g mL}^{-1}$) and tetracycline (5 $\mu\text{g mL}^{-1}$). Plants received a weekly dose of modified Long Ashton nutrient solution with no N and 5 mM NaH_2PO_4 , as described by Foo et al. (2013). Nine days after inoculation, root segments from the root tip to the first visible nodule (approximately 3–6 cm) or entire lateral roots were harvested and fixed in 25% glutaraldehyde (v/v) in wash buffer (100 mM sodium phosphate at pH 7, 10 mM KCl, and 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), then twice washed in wash buffer, and stained overnight in the dark in wash buffer containing 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, 5 mM $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$, and 800 $\mu\text{g mL}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside acid. Root segments were viewed with a Zeiss Axiolab light microscope with a 20× objective, and images were taken with a Nikon Digital Sight DS-Fi2 camera. The root length, number of blue-stained infection threads, developing nodules (nodules not visible with the naked eye but visible under a 20× objective; Fig. 1), and, for Figure 4 and Supplemental Figure S1, the number of nodules visible to naked eye were counted in one to 10 roots of six to 15 plants per genotype. Total nodule number is the sum of developing and visible nodules. Root samples were scored blind, meaning that roots were scored without the scorer knowing the genotype.

Nodule Function Studies

For the acetylene reduction assay, plants were grown and inoculated with *R. leguminosarum* bv *viciae* (RLV248) as described previously (Foo and Davies, 2011). A whole-root system of mature nodulated plants was placed in 100-mL bottles sealed with a gas-tight lid fitted with a septum, with four replicate bottles per genotype. Acetylene was added to each bottle to make a final concentration of 1% (v/v), and the roots were incubated for 4 h at room temperature. The amount of ethylene generated from the reduction of acetylene via nitrogenase was measured by gas chromatography-mass spectrometry as described by Foo et al. (2006), except that no cryotrap was used. Injections were split 20:1, and the oven temperature was 50°C. A standard of mixed ethylene and acetylene (1% [v/v] of each) was analyzed between each replicate so that the concentration of ethylene in the samples could be calculated. After analysis, the dry weight of nodules was measured, and ethylene evolved was expressed on a per gram dry weight of nodules basis.

Hormone and Nod LCO Application Studies

For hormone application studies with blue lupin (*Lupinus angustifolius*), the plants were grown and inoculated as described previously (Foo et al., 2016b). Pea plants were inoculated on day 7 as described previously (Foo and Davies, 2011). For both pea and lupin, pots were then treated on days 9, 13, 15, and 18 with 75 mL of control (water), 2 × 10⁻⁵ M (+)-GR24 (also referred to as GR24^{SDS}; Scaffidi et al., 2014), or 1.5 × 10⁻⁵ M AVG (Sigma-Aldrich). Chemicals were dissolved in dimethyl sulfoxide, and control plants received an equal concentration of dimethyl sulfoxide in water only. On day 28, plants were harvested, and the number of nodules and root dry weight were recorded. The effects of GR24 application on infection thread formation and nodule development were scored as described above.

To study the effects of Nod LCO on root architecture, 4-d-old sterile pea seedlings were transplanted to slants (20 mL of one-half-strength modified Long Ashton nutrient solution with 0.8 mM KNO_3 and 0.25 mM NaH_2PO_4 solidified with 5 g L^{-1} Phytigel [Sigma-Aldrich]). While the medium was molten, Nod LCO (CO-IV [C18:1 Δ 11Z, Ac]) was added to a final concentration of 1 × 10⁻⁸ M. Nod LCO was dissolved in a minimal volume of 50% acetonitrile (v/v), and control slants received an equal concentration of acetonitrile. On day 18, plants were harvested, and tap root length, lateral root number, and the length of the longest lateral root were recorded.

Hormone Analysis

For SL quantification, approximately 2 g (fresh weight) of whole-root tissue (three to four plants per replicate) was harvested from 3- to 4-week-old plants, and SLs were purified and measured as described by Foo et al. (2013) with the

inclusion of labeled SL standards ($[6\text{-}^2\text{H}_1]$ fabacyl acetate, $[6\text{-}^2\text{H}_1]$ orobanchol, and $[6\text{-}^2\text{H}_1]$ orobanchyl acetate). For ethylene quantification, plants were grown in sterile vermiculite, three per 250-mL glass jar in a growth cabinet (as described in "Plant Material and Growth Conditions") for 12 d, and ethylene evolution from whole plants was performed from four replicate jars, as described by Foo et al. (2016c).

Gene Expression

For the gene expression studies in Figure 5 and Supplemental Figures S3 and S4, plants were grown for 10 d and root tip tissue was harvested (day 0). The remaining plants were inoculated with a 10% solution (v/v) of a 3-d-old culture of RLV248, and 2 and 4 d later, root tip tissue was harvested. For the gene expression studies in Figures 6 and 7A, whole-root tissue (three to four plants per replicate) was harvested from 3- to 4-week-old plants, and after grinding, a subset was taken for gene expression analysis and the remainder was processed for hormone analysis (see above). For the gene expression studies in Figure 7B, after ethylene levels were measured, plants were removed from jars and the whole-root tissue (three plants per replicate) was harvested.

Tissue was ground and RNA was extracted from approximately 100 mg of tissue using the ISOLATE II RNA Mini Kit (Bioline). cDNA was synthesized from 1 μ g of RNA using the SensiFAST cDNA Synthesis Kit (Bioline). cDNA was diluted, and duplicate, real-time PCRs were performed in a Rotor Gene 2000 (Corbett) using the SensiFAST SYBR Hi-ROX Kit (Bioline) and 100 to 200 pmol of a primer pair. Primer pairs for genes analyzed in this study were as follows: *PsD27* (forward, 5'-CAAGCAGCAACAGGAATCAG-3', and reverse, 5'-TTGATGGTGGCATCACTCTC-3'), *PsCCD7* (*RMS5* forward and reverse; Johnson et al., 2006), *PsCCD8* (*RMS1* forward and reverse; Johnson et al., 2006), *PsENOD12a* (Foo et al., 2016b), *PsENOD12b* (forward, 5'-TGAACCACCAGTGAATGAGC-3', reverse, 5'-TGATGTTAATGTTCCGCTGT-3'), *PsENOD40* (Foo et al., 2016b), *PsACS1* (Foo et al., 2006), *PsACOX* (Foo et al., 2006), the defense markers *PsPAL1*, *PsHmi6* and *PsB-Gluc* (Blake et al., 2016) and the housekeeping gene *ACTIN* (Foo et al., 2005). Standard curves were created for each gene using serially diluted plasmids containing cloned fragments of each amplicon. The average concentration of technical replicates was calculated. The relative gene expression of four biological replicates was determined by comparing the concentration of the gene of interest with the *ACTIN* concentration of that sample.

Statistical Analysis

For pairwise comparisons, Student's *t* tests were performed in Excel. For other experiments, one- or two-way ANOVAs were performed in R version 3.2.2 (R Core Team), followed by Tukey's honest significant difference posttests where appropriate. When appropriate, the data were log transformed prior to analysis.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Nodule development in wild-type, SL-deficient *ccd8-2*, and SL-insensitive *d14-1* plants infected with *lacZ*-labeled *R. leguminosarum* bv *viciae*.

Supplemental Figure S2. Nodule number in blue lupin following treatment with (+)-GR24 or solvent control.

Supplemental Figure S3. Expression of the SL biosynthesis genes *CCD7*, *CCD8*, and *D27* at 0, 2, and 4 d following inoculation with *R. leguminosarum* bv *viciae* in wild-type cv *Parvus*.

Supplemental Figure S4. Expression of the disease marker genes at 0, 2, and 4 d following inoculation with *R. leguminosarum* bv *viciae* in wild-type cv *Parvus* and *ccd8-1* plants.

Supplemental Figure S5. Root development in wild-type cv *Parvus* and SL-deficient *ccd8-1* mutant plants following treatment with 0.1 μ M Nod LCO or solvent control.

Supplemental Figure S6. Fold change relative to the respective wild type of the canonical SLs orobanchol and orobanchyl acetate in various pea symbiosis mutants.

Supplemental Table S1. Putative flavonoid ions monitored via selected ion monitoring compound retention times and UV absorbance data for stronger signals.

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