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Running head: The role of strigolactones in nodulation

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Determining the site of action of strigolactones during nodulation^

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One-sentence summary:

The plant hormone strigolactone promotes infection thread formation but does not appear to influence other stages of nodulation, including nitrogen-fixation.

Footnotes:

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List of author contributions

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. synthesised the Nod LCO; E.L.M and E.F. analysed the data; E.F. conceived the project and wrote the article with contributions from J.B.R. and E.L.M.

ABSTRACT

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* L.) mutant forms significantly less 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 lipochito-oligosaccharides (LCOs) and SL-deficient nodules appear to fix nitrogen at a similar rate to wild type plants. In contrast, a SL receptor mutant displayed no decrease in infection thread formation or nodule number, suggesting SL-deficiency may influence the bacterial partner. We found this influence of SL-deficiency was not due to altered flavonoid exudation or 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 (*ENOD*) genes. Importantly, SL synthesis is down-regulated by mutations in genes of the Nod LCO signalling 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.

INTRODUCTION

Nodulation results from the intimate relationship of nitrogen (N)-fixing rhizobacteria and leguminous plants. The uptake of rhizobia and organogenesis of the nodule in which the bacteria are hosted only occurs 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 lipochito-oligosaccharides (LCOs) (e.g. Peters et al., 1986). Following recognition of these Nod LCOs by receptor-like kinases, including the Nod LCO receptors NFP and LYK3, there is an induction of oscillations in nuclear-associated calcium levels via the action of DMI2 and DMI1. This is sensed by DMI3, that along with parallel pathways influence a range of transcription factors (e.g. NIN, NSP1, NSP2, IPD3) that in turn coordinate the expression of nodulation-associated genes such as *ENOD*s (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 colonised 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 synthesised from a carotenoid precursor via 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 play important roles in shoot and root development and during the interaction of plants with microorganisms (Foo and Reid, 2013; Smith and Li, 2014; Lopez-Raez et al., 2017). For example, SLs are exuded from plant roots and act as a rhizosphere signal to promote symbioses of plants with phosphorus (P)-acquiring arbuscular mycorrhizal (AM) fungi by promoting spore germination and hyphal branching (Akiyama et al., 2005). This AM signalling 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 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., 2014).

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* genes, leads to a significant reduction in the number of nodules in species that form both indeterminate (pea) 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 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., 2014) and *D27 RNAi* lines in this species did not have altered nodulation (van Zeiji 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 Zeiji et al., 2015). In the case of *CCD8*, promoter-fusion studies revealed that this expression was specifically in infected root hairs and developing nodule primordia (Breakspear et al., 2014) and in mature nodules the expression of *D27*, *CCD7* and *CCD8* becomes restricted to the meristem and distal infection zone (van Zeiji et al., 2015). Overall these mutant and gene expression studies suggest 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 signalling (Soto et al. 2010; Moscatiello 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 non-legumes and some, but not all, legume hosts were shown to induce swarming behavior (Tambalo et al., 2013). One possibility yet to be explored is that SL status may influence other root exudates, including flavonoids.

Outputs of the Nod LCO signalling cascade include changes to hormone biosynthesis and/or perception. There is some indication that the Nod LCO signalling pathway may influence SL biosynthesis. *M. truncatula* mutants with lesions in *NSP1* and/or *NSP2* (transcription factors downstream of Nod LCO signalling) 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 Zeiji et al. (2015) found that application of Nod LCOs induced 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 this 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; Guinell, 2015). 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., 2014). This suggests SLs may act upstream of ethylene action during nodulation but further studies are required to fully test this hypothesis.

In this study, we pin point the stage at which SLs appear to influence nodulation and examine interactions between SLs, the Nod LCO signalling pathway and ethylene during nodulation, in pea (*Pisum sativum* L.). 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 a SL receptor mutant, *d14*, which suggests 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 early nodulation (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 signalling 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 (e.g. 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., 2005; 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 level of flavonoids between two null SL-deficient mutants, *ccd8-1* and *ccd8-2*, and their respective progenitor lines when analysed 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 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 was also 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*-labelled 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 a 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 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 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 30-40% reduction in infection thread formation in *ccd8-1* mutants was mirrored in a similar significant reduction in the number of developing nodules compared with wild type (Fig. 3 C, D; $P < 0.05$). Both reductions in infection thread formation and developing nodules in *ccd8-1* are consistent with the 30-40% decrease in mature nodules previously reported 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 *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 (Suppl. Fig. 1A, B). In contrast, we found no decrease in the number of infection threads or nodules formed on *d14* mutants compared with wild type, indicating that the D14 SL receptor is not required for the

effect of the *CCD8* product on nodule development. As observed previously (Urquart et al., 2015) both *ccd8-2* and *d14* mutants have somewhat shorter lateral roots than wild type, consistent with the reported SL-deficiency and insensitivity of these mutants (Suppl. Fig. 1C).

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, Suppl. Fig. 1A), infection thread formation was significantly reduced in *ccd8* mutants compared with 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 2-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 was 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 affects of SLs on the number of visible nodules seen in pea (Foo and Davies, 2011).

Some legume species such as lupin undergo infection not through root hairs but via cracks in the epidermis (e.g. 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* (Suppl. Fig. 2), while similar doses of GR24 have been shown to alter nodulation in pea, *M. truncultula* and *M. sativa* (Soto et al., 2010; Foo and Davies, 2011; De Cuyper et al., 2014). 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 levels were approximately 4 times lower than surrounding mature root (the level of fabacyl acetate in nodule tissue was 0.05ng/g FW, compared with 0.19 ng/g FW 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 to comparable wild type nodules, indicating SL-deficient nodules can fix nitrogen.

Gene expression during early nodulation in a SL-deficient mutant

During early interactions with rhizobia, the expression of a suite of early nodulation genes (*ENOD*s) are induced, via the action of the Nod LCO signal transduction pathway. In pea, this includes *ENOD12a*, *ENOD12b* and *ENOD40* (e.g. 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* (e.g. Schneider et al., 1999). We found that in the days following inoculation with rhizobia, the induction of some of these genes was significantly reduced 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 wild type (Fig. 5, $P < 0.01$). We also found that in wild type plants the expression of the SL biosynthesis gene *D27* was significantly elevated following challenge with the rhizobia (Suppl. Fig. 3, $P < 0.05$), consistent with similar reports in *M. truncutula* (van Zeiji et al. 2015). Recent reports suggest SLs may also influence disease development in some systems (Lopez-Raez 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

(Suppl. Fig. 4) and callose and lignin deposition was examined in *ccd8* and wild type plants exposed to rhizobia. No trends of consistently elevated or reduced gene expression (Suppl. Fig. 4), or callose or lignin deposition was observed between *ccd8* and wild type roots.

In addition to inducing nodulation specific molecular and physical changes, rhizobia (via Nod LCO signalling) also induce changes in root architecture (e.g. Olah et al., 2005). Given that SLs have been implicated in the 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. 2-way ANOVAs showed that Nod LCO treatment significantly affected lateral root number and length (Suppl. Fig. 5, $P < 0.01$). However, there was no significant interaction between genotype and treatment, indicating *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. truncutula* have identified SL biosynthesis and/or expression of SL biosynthesis genes is in part dependent on some elements of the LCO signalling pathway (Liu et al., 2011; Breakspear et al., 2014; van Zeiji 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 signalling pathway as a whole influences SL biosynthesis. To address this question we systematically examined SL levels in non-nodulated roots of pea mutants disrupted in Nod LCO receptors (*nfp* and *lyk3*), LCO signalling 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 significantly reduced in mutants disrupted in one Nod LCO receptor (*lyk3*), signalling 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 were also detected in some experiments, and these were also reduced in *dmi3* (Suppl. Fig. 6). 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 signalling 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 (e.g. Beveridge et al., 1997).

It is interesting to note that in some cases this reduction in SL levels was accompanied by significant reductions in the expression of SL biosynthesis genes *D27* and *CCD8*, but in many cases the expression of these genes did not reflect the level of the hormone 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 (e.g. Foo et al., 2005: Umehara et al., 2008) and is consistent with other hormones, where expression of hormone biosynthesis genes is often not an accurate proxy for hormone levels (Symons and Reid, 2008).

Strigolactones act largely independently of ethylene to influence nodulation

It has recently been suggested that ethylene may act downstream of SLs during nodulation (De Cuyper et al., 2014) 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., 2016b) 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 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 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 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 signalling component *EIN2* and comparing these double mutant plants to wild type, *ein2* and *ccd7* segregants from the cross between *ein2* and *ccd7* (Fig. 8 B, C). We found that like *ein2* single mutant plants, the *ein2 ccd7* double mutants formed significantly more visible nodules than 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 *ein2* single mutant is consistent with an approximate 40% significant reduction in nodule number of *ccd7* single mutant compared with wild type ($P < 0.05$). Taken together with the application studies, this 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; Li and Smith, 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 (reviewed by Lopez-Raez et al., 2017). In this study we show that infection thread formation is specifically reduced in SL-deficient *ccd8* mutants during nodulation and that this affect may occur due to 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 in infection thread formation. Application and genetic studies indicate that this influence of SLs early in nodulation appears to be independent of ethylene signalling. Disruption of infection thread formation in a SL-deficient mutant is accompanied by reduced expression of some early nodulation genes, indicating that SLs result in a modification of the Nod LCO signalling pathway. Further, we show that SL levels are down-regulated by mutations in the Nod LCO signalling 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 (Fig. 3A, 4A, Suppl. Fig. 1), 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 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. truncutula* that found the expression of the SL biosynthesis gene *CCD8* was specifically upregulated in infected root hairs and developing nodule

primordia (Breakspear et al., 2014). Other studies in *M. trucutula* also found that later in development, 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 Zeiji 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 can also fix atmospheric nitrogen (Fig. 3E).

In addition to infection via root hairs, many other legume-rhizobial interactions involve uptake of rhizobia through cracks in the epidermis. Interestingly, when we examined the influence of SLs on 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 (Suppl. Fig. 2). This is in contrast to reports of effects of GR24 on nodule number in a range of root-hair entry species (pea, *M. truncutula, M. sativa* and *L. japonicus*; Soto et al., 2010; Foo and Davies, 2011; Liu et al., 2013; De Cuyper et al., 2014). 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 (Suppl. Fig. 1). This is consistent with previous studies that showed that pea *max2* mutants, disrupted in another key SL signalling component, also did not display reduced nodulation (Foo et al., 2013a). 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 SL-deficient *ccd8* root exudates had a similar positive influence on bacterial growth as wild type root exudates (Fig. 2B), suggesting that products resulting from CCD8 action do not influence nodulation by directly effecting bacterial growth. This is consistent with

previous studies showing that application of the synthetic SL GR24 does not influence 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 discreet 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 dialog 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 signalling pathway (Fig. 1). Indeed, previous studies have indicated that the Nod LCO signalling 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 Zeiji et al., 2015). We examined this systematically, quantifying the level of SLs produced in pea mutants with lesions in each step of the signalling pathway (Fig. 6, Suppl. Fig. 6). We found that SL levels are reduced in mutants in core elements of the Nod LCO pathway, including a Nod LCO receptor (LYK3), and signalling elements (DMI1, DMI2 and DMI3). This positive influence on SL levels in wild type plants requires the downstream transcription factor NSP2 but not NIN. Non-mycorrhizal species such as Arabidopsis 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 non-canonical SL levels, since recent reports indicate 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 to note 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 non-plant roles for SLs during this process in the future.

The promotion of SL levels by the Nod LCO signalling 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 Zeiji et al., 2015), suggests Nod LCOs may induce SL biosynthesis. However, when the SL levels in the root infection zone (1-2cm behind the root tip) of wild type peas were measured 1, 2, 3 and 7 days 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 root hair expression of *CCD8* in *M. truncultula* (Breakspear et al., 2014), such localised 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 significantly altered 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 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 have also 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, 2016), reflecting the common phenomena that plant hormone interactions are often specific to a given developmental process (Vanstraelen and Benkova, 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 signalling 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 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 phosphorous (Foo et al., 2013a). 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 *Pisum sativum* L. lines used were the strigolactone-deficient line *ccd8-1* (also known as *rms1-1*, Beveridge et al. 1997) derived from wild-type cv. Parvus; the strigolactone 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; the symbioses 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*; Levy et al., 2004) and *nsp2* (*sym7*; Kalo 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 Fig 2. The *ccd8-2* line has also been backcrossed to cv. Torsdag (also known as *rms1-2*T; Foo et al., 2013a) and this line is employed in Fig. 4, and in Fig. 8 to allow direct comparison to *ein2* and in Suppl. Fig. 1 to allow direct comparison to *d14*. The double mutant *ein2 ccd7* was derived from a cross between *ccd7-3* (also known as *rms5-3*T, Foo et al., 2013a) and *ein2*. In other cases comparisons were made between the mutant line and its wild type progenitor line. Seeds were surface sterilised with 70% EtOH and grown in growth cabinets (18 h photoperiod, 20°C day, 15°C night, under cool-white fluorescent tubes [100 μmol m^{-2} s^{-1}]), two per pot in vermiculite, under conditions to exclude rhizobial bacteria, unless otherwise stated.

Flavonoid analysis and bacterial growth assays

588 Plants were grown for 21 d as described in Foo et al. (2016b), removed from pots,
589 placed on damp paper and five 10 x 10mm filter papers placed 1cm from root tips.
590 After 2 h, papers were pooled from 20 plants per genotype and flavonoids extracted in
591 1:1 ethyl acetate:methanol overnight at 4°C. The solvent was dried and resuspended
592 in 100 µL 0.4% acetic acid. 10 µl was injected onto an Acquity UPLC BEH C_{18}
593 column using an Acquity H-series UPLC coupled to an Acquity PDA detector
594 (Waters, Australia) in series with a Xevo triple quadrupole mass spectrometer. The
595 column was held at 35^{o}C, flow rate was 0.35 mL/min, with 100% A (1% acetic acid) :
596 0% B (acetonitrile) increasing to 90% A and 10% B at 0.5 minutes and 70% A : 30%
597 B at 20 minutes and the PDA was monitored continuously over the range 230 to
598 500nm. Initial selection of targeted flavonoids was based on peaks with UV λ_{max}
599 between 325 nm and 380 nm, supported by subsequent positive and negative ion
600 MS/MS spectra and where possible by characteristic flavonoid full UV spectra.
601
602 The mass spectrometer was operated in several different modes in separate injections.
603 Initially negative ion full scan 'survey scans' were acquired over the range *m/z* 200 to
604 1700 every 0.3 seconds, with a cone voltage of 40V followed by Scanwave daughter
605 scans at 32V and 45V collision energy (CE) at 2000 m/z per second were
606 automatically acquired from the strongest ions. Subsequent runs targeted some of the
607 stronger putative flavonoids – e.g. m/z 771, 787, and 1023 to acquire higher quality
608 scanwave daughters with a CE of 40V. The ion source temperature was 130°C, the
609 desolvation gas was nitrogen at 950 L/hr the desolvation temperature was 450°C and
610 the capillary voltage was 2.7KV in all cases. Positive ion MS/MS data were also
611 acquired in 'precursor scan' mode using *m/z* 287 and 303 as precursor ions (targetting
612 kaempferol and quercetin containing flavonoids respectively). This indicated the
613 majority of flavonoids present were highly glycosylated and acylated, consistent with
614 the finding of kaempferol and quercetin sophorotriosides and several acylated
615 versions of these in pea shoots (Ferreres et al., 1995). While the molecular weights of
616 five of the compounds found in pea roots through this process (772, 788, 934, 950 and
617 964 Da) were the same as reported for these pea shoot flavonoid sophorotriosides,
618 there was insufficient sample to determine their structures. Peaks identified as
619 probable flavonoids through this method were then targeted by selected ion
620 monitoring, using dwell time of 25ms per ion (Suppl. Table 1).

For bacterial growth assays, root exudates were collected and extracted as described above and resuspended in 1 ml of H_2O. 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 H_2O 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 absorbance at 600 nm of 1 ml of culture on an Spectrostar Nano spectrophotometer (BMG Labtech, Germany).

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 x 10^{-6}M Nod LCO (CO-IV (C18:1 Δ11*Z*, Ac)) or 10% solution of a 3 d old culture of *Rhizobium leguminosarum* bv. *viciae* (RLV248) grown in yeast-mannitol broth (an equal volume of sterile yeast-mannitol broth and/or solvent (50% acetonitrile) was also included in all treatments/controls). Nod LCO was prepared by a chemoenzymatic approach combining biotechnological synthesis of the saccharidic backbone in transgenic *E.coli* followed by chemical acylation with cis-vaccenic acid (Rasmussen et al 2004; Samain et al 1999; Chambon et al 2015). Five d after treatment, 6-10 roots per plant were stained briefly with toluidine blue and examined under 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 of a 3 d old culture of *R. leguminosarum* bv. *viciae* (RLV3841) carrying pXLGD4 (carrying the *lacZ* reporter gene; supplied by John Innes Centre, United Kingdom) grown in TY medium with streptomycin (200 µg/mL) and tetracycline (5 µg/mL). 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., (2013a). Nine d after inoculation root segments from the root tip to the first visible nodule (approx. 3 to 6 cm) or entire lateral root were harvested and fixed in 25% gluteraldehyde in wash buffer (100 mM sodium phosphate at pH 7.0, 10 mM KCl and

1 mM $MgCl_2 \cdot 6H_2O$), then twice washed in wash buffer and stained overnight in the dark in wash buffer containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6] \cdot 3H_2O$ and 800 μg mL^{-1} X-gal. Root segments were viewed with a Zeiss Axiolab light microscope (Göttingen, Germany) with a 20x objective and images taken with a Nikon Digital Sight DS-Fi2 camera (Melville, NY, USA). The root length, number of blue stained infection threads, developing nodules (nodules not visible with the naked eye, but visible under a 20x objective, Fig. 1) and for Fig. 4 and Suppl. Fig. 1 number of nodules visible to naked eye was counted in 1-10 roots of 6-15 plants per genotype. Total nodule number is the sum of developing and visible nodules. Root samples were scored blind, meaning roots were scored without the scorer knowing the genotype.

Nodule function studies

For the acetylene reduction assay, plants were grown and inoculated with *Rhizobium leguminosarum* bv. *viciae* (RLV248) as previously described (Foo and Davies, 2011). A whole root system of mature nodulated plants were 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 incubated for 4 h at room temperature. The amount of ethylene generated from the reduction of acetylene via nitrogenase was measured by GC-MS as described by Foo et al. (2006) except that no cyrotrap 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 analysed 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 g dry weight (DW) 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., 2015). Pea plants were inoculated on d 7 as previously described (Foo and Davies, 2011). For both pea and lupin, pots were then treated on d 9, 13, 15 and 18 with 75ml of control (water) or 2 x 10^{-5}M (+)-GR24 (also referred to as $GR24^{5DS}$; Scaffadi *et al*., 2014) or 1.5 x 10^{-5}M AVG (Sigma-Aldrich, Australia). Chemicals were dissolved in DMSO and control plants received an equal concentration of DMSO in water only. On d 28 plants were

harvested and the number of nodules and root DW 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 effects on root architecture 4 d old sterile pea seedlings were transplanted to slants (20ml of half-strength modified Long Ashton nutrient solution with 0.8mM KNO_3 and 0.25mM NaH_2PO_4 solidified with 5g/L Phytagel (Sigma Aldrich Pty Ltd.) While the media was molten, Nod LCO (CO-IV (C18:1 Δ11Z, Ac)) was added to a final concentration of 1 x 10^{-8}M. Nod LCO was dissolved in a minimal volume of 50% acetonitrile and control slants received an equal concentration of acetonitrile. On d 18 plants were harvested and tap root length, lateral root number and the length of longest lateral were recorded.

Hormone analysis

For strigolactone quantification, approximately 2 g (fresh weight) of whole root tissue (3 to 4 plants per replicate) was harvested from 3-4 week old plants and SLs were purified and measured as described by Foo et al. (2013a) with the inclusion of labelled strigolactone standards ([6'-$^2H_1$]fabacyl acetate, [6'-$^2H_1$]orobanchol and [6'-$^2H_1$]orobanchyl acetate). For ethylene quantification plants were grown in sterile vermiculite, 3 per 250 ml glass jar in a growth cabinet (as described in *plant material and growth conditions* section) for 12 d and ethylene evolution from whole plants was performed from 4 replicate jars, as described by Foo et al. (2016b).

Gene expression

For the gene expression studies in Fig. 5 and Suppl. Fig. 3 and 4, plants were grown for 10 d and root tip tissue harvested (d 0). The remaining plants were inoculated with a 10% solution of a 3 d old culture of RLV248 and 2 and 4 d later root tip tissue was harvested. For gene expression studies in Fig. 6 and Fig. 7A, whole root tissue (3 to 4 plants per replicate) was harvested from 3-4 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 gene expression studies in Fig. 7B, after ethylene

levels were measured plants were removed from jars and the whole root tissue (3 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, Alexandria, Australia). cDNA was synthesised from 1 μg of RNA using the SensiFAST^{TM} cDNA Synthesis Kit (Bioline). cDNA was diluted and duplicate, real-time PCRs were performed in a Rotor Gene 2000 (Corbett, USA) using the SensiFAST^{TM} SYBR® Hi-ROX Kit (Bioline) and 100-200 pmol of a primer pair. Primer pairs for genes analysed in this study were as follows: *PsD27* (F 5'-CAAGCAGCAACAGG AATCAG-3', R 5'-TTGATGGTGGCATCACTCTC-3'), *PsCCD7* (*RMS5* F and R; Johnson et al. 2006), *PsCCD8* (*RMS1* F and R; Johnson et al., 2006), *PsENOD12a* (Foo et al., 2015), *PsENOD12b* (F 5'-TGAACCACCAGTGAATGAGC-3', R 5'-TGGATGTTATGTTCCGCTGT-3'), *PsENOD40* (Foo et al., 2015), *PsACS1* (Foo et al., 2006), *PsACOX* (Foo et al., 2006) 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, Vienna, Austria), followed by Tukey's HSD post-tests where appropriate. When appropriate the data was log transformed prior to analysis.

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Figure legends

Figure 1. Scheme of physical and molecular changes during nodule development in pea, including the proposed position of genes in the Nod LCO signalling pathway that influence a complex non-linear network of transcription factors (TFs). (A) Root hair curling, (B) infection thread formation, (C) developing nodule not visible to naked eye, (D) maturing nodules and (E) young mature nodule. For (A-E) photos of pea roots colonised with blue stained *lacZ*- labelled rhizobia.

Figure 2. The influence of wild type (WT) and strigolactone-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 Parvus or *ccd8-1* root exudates compared with a solvent control (n=4) and (C) percentage of root hairs curled in wild type 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 mean ± s.e. and analysis of each experiment by ANOVA indicated no significant differences between *ccd8* and its WT progenitor.

779 Figure 3. Nodule development in wild type cv. Parvus (WT) and strigolactone-
780 deficient *ccd8-1* plants infected with *lacZ* labeled *Rhizobium leguminosarum* bv.
781 *viciae*. (A) Number of infection threads per cm of root (4-10 root segments from n =
782 6-11 plants) in two independent experiments, (B) photo of stained roots showing
783 infection threads in WT and *ccd8-1* plants (scale bars are 0.1 mm), (C) photo of
784 stained root showing nodules in WT and *ccd8-1* plants (scale bar is 5 mm), (D)
785 number of developing nodules per cm of root (10 root segments from n = 15 plants)
786 and (E) ethylene evolution per gram nodule dry weight (DW) from acetylene
787 reductase assay (n=4). Values are mean ± s.e. and values statistically different from
788 WT are indicated by * ($P<0.05$). In (B) and (C) *R. leguminosarum* is stained blue.
789
790 Figure 4. Nodule development in wild type cv. Torsdag (WT) and strigolactone-
791 deficient *ccd8-2* plants infected with *lacZ* labeled *Rhizobium leguminosarum* bv.
792 *Viciae* treated with synthetic strigolactone (+)-GR24 or solvent control. (A) Number
793 of infection threads per cm of root and (B) total number of nodules per cm of root.
794 Values are mean ± s.e., (1-2 root segments from n = 12-13 plants) and values with
795 different letters are significantly different ($P<0.05$).
796
797 Figure 5. The expression of early nodulation (*ENOD)* genes 0, 2 and 4 days following
798 inoculation with *Rhizobium leguminosarum* bv. *viciae* in wild type (WT) cv. Parvus
799 and the strigolactone-deficient *ccd8-1* mutant. Values are mean ± s.e, n=3, n.d. is not
800 done. For day 2 and day 4, values with different letters are significantly different
801 ($P<0.05$).
802
803 Figure 6. Strigolactone levels and expression of strigolactone biosynthesis genes in
804 root tissue of various symbiosis mutants. (A) Fold-change relative to respective wild
805 types (WT) of the major canonical strigolactone in pea, fabacyl acetate, in root tissue
806 of various pea symbiosis mutants and (B) the fold-change relative to respective WT of
807 the expression of strigolactone biosynthesis genes *CCD7, CCD8* and *D27* in root
808 tissue of pea symbiosis mutants. Values statistically different from WT are indicated
809 by * ($P<0.05$), **($P<0.01$) and ***($P<0.001$). Values are means ± 95% c.i. (n=4).
810
811 Figure 7. Strigolactone and ethylene levels and expression of biosynthesis genes in

812 strigolactone-deficient *ccd8-1* or ethylene-insensitive *ein2* plants compared with their
813 respective wild types (WT). (A) Levels of three strigolactones, fabacyl acetate,
814 orobanchol and orobanchyl acetate and relative expression of strigolactone
815 biosynthesis genes *CCD7, CCD8* and *D27* in root tissue of WT (cv. Torsdag) and *ein2*
816 mutant plants and (B) ethylene evolution from whole plants of WT (cv. Parvus) and
817 *ccd8-1* and expression of ethylene metabolism genes *ACS1* and *ACOX* in roots of
818 these plants. Values are mean ± s.e, n=4 and analysis of each parameter by t-test
819 indicated no significant differences between mutant and WT.
820
821 Figure 8. Interactions between ethylene and strigolactone during nodulation. (A)
822 Nodule number in wild type cv. Torsdag (WT), strigolactone-deficient *ccd8-2* or
823 ethylene-insensitive *ein2* pea mutants treated with synthetic strigolactone (+)-GR24,
824 ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) or solvent control (n=6-
825 11) n.d. is not done, (B) nodule number in WT, strigolactone-deficient *ccd7*, *ein2* and
826 double mutant *ccd7 ein2* segregants (n=5-7) and (C) photo of nodules on secondary
827 roots (tertiary roots have been removed, scale bar =5mm). Values are mean ± s.e. For
828 (A) separate two-way ANOVAs were performed for the *ccd8* and *ein2* comparisons,
829 and for (B) a one-way ANOVA was performed and values with different letters are
830 significantly different ($P<0.05$).
831
832 SUPPLEMENTAL DATA
833 Supplementary Figure 1. Nodule development in wild type (WT), strigolactone-
834 deficient *ccd8-2* and strigolactone-insensitive *d14-1* plants infected with *lacZ*-
835 labelled *Rhizobium leguminosarum* bv. *viciae*.
836 Supplementary Figure 2. Nodule number in blue lupin (*Lupinus angustifolius*)
837 following treatment with (+)-GR24 or solvent control.
838 Supplementary Figure 3. The expression of strigolactone biosynthesis genes *CCD7,*
839 *CCD8* and *D27* 0, 2 and 4 days following inoculation with *Rhizobium leguminosarum*
840 bv. *viciae* in wild type cv.
841 Supplementary Figure 4. The expression of disease marker genes 0, 2 and 4 days
842 following inoculation with *Rhizobium leguminosarum* bv. *viciae* in wild type cv.
843 Supplementary Figure 5. Root development in wild type cv.
844 Supplementary Figure 6. Fold-change relative to respective wild type (WT) of the

canonical strigolactones (A) orobanchol and (B) orobanchyl acetate in various pea symbiosis mutants.

Supplemental Table 1. Putative flavonoid ions monitored via selected ion monitoring compound retention times and UV absorbance data for stronger signals.

Supplementary Figure 1. Nodule development in wild type (WT), strigolactone-deficient *ccd8-2* and strigolactone-insensitive *d14-1* plants infected with *lacZ*-labelled *Rhizobium leguminosarum* bv. *viciae*. All plants were on a Torsdag background. (A) Number of infection threads per cm of rot, (B) number of nodules (sum of nodules visible only under magnification and those visible to the naked eye) per cm of root and (C) length of roots. Values are mean ± s.e., n=12 plants with 3 lateral roots measured for each plant. Values with different letters are significantly different ($P<0.05$).

Supplementary Figure 2. Nodule number in blue lupin (*Lupinus angustifolius*) following treatment with (+)-GR24 or solvent control. Values are mean ± s.e, (n=13-14). Analysis by t-test indicated no significant difference between treatments.

Supplementary Figure 3. The expression of strigolactone biosynthesis genes *CCD7, CCD8* and *D27* 0, 2 and 4 days following inoculation with *Rhizobium leguminosarum* bv. *viciae* in wild type cv. Parvus (WT). Values are mean ± s.e, n=3, n.d. is not done. For WT, values with different letters are significantly different ($P<0.05$).

Supplementary Figure 4. The expression of disease marker genes 0, 2 and 4 days following inoculation with *Rhizobium leguminosarum* bv. *viciae* in wild type cv. Parvus (WT) and *ccd8-1* plants. Values are mean ± s.e, n=3, values with different letters are significantly different ($P<0.05$).

Supplementary Figure 5. Root development in wild type cv. Parvus (WT) and strigolactone-deficient *ccd8-1* mutant plants following treatment with 0.1 μM Nod

LCO or solvent control. (A) Number of secondary lateral roots and (B) average length of secondary roots. Values are mean ± s.e, n=10-19. 2-way ANOVAs showed that NodLCO treatment significantly affected lateral root number and length ($P<0.01$). However, there was no significant interaction between genotype and treatment.

Supplementary Figure 6. Fold-change relative to respective wild type (WT) of the canonical strigolactones (A) orobanchol and (B) orobanchyl acetate in various pea symbiosis mutants. Values are means ± 95% c.i. (n=4). Values statistically different from WT are indicated by * ($P<0.05$).

Supplemental Table 1. Putative flavonoid ions monitored via selected ion monitoring compound retention times and UV absorbance data for stronger signals. Please note, where there are two peaks at the same *m/z* value, the UV data is for the one in bold.

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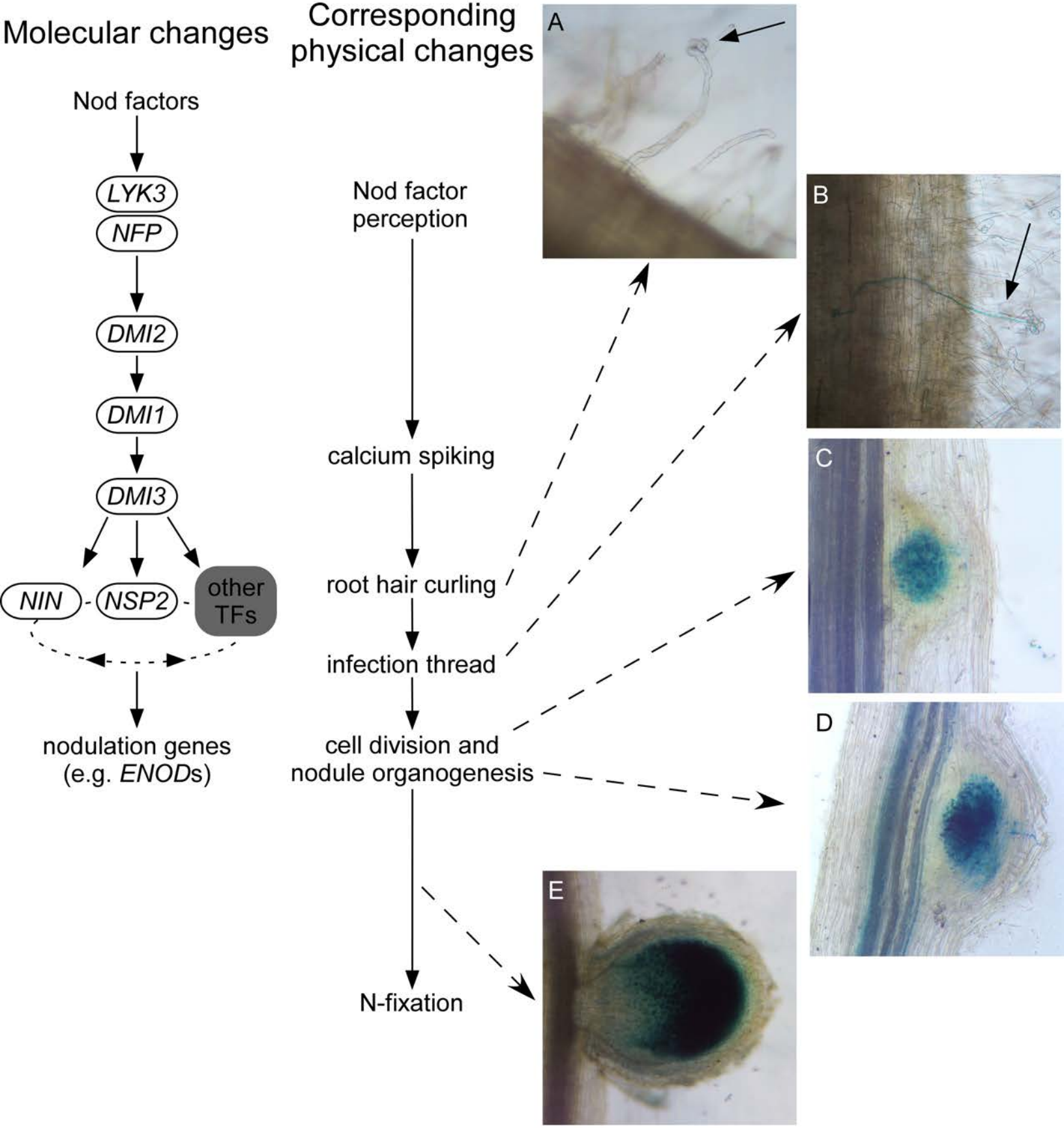

Figure 1. Scheme of physical and molecular changes during nodule development in pea, including the proposed position of genes in the Nod factor signalling pathway that influence a complex non-linear network of transcription factors (TFs). (A) Root hair curling, (B) infection thread formation, (C) developing nodule not visible to naked eye, (D) maturing nodules and (E) young mature nodule. For (A-E) photos of pea roots colonised with blue stained *lacZ*- labelled rhizobia.

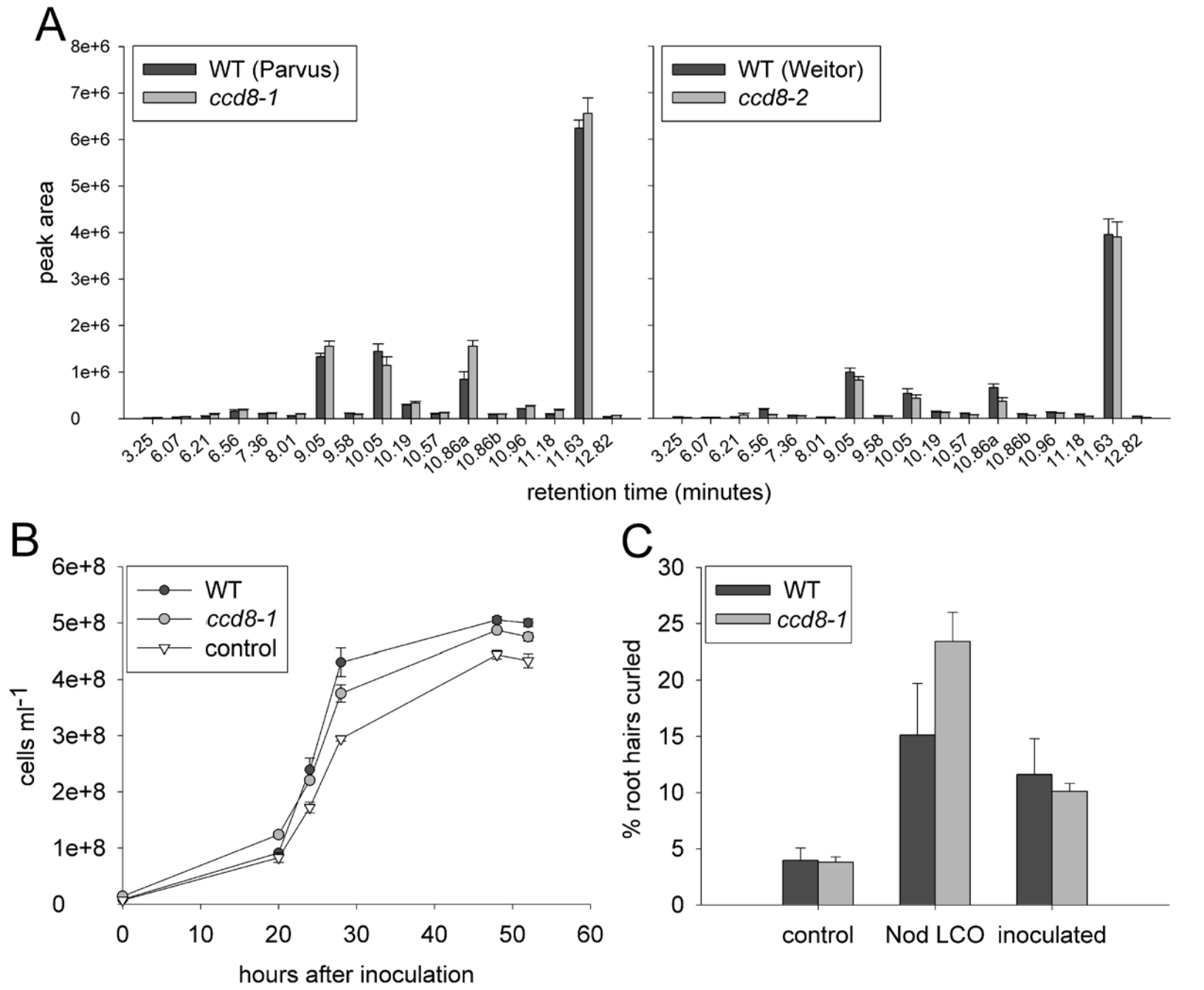

Figure 2. The influence of wild type (WT) and strigolactone-deficient *ccd8* mutants on early events in nodulation. (A) Flavonoid profile in root exudate of *ccd8-1* and *ccd8-2* mutants and their respective wild types, cv. Parvus and cv. Weitor (n=4), (B) growth of *Rhizobium leguminosarum* bv. *viciae* (RLV248) over time after treatment with wild type Parvus or *ccd8-1* root exudates compared with a solvent control (n=4) and (C) percentage of root hairs curled in wild type 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 mean ± s.e. and analysis of each experiment by ANOVA indicated no significant differences between *ccd8* and its WT progenitor.

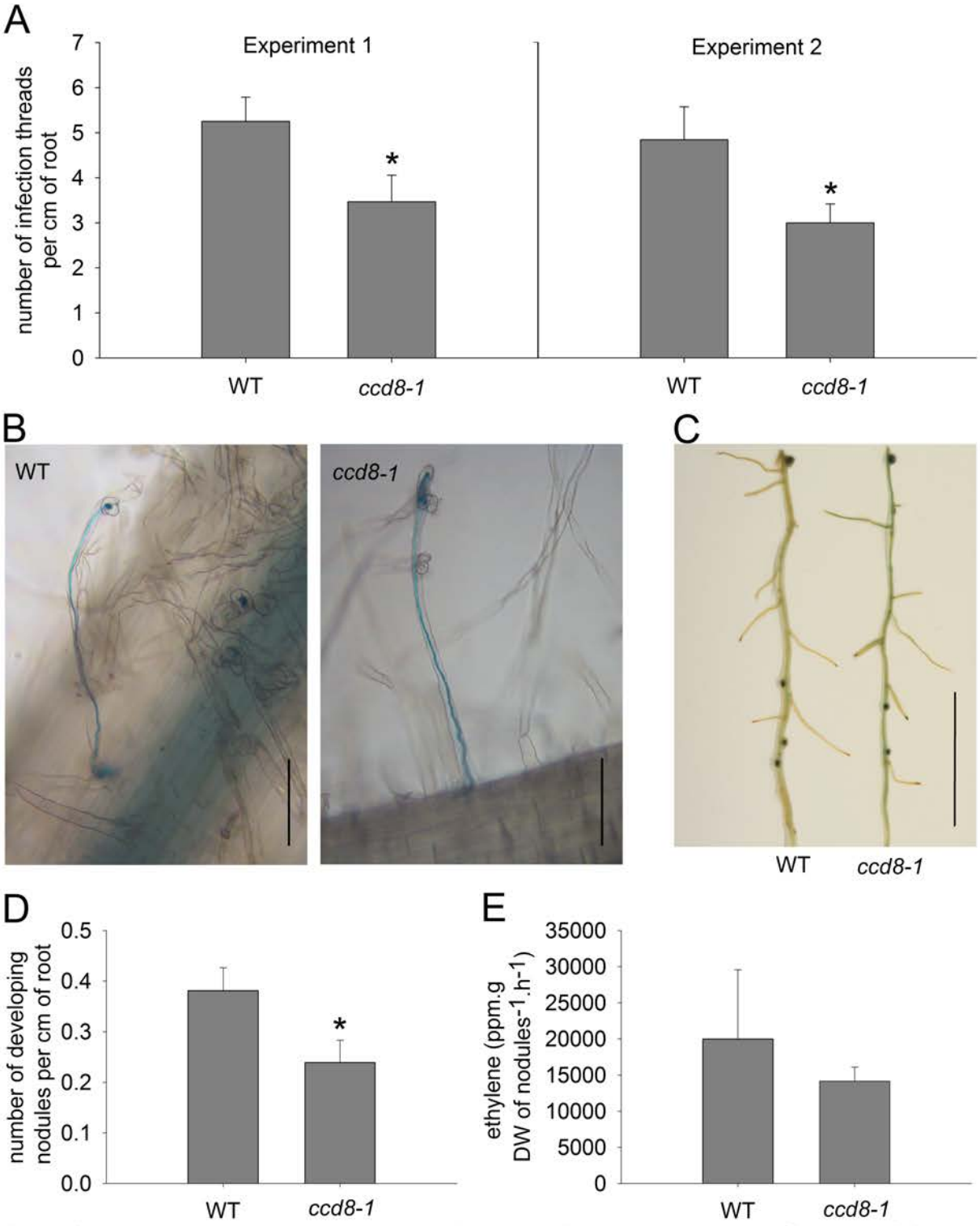

Figure 3. Nodule development in wild type cv. Parvus (WT) and strigolactone-deficient *ccd8-1* plants infected with *lacZ*- labeled *Rhizobium leguminosarum* bv. *viciace*. (A) Number of infecton threads per cm of root (4-10 root segments from n=6-11 plants) in two independent experiments, (B) photo of stained roots showing infection threads in WT and *ccd8-1* plants (scale bars are 0.1 mm), (C) photo of stained root showing nodules in WT and *ccd8-1* plants (scale bar is 5 mm), (D) number of developing nodules per cm of root (10 root segments from n=15 plants) and (E) ethylene evolution per gram nodule dry weight(DW) from acetylene reductase assay (n=4). Values are mean ± s.e. and values statistically different from WT are indicated by * (P<0.05). In (B) and (C) *R. leguminosarum* are stained blue.

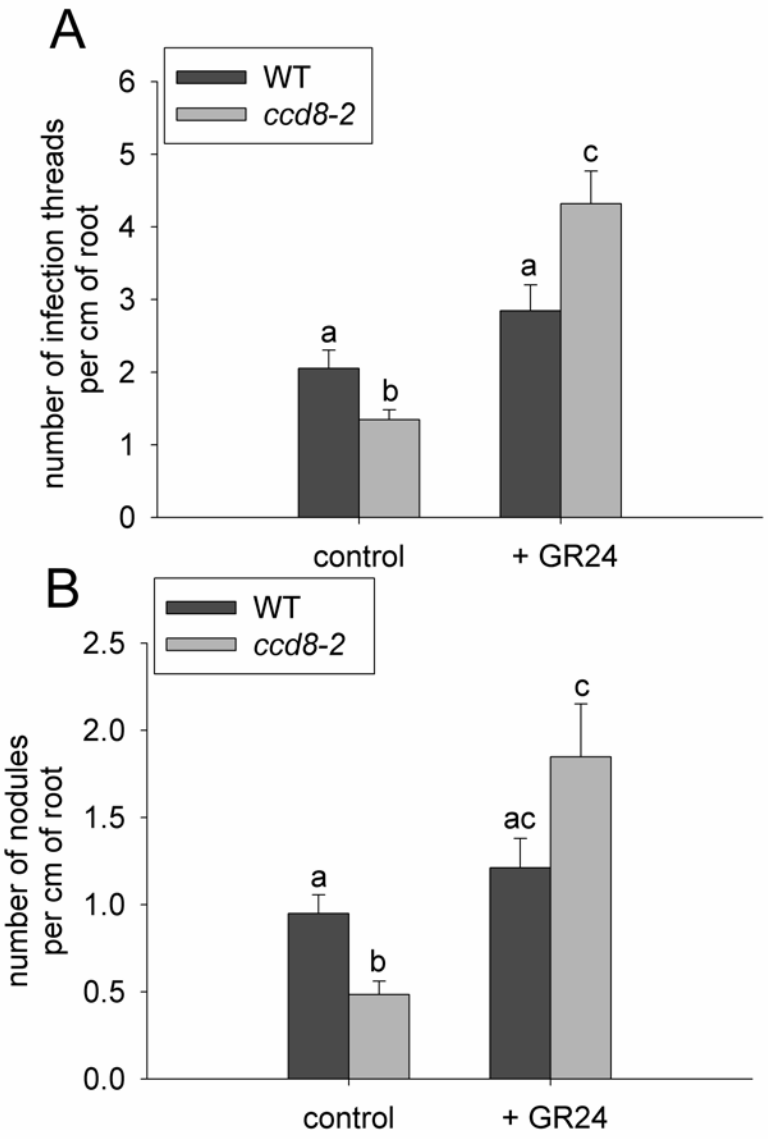

Figure 4. Nodule development in wild type cv. Torsdag (WT) and strigolactone-deficient *ccd8-2* plants infected with *lacZ*-labeled *Rhizobium leguminosarum* bv. *viciae* treated with synthetic strigolactone (+)-GR24 or solvent control. (A) Number of infection threads per cm of root and (B) total number of nodules per cm of root. Values are mean ± s.e., (1-2 root segments from n = 12-13 plants) and values with different letters are significantly different ($P<0.05$).

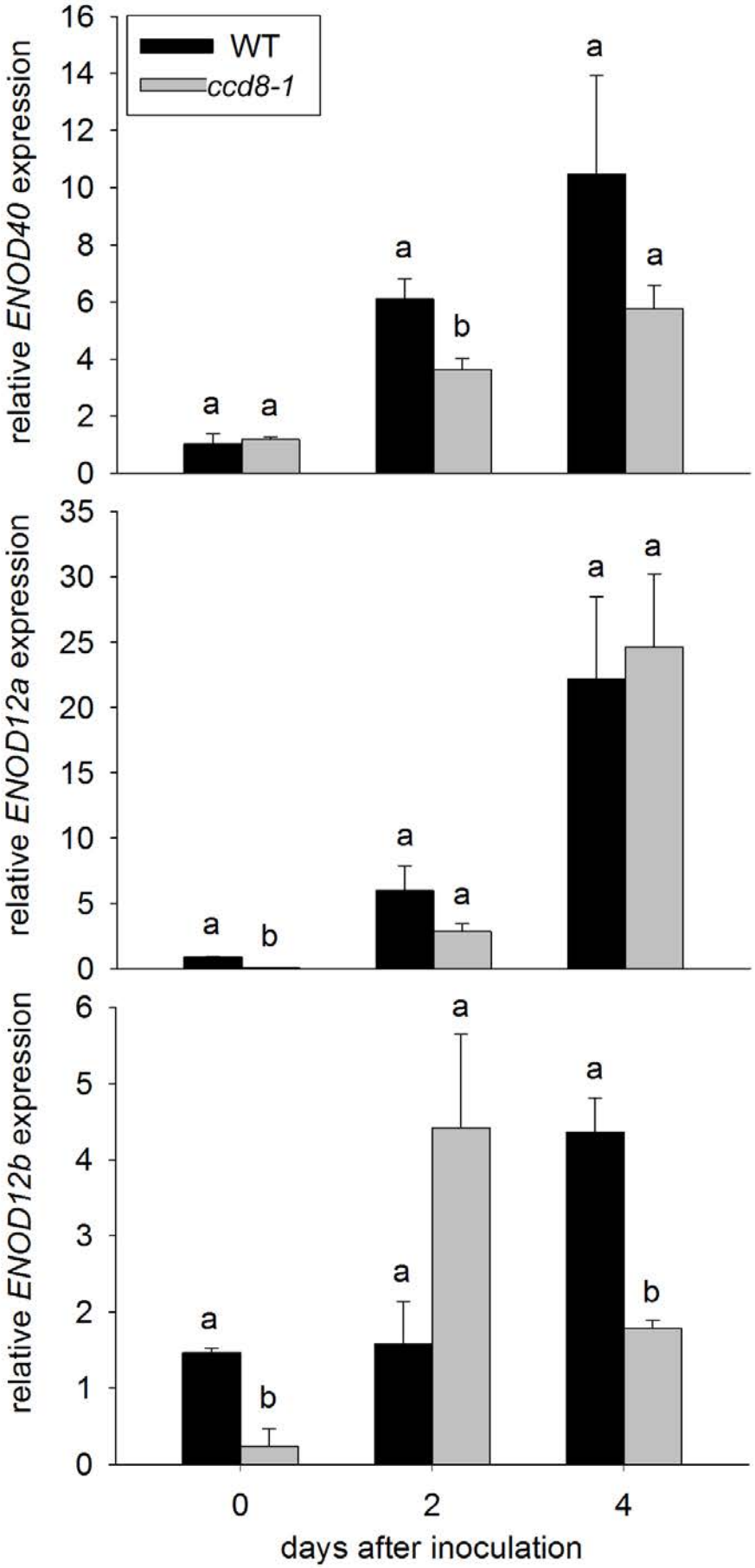

Figure 5. The expression of early nodulation (*ENOD*) genes 0, 2 and 4 days following inoculation with *Rhizobium leguminosarum* bv. *viciae* in wild type (WT) cv. Parvus and strigolactone-deficient *ccd8-1* mutant plants. Values are mean ± s.e, n=3. Values with different letters are significantly different (P<0.05).

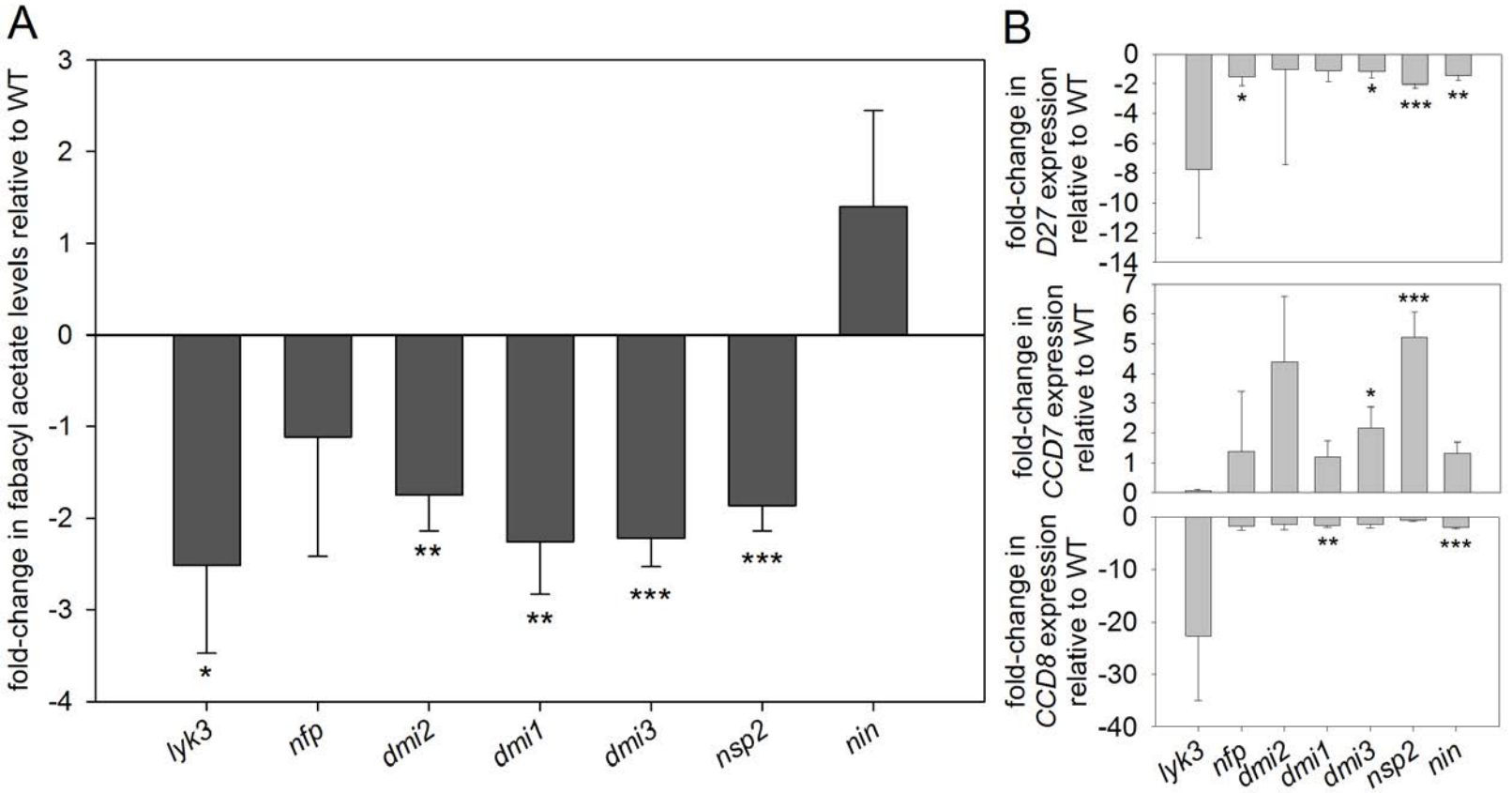

Figure 6. Strigolactone levels and expression of strigolactone biosynthesis genes in root tissue of various symbiosis mutants. (A) Fold-change relative to respective wild types (WT) of the major canonical strigolactone in pea, fabacyl acetate, in root tissue of various pea symbiosis mutants and (B) the fold-change relative to respective WT of the expression of strigolactone biosynthesis genes *CCD7*, *CCD8* and *D27* in root tissue of pea symbiosis mutants. Values statistically different from WT are indicated by * (P<0.05), **(P<0.01) and ***(P<0.001). Values are means ± 95% c.i. (n=4).

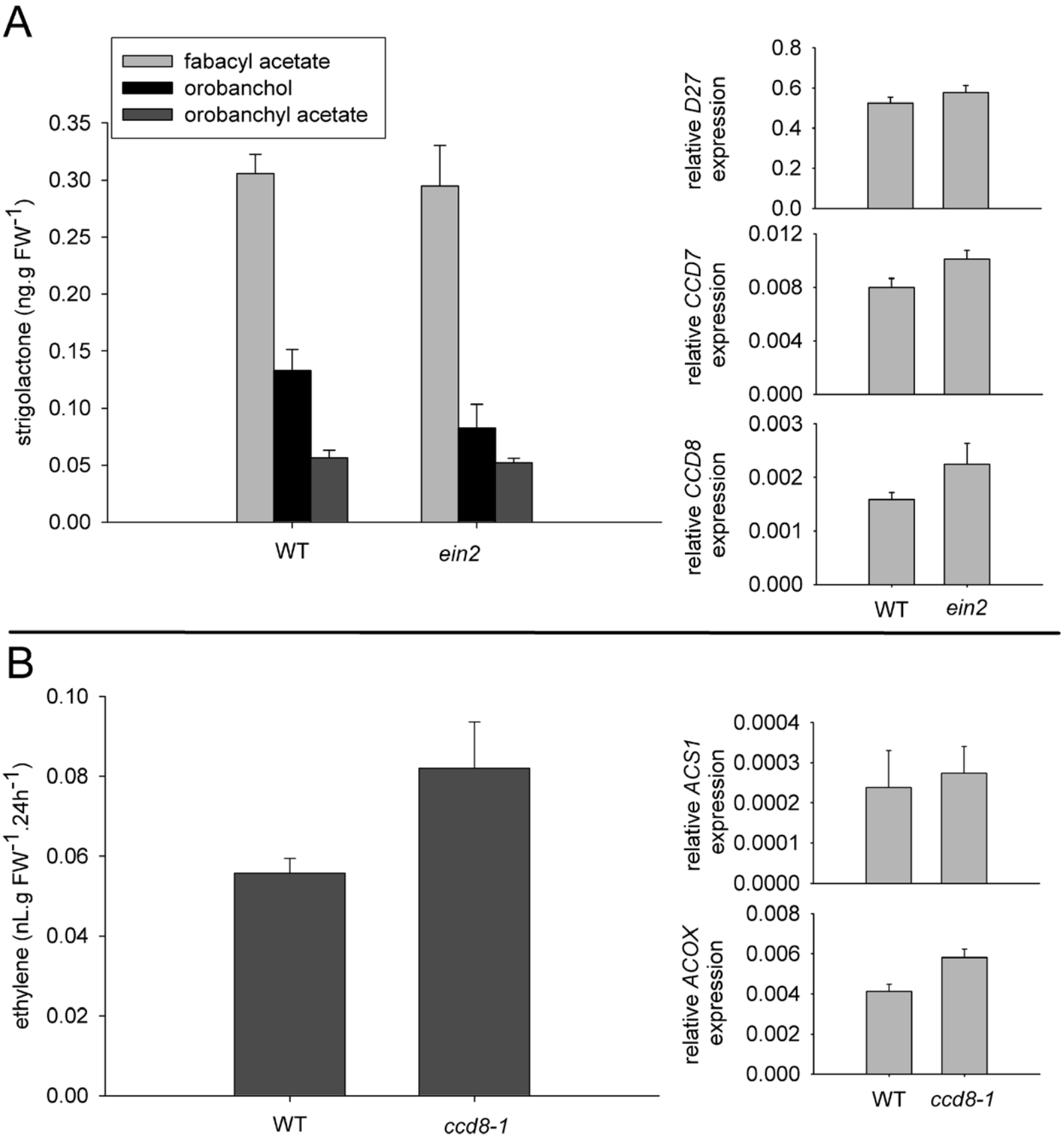

Figure 7. Strigolactone and ethylene levels and expression of biosynthesis genes in strigolactone-deficient *ccd8-1* or ethylene-insensitive *ein2* plants compared with wild type cv. Torsdag (WT). (A) Levels of three strigolactones, fabacyl acetate, orobanchol and orobanchyl acetate and relative expression of strigolactone biosynthesis genes *CCD7*, *CCD8* and *D27* in root tissue of WT and *ein2* mutant plants and (B) ethylene evolution from whole plants of WT and *ccd8-1* and expression of ethylene metabolism genes *ACS1* and *ACOX* in roots of these plants. Values are mean ± s.e, n=4 and analysis of each parameter by t-test indicated no significant differences between mutant and WT.

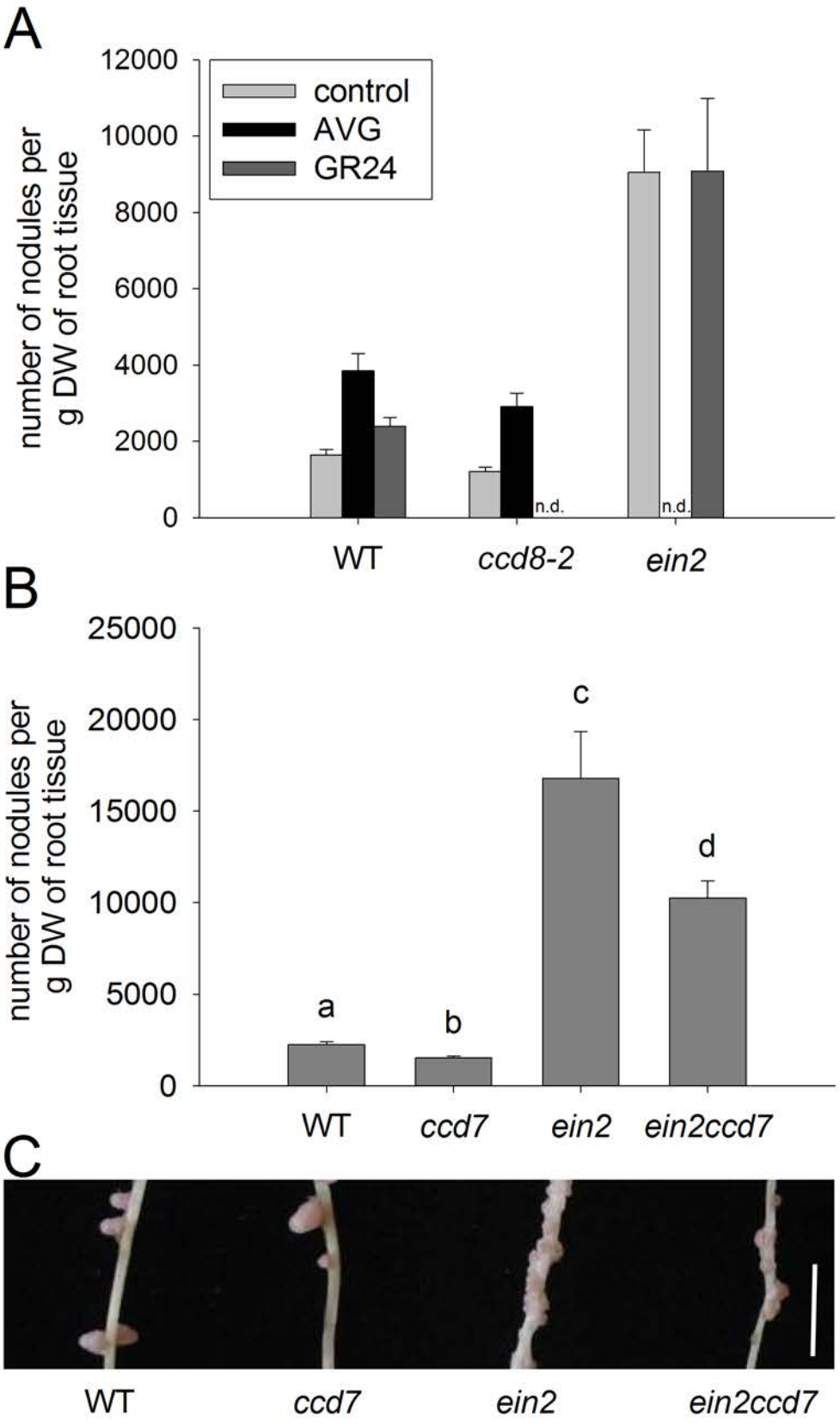

Figure 8. Interactions between ethylene and strigolactone during nodulation. (A) Nodule number in wild type cv. Torsdag (WT), strigolactone-deficient *ccd8-2* or ethylene-insensitive *ein2* pea mutants treated with synthetic strigolactone (+)-GR24, ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) or solvent control (n=6-11) n.d. is not done, (B) visible nodule number in WT, strigolactone-deficient *ccd7*, *ein2* and double mutant *ccd7 ein2* segregants (n=5-7) and (C) photo of nodules on secondary roots (tertiary roots have been removed, scale bar =5mm). Values are mean ± s.e. 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).

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