From lateral root density to nodule number, the strigolactone analogue GR24 shapes the root architecture of *Medicago truncatula*

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For the first time, strigolactones are shown to influence lateral root development and nodulation in *Medicago truncatula.* Nodulation is affected at early stages and crosstalks with ethylene signalling are involved.

Abstract

In the rhizosphere, strigolactones not only act as crucial signalling molecules in the communication of plants with parasitic weeds and arbuscular mycorrhiza, but they also play a key role in regulating different aspects of the root system. Here, we investigated how strigolactones influence the root architecture of *Medicago truncatula*. We provide evidence that addition of the synthetic strigolactone analogue GR24 has an inhibitory effect on the lateral root density. Moreover, GR24 treatment of *Sinorhizobium meliloti*-inoculated *M. truncatula* plants affects the nodule number both positively and negatively, depending on the concentration. Plants treated with 0.1 µM GR24 had a slightly increased number of nodules, whereas concentrations of 2 µM and 5 µM strongly reduced it. This effect was independent of the autoregulation of nodulation mechanism that is controlled by SUPER NUMERIC NODULE. Furthermore, we demonstrate that GR24 controls the nodule number through crosstalk with the SICKLE-dependent ethylene signalling. Moreover, because the expression of the nodulation marker *EARLY NODULATION11* was strongly reduced in GR24-treated plants, we concluded that strigolactones influence nodulation at a very early stage of the symbiotic interaction.

Introduction

Strigolactones, originally characterized as rhizosphere molecules, are a group of terpenoid lactones that stimulate germination of root-parasitic weeds (Cook *et al*., 1966; Xie *et al*., 2010) and induce hyphal branching of arbuscular mycorrhizal fungi (Akiyama *et al*., 2005). Strigolactones were also found to act as endogenous hormones involved in the regulation of shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Meanwhile, these molecules have become a cutting-edge topic in plant biology because they affect many aspects of plant development, such as light-regulated development (Tsuchiya *et al*., 2010), secondary growth processes (Agusti *et al*., 2011), branching of moss protonema (Proust *et al*., 2011), and seed germination of nonparasitic plants (Toh *et al*., 2012).

These hormones also play a key role in the regulation of the root architecture (Rasmussen *et al*., 2013). Indeed, a potential role for strigolactones on the primary root length has been demonstrated, albeit with various results. In rice (*Oryza sativa*), the synthetic strigolactone analogue GR24 induces root elongation at concentrations as high as 10 µM (Arite *et al*., 2012), whereas in *Arabidopsis thaliana* the primary root length is inhibited by GR24 at concentrations higher than 2.5 µM but induced at lower concentrations (Ruyter-Spira *et al*., 2011). GR24 also affects the development of lateral roots. In *Arabidopsis*, the lateral root density of wild-type plants and strigolactone-deficient mutants decreases after GR24 treatment (Kapulnik *et al*., 2011a). This inhibitory effect was only observerd under phosphate-sufficient conditions, whereas it diminished under phosphate-limiting conditions (Ruyter-Spira *et al*., 2011). Strigolactones are also involved in root hair elongation. In *Arabidopsis*, treatment with low GR24 concentrations increases the root hair length (Kapulnik *et al*., 2011a), but with very high concentrations inhibits root hair elongation in tomato (*Solanum lycopersicum*) (Koltai *et al*., 2010). Another effect of GR24 on the root system is the inhibition of adventitious root formation. For example, this phenotype was observed in stem cuttings of pea (*Pisum sativum*) (Rasmussen *et al*., 2012), tomato (Kohlen *et al*., 2013), and in etiolated hypocotyls of *Arabidopsis* (Rasmussen *et al*., 2012).

Nodulation, another important aspect of legume root architecture that occurs under nitrogen deficiency, is also affected by strigolactones (Soto *et al*., 2010; Foo

and Davies, 2011). *Medicago truncatula* develops novel root organs, nodules, as a result of a symbiotic interaction with the soil-borne bacteria, *Sinorhizobium meliloti*. Inside these nodules, the bacteria fix atmospheric nitrogen to ammonia (NH_3) that is subsequently converted to ammonium (NH_4), which is assimilated by the plant. In return, the microsymbiont receives carbon sources and a protective niche (Oldroyd, 2013). Nodule formation is a complex developmental process that is initiated by a chemical crosstalk between the macro- and microsymbionts and involves the rhizobial secretion of lipo-chitooligosaccharides, known as Nod factors (NFs) (Dénarié *et al*., 1996). Perception of NFs activates the *EARLY NODULATION (ENOD)* genes, which, in turn, trigger several signalling pathways that subsequently initiate infection and nodule primordium formation (Mortier *et al*., 2012). In *M. truncatula*, the *ENOD11* gene has been shown to be a powerful marker for both bacterial infection and NF signalling at the epidermis (Journet *et al*., 2001; Charron *et al*., 2004; Cerri *et al*., 2012).

As nodulation is an energy-consuming process, legumes have developed several mechanisms to strictly control the number of nodules. One mechanism acts locally on the rhizobial infection and involves ethylene signalling (Oldroyd *et al*., 2001), whereas another mechanism, the autoregulation of nodulation (AON), comprises systemic signal exchanges between roots and shoots (Mortier *et al*., 2012). Nitrate availability in the soil also negatively regulates nodule numbers and nitrate, at least in part, acts on the AON pathway to exert its control (Mohd-Radzman *et al*., 2013).

In addition to environmental factors, hormones play a central role during nodulation. Cytokinin and auxin are especially important for nodule development, but there is also evidence for the involvement of other hormones, such as abscisic acid, ethylene, gibberellin and jasmonic acid, during the initiation and/or the coordination of nodulation (Ryu *et al*., 2012). During nodulation, strigolactones seem to act mainly as hormones and not as rhizosphere signalling molecules because exogenous treatment with GR24 does not change bacterial growth, NF production, and bacterial calcium spiking (Moscatiello *et al*., 2010; Soto *et al*., 2010). Hence, in the rhizosphere, strigolactones are a recognition signal only for parasitic plants (Xie *et al*., 2010) and arbuscular mycorrhizal fungi (Akiyama *et al*., 2005; Genre *et al*., 2013), but not for rhizobia (Foo and Davies, 2011). Nevertheless, application of low

concentrations of GR24 significantly increases the number of nodules in alfalfa (*Medicago sativa*) (Soto *et al*., 2010). Furthermore, the strigolactone-deficient *ramosus1* (*rms1*) mutant in pea, affected in the strigolactone biosynthesis gene *CAROTENOID CLEAVAGE DIOXYGENASE8* (*CCD8*) carries fewer nodules than wild-type plants (Foo and Davies, 2011). Additionally, treatment with GR24 can revert the *rms1* phenotype, in accordance with the observations made in alfalfa. Recently, a similar phenotype was observed for the strigolactone-deficient *Lotus japonicus CCD7*-silenced plants (Liu *et al*., 2013). Together, these data demonstrate a positive role for strigolactones in both determinate (*L. japonicus*) and indeterminate (pea and *M. sativa*) nodule development. As the strigolactone-deficient *rms1* mutant could still form functional nodules, strigolactones might not be essential for nodule formation, but rather for the determination of the optimal nodule number. However, *rms4* mutants affected in strigolactone signalling carry more nodules than wild type plants, in contrast to the phenotype to *rms1* (Foo *et al*., 2013). This discrepancy demonstrates that there is still much to be discovered about strigolactone signalling during nodulation.

Here, the role of GR24 on the root architecture of *M. truncatula* was studied for the first time. Besides its effect on both primary and lateral root development, GR24 was shown to influence nodulation on *M. truncatula* as well. New insights into the mechanism used by strigolactones to interfere with the nodulation process are provided.

Materials and methods

Biological material

Medicago truncatula Gaertn. cv. Jemalong J5, the *sickle (skl)* and *super numeric nodules4 (sunn-4)* mutants (Penmetsa and Cook, 1997; Schnabel *et al*., 2005), and the *pENOD11:*β-glucuronidase (*GUS)* transgenic plants (Journet *et al*., 2001) were grown and inoculated as described (Mergaert *et al*., 2003). *Sinorhizobium meliloti 2011* pHC60-GFP (Cheng and Walker, 1998) were cultured at $28^{\circ}C$ in yeast extract broth medium (Vervliet *et al*., 1975), supplemented with 10 mg L^{-1} tetracycline.

In vitro *application of GR24*

The racemic synthetic strigolactone analogue GR24 was dissolved in acetone to a 10 mM concentration and further diluted in the sterile medium. As control, acetone without supplemented hormones was used.

For root development studies, plants were grown *in vitro*, in small square Petri dishes (12x12 cm) on M medium (Bécard and Fortin, 1988), but without sucrose and vitamins and with or without GR24 at a final concentration of 0.1 μM or 2 μM. Plants were cultured at 24°C, with a 16-h/8-h photoperiod under $70\ \mu E\ m^{-2}\ m^{-1}$ light intensity per day. At 5 days post germination (dpg) and at 12 dpg, plants were transferred to large Petri dishes (24x24 cm) on an identical medium as described above to renew the medium. At 19 dpg, lateral root numbers were counted and the main root lengths were measured by means of the ImageJ 1.45 software (http://rsb.info.nih.gov/ij/index.html).

For nodulation experiments, plants were grown *in vitro*, in small square Petri dishes (12x12 cm) on nitrogen-poor agar-containing SOLi medium (Blondon, 1964) supplemented with or without GR24 at a final concentration of 0.1 μM, 0.2 μM, 2 μM, or 5 μM. Plants were cultured under the same conditions as described above. Before inoculation, the root hair length in the susceptible root zone for nodulation (Sieberer and Emons, 2000; Gage, 2004) was measured using the ImageJ 1.45 software. Next, plants were inoculated at 5 dpg with *Sm2011* pHC60-GFP by immersing the roots in a bacterial solution at OD_{600}=0.01. After inoculation and at 12 dpg, plants were transferred to large Petri dishes (24x24 cm) on an identical medium. At 14 days post inoculation (dpi), nodule numbers were counted and main root lengths were measured with the ImageJ 1.45 software. Nodules appeared on both the main root and on some lateral roots; all nodules were taken into account for the analysis.

For nodulation experiments in perlite, 2-day-old seedlings were transferred to perlite and grown for 5 days in nitrogen-poor SOLi medium before inoculation. The nodule number was counted at 14 dpi. Every 2 or 3 days, new SOLi medium supplemented with GR24 at the adequate concentration were added to the pots to establish a continuous GR24 amount.

For quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) analysis, plants were grown and inoculated as described above for the nodulation. Before and 1, 2, and 4 dpi, the root zone susceptible for rhizobial infection was harvested. Four independent biological repeats were done.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. Genomic DNA was removed by DNase treatment and RNA was purified through NH_4Ac (5 M) precipitation. Samples were quality-controlled and quantified with a Nano-Drop Spectrophotometer (Isogen). One microgram of RNA was reverse-transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad) and subsequently diluted 25 times. Real-time qRT-PCR was done on a LightCycler 480 (Roche Diagnostics) with SYBR Green for detection, in triplicate on a 384-multiwell plate, in a total volume of 5 μL and a cDNA fraction of 10%. Cycle threshold values were obtained with the accompanying software and analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533, MGI). Primers used (Supplementary Table S1 available at *JXB* online) were unique in the MGI version 9.0 and the Medicago EST Navigation System database (Journet *et al.*, 2001).

Histochemical localization of GUS activity

GUS activity in *pENOD11:GUS* transgenic roots was analyzed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate as described (Van den Eede *et al.*, 1992). Roots were vacuum-infiltrated for 20 min and subsequently incubated for 12 h in GUS buffer at 37°C. After staining, roots were fixed and dehydrated in 70% ethanol. Photographs were taken with a stereomicroscope MZFLII (Leica Microsystems, Wetzlar, Germany).

Results

Influence of GR24 on the M. truncatula lateral root density

As an inhibitory or stimulating role of GR24 on both primary root growth and lateral root development had been shown (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011; Arite *et al*., 2012; Sun *et al*., 2014), the impact of GR24 on the root development in *M. truncatula* was investigated. Different concentrations of GR24 were supplied exogenously to the M growth medium of 2-day-old seedlings. After 19 days of growth, the main root length was measured (Fig. 1A) and the number of emerged lateral roots was counted (Fig. 1B). Both parameters were further used to determine the lateral root density (Fig. 1C). Treatment with 0.1 µM and 2 µM GR24 resulted in an average main root length of 12.98 ± 0.39 cm and 13.29 ± 0.37 cm, respectively, not significantly different from the average main root length of 13.01 ± 0.38 cm measured for control mock-treated plants ($P = 0.943$ and $P = 0.608$; Student's *t*-tests) (Fig. 1A). In contrast, the average lateral root number was significantly reduced when plants were grown in the presence of 0.1 µM or 2 µM GR24: on average, 3.95 ± 0.27 and 2.89 ± 0.22 lateral roots were counted, respectively, versus 4.95 ± 0.27 lateral roots in control mock-treated plants ($P < 0.01$ and $P < 0.001$; Student's *t*-tests) (Fig. 1B). Finally, plants treated with 0.1 µM or 2 µM GR24 had significantly lower lateral root density (0.31 ± 0.02 and 0.22 ± 0.02 lateral roots per cm, respectively) than the control plants (0.39 ± 0.02 lateral roots per cm) ($P < 0.01$ and $P < 0.001$; Student's *t*-tests) (Fig. 1C). Hence, in agreement with data published for *Arabidopsis* (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011), exogenous treatment of GR24 negatively regulated the lateral root density in *M. truncatula*.

Influence of GR24 on the development of inoculated roots

Nodule formation in alfalfa and pea had been shown to be stimulated in the presence of GR24 (Soto *et al*., 2010; Foo and Davies, 2011). Therefore, to examine the effect of GR24 on *M. truncatula* nodulation, 2-day-old seedlings were grown *in vitro* on nitrogen-poor SOLi medium supplemented with increasing concentrations of GR24. Treatment with 0.1 µM GR24 resulted in a weak, but significant, stimulation of nodulation. On average 12.21 ± 0.55 nodules per plant were counted versus 10.67 ± 0.50 nodules on the corresponding mock-treated plants ($P < 0.05$; Student's *t*-test)

(Fig. 2A). In contrast, when plants were treated with increased hormone concentrations, a reduction in nodule number was observed. Addition of 0.2 μM GR24 caused a small, but significant, reduction in nodule number of approximately 7.46 ± 0.46 nodules per plant versus 8.91 ± 0.39 nodules on the control mock-treated plants (P < 0.05; Student's t-test) (Fig. 2B). The nodulation repression was significantly enhanced when plants were grown in the presence of 2 μM or 5 μM GR24: on average, 2.46 ± 0.22 and 2.15 ± 0.25 nodules per plant, respectively, when compared to 6.53 ± 0.33 and 6.59 ± 0.30 nodules observed on their matching control mock-treated plants (P < 0.001; Student's t-tests) (Fig. 2C, D).

As light influences strigolactone biosynthesis in tomato roots (Koltai *et al*., 2011), our experiments were repeated with plants grown in perlite, in which the roots grow in the dark (Supplementary Fig. S1 available on *JXB* online). The results were comparable with those obtained when the plants were grown on plate. Hence, because light exposure of the roots did not change our observations, plates could be further used as growth system to study the effect of strigolactones during nodule development.

As nodulation is controlled by the AON pathway, the nodule number does not strictly correlate with the root length, but it would still be hampered by very short roots. Hence, to be sure that the effect of GR24 on the nodule number could not be attributed to a side effect on the root growth, we measured the corresponding main root length in nodulated plants (Fig. 2). Similar to the results obtained for plants growing in M medium (Fig. 1), treatment with 0.1 μM GR24 did not affect root growth. The average main root length of 18.02 ± 0.36 cm did not differ significantly from the root length of 18.57 ± 0.54 cm measured for the matching control plants (P = 0.398; Student's t-test) (Fig. 2A). However, treatment with higher concentrations resulted in a weak reduction in the main root length: on average, 16.02 ± 0.38 cm, 13.69 ± 0.45 cm, and 14.94 ± 0.50 cm were measured for plants treated with 0.2 μM, 2 μM, and 5 μM GR24, respectively, which is significantly different from the average main root length of 17.60 ± 0.39 cm, 16.98 ± 0.39 cm, and 16.98 ± 0.39 cm (P < 0.01 and P < 0.001; Student's t-test) of the corresponding control plants (Fig. 2, B-D). As treatment with 2 μM or 5 μM GR24 resulted in a decrease of the main root length of approximately 10%, and a reduction in nodule number of approximately

60%, we conclude that the minor effect on the main root length cannot be the cause of the major effect observed on the nodule number.

The effect of GR24 on the main root length of inoculated plants did not correspond to the results observed for non-inoculated plants. To investigate whether this difference might be due to the ammonium supplied by the bacteria, the average main root length was measured with or without addition of NH_4NO_3 (1 mM) in the medium (Supplementary Fig. S2 available on *JXB* online).Treatment with 0.1 μM or 2 μM GR24 did not affect the main root length of plants growing in the absence of NH_4NO_3 (Supplementary Fig. S2A available on *JXB* online). However, when NH_4NO_3 was supplemented to the medium, GR24 had a negative impact on the main root length (Supplementary Fig. S2B available on *JXB* online), similarly to what was observed for inoculated plants (Fig. 2C). Hence, the effect of GR24 on the main root length might depend on the presence of ammonium.

In conclusion, strigolactones influence nodulation on *M. truncatula* in a concentration-dependent manner. Whereas treatment with 0.1 μM of exogenous strigolactones positively regulates nodule number, treatment with higher concentrations represses nodule formation. Moreover, GR24 negatively regulates the main root length upon nodulation or in NH_4NO_3-supplemented SOLi medium.

GR24 does not influence the root hair length in the nodulation susceptible zone

In *Arabidopsis*, the treatment with GR24 enhances root hair length, whereas in tomato it inhibits it (Koltai *et al*., 2010; Kapulnik *et al*., 2011a, 2011b). Because aberrant root hair length could prevent the interaction between the plant and its symbiont, the impact of GR24 on root hair development was assessed before inoculation.

Two-day-old seedlings were grown *in vitro* on nitrogen-poor SOLi medium supplemented with GR24. At 5 dpg, just before inoculation, the root hair length in the zone susceptible for rhizobial infection was measured (Fig. 3). Treatment with GR24 did not change the root hair length: an average length of 0.398 ± 0.012 cm observed for control plants did not differ significantly from the average length of 0.402 ± 0.010 cm and 0.399 ± 0.014 cm measured for plants grown in the presence of 0.1 μM or 2 μM GR24, respectively ($P = 0.844$ and $P = 0.958$; Student's *t*-tests).

Thus, GR24 does not seem to affect root hair length in *M. truncatula* and the impact of strigolactones on nodulation cannot be explained by a defect in root hair development.

ENOD11 promoter activity is differently regulated during nodulation upon GR24 treatment

To assess whether early nodulation signalling events still take place in roots exogenously treated with GR24, the expression of the early epidermal nodulation marker *ENOD11* was analyzed using the transcriptional reporter line p*ENOD11*:GUS (Journet *et al.*, 2001). At 5 dpi, roots were stained with GUS and analyzed by stereomicroscopy (Fig. 4A). Without exogenous GR24, the *ENOD11* expression pattern was in agreement with that reported previously (Journet *et al.*, 2001). GUS staining was visible in the root hairs and the epidermis and, at some places, intensive blue-stained spots were seen, corresponding to successful infections (Fig. 4A). When plants were grown in the presence of 0.1 μM GR24, no remarkable differences in staining patterns were detected (data not shown). However, in plants treated with 2 μM GR24, blue staining was strongly reduced and clear infection spots were scarce (Fig. 4A).

To confirm this effect, *ENOD11* transcript levels were analyzed by qRT-PCR before and at different time points after inoculation (Fig. 4B and Supplementary Fig. S3 available on *JXB* online). Starting at 2 days post rhizobial infection, gene expression increased in both mock-treated control roots and roots grown in the presence of 2 μM GR24. However, the induction of *ENOD11* expression in GR24-treated roots was reduced upon infection when compared to control plants. In general, a 50% decrease in transcript level was observed compared to the increase in expression measured in control roots. These data are in agreement with the observed GUS pattern and indicate that addition of 2 μM of GR24 represses nodulation at very early stages upstream from or at the level of the *ENOD11* induction.

Involvement of SICKLE (SKL) in the strigolactone control of nodule number

The fewer infection foci observed in GR24-treated p*ENOD11*:GUS roots might hint at a less effective infection. Several hormones play an important role in controlling the epidermal responses during the nodulation process (Ding and Oldroyd, 2009; Murray, 2011). For instance, studies on the *M. truncatula skl* mutant, affected in ETHYLENE INSENSITIVE2, have shown that ethylene is a major signal that negatively regulates infection (Oldroyd *et al.*, 2001). Indeed, *skl* is characterized by a high number of successful rhizobial infections, resulting in a supernodulation phenotype (Penmetsa and Cook, 1997). Moreover, ethylene had been shown to repress early nodulin genes, such as *ENOD11* (Oldroyd *et al.*, 2001). To analyze whether SKL plays a role in the effect of GR24 on nodulation, we analyzed the *skl* mutants.

The nodule numbers counted in the corresponding mock-treated *skl* mutants were equal to those of the plants grown in the presence of either 0.1 μM or 2 μM GR24 (Fig. 5A). Treatment with 0.1 μM GR24 resulted on average in 27.31 ± 1.96 nodules per plant, not significantly different from the 26.68 ± 1.44 nodules per plant in the control plants ($P = 0.795$; Student's *t*-test). When *skl* mutants were grown in the presence of 2 μM GR24, an average of 36.49 ± 1.73 nodules were counted per plant, also not significantly different from the average of 37.53 ± 2.11 nodules on the control mock-treated plants ($P = 0.705$; Student's *t*-test). Additionally, no reduction in the main root length could be observed in nodulated *skl* mutants when grown on medium supplemented with 0.1 μM or 2 μM GR24 (Fig. 5B). On average, main root lengths of 19.38 ± 0.51 cm and 14.92 ± 0.56 cm were not significantly shorter than those of 18.95 ± 0.43 cm and 14.91 ± 0.46 cm measured for the corresponding control mock-treated plants ($P = 0.521$ and $P = 0.991$; Student's *t*-tests). The results observed in a *skl* mutant background demonstrate that the SKL-mediated ethylene signalling might play an important role in the GR24 control on the root system in *M. truncatula*, both to regulate nodule number and to influence primary root length.

Repression of nodulation by GR24 is independent of SUNN

Nodule formation is under the control of AON, a systemic response that involves shoot-controlled factors (Magori and Kawaguchi, 2009). As strigolactones have been suggested to move upwards in plant stems and to act as a long-distance messenger

for auxin (Brewer *et al*., 2009), their possible involvement in the AON process to control nodule number via the SUNN receptor (Schnabel *et al*., 2005) was investigated. Therefore, the impact of GR24 on nodule number was tested too in a supernodulating *sunn-4* background.

Treatment with 0.1 µM GR24 resulted on average in 41.39 ± 2.43 nodules per plant, a weak, but not significant, stimulation of nodulation compared to 36.13 ± 2.15 nodules per plant observed for *sunn-4* mutants grown under control conditions ($P = 0.107$; Student's *t*-test) (Fig. 6). However, in the presence of 2 µM GR24, the number of nodules in the *sunn-4* mutants dramatically decreased (Fig. 6), similarly to what occurred in wild-type plants (Fig. 2C). On average, 15.44 ± 1.47 nodules were counted per plant, which was significantly different from the number of nodules counted on control mock-treated plants ($P < 0.001$; Student's *t*-test) (Fig. 6). As the reduction in nodule number was still observed in nodulated *sunn-4* mutants after GR24 treatment, we concluded that the effect of GR24 on nodulation is independent of the AON pathway that is controlled by the *SUNN* receptor.

Discussion

Here, the effect of strigolactones on the root architecture of *M. truncatula* was studied for the first time. Addition of GR24 reduced lateral root development, whereas the impact on nodulation was either positive or negative depending on the hormone concentration. As the expression of *ENOD11* was repressed by GR24 and the ethylene signalling mutant, *skl* was insensitive to GR24, this study revealed that nodulation was affected at a very early stage. Furthermore, a crosstalk with ethylene signalling was shown to be involved in the control of the nodule number by GR24.

A major determinant of the root architecture is the development of lateral roots. Here, an inhibitory effect of GR24 on the lateral root density was observed, consistent with previous studies. Indeed, in *Arabidopsis*, treatment with GR24 reduced lateral root formation (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011). Also in rice, exogenous treatment of GR24 has been shown to reduce the lateral root density (Sun *et al*., 2014). The molecular mechanisms underlying this observation are currently unknown. In *Arabidopsis*, the endogenous auxin levels, which also tightly control lateral root development (Lavenus *et al*., 2013), have been proposed

to regulate this effect (Ruyter-Spira *et al*., 2011). It will be interesting to investigate whether a similar hypothesis could be made for *M. truncatula*. To get better insights, genome-wide analyses, as well as genetic studies should be performed.

The main root length is another important parameter of root architecture and here, we have observed that GR24 has a varying effect depending on the medium and conditions used. In the present study, GR24 had no effect on the main root length of uninoculated plants grown on SOLi medium or grown on M medium. However, GR24 treatment of inoculated plants growing on the SOLi medium resulted in a reduction of the main root length. A similar result was obtained when plants were grown on SOLi medium supplemented with 1mM NH_4NO_3. Since M medium has a higher N amount (through nitrate) compared to the SOLi medium, we can hypothesize that the observed difference is likely due to the presence of high ammonium levels obtained through nitrogen fixation or through exogenous addition. Also in previous publications in *Arabidopsis* and rice, the effect of GR24 on root development was shown to depend on the available nutrient conditions (Ruyter-Spira *et al*., 2011; Sun *et al*., 2014). It will be interesting to test our hypothesis via the use of different media compositions, bacterial nitrogen fixation mutants and the use of plant mutants affected in nitrogen metabolism.

In *M. truncatula*, nodulation is another important aspect of root architecture and this study showed that GR24 also affects the number of nodules. Exogenous GR24 had a dose-dependent effect on nodule formation. Indeed, the number of nodules was strongly reduced at a concentration of 2 μM and 5 μM GR24, but slightly increased in plants grown in the presence of 0.1 μM GR24. These data present some similarities with previous observations in other legume species, but also show some differences. Low levels of GR24 have a promoting effect in alfalfa (Soto *et al*., 2010) and, in agreement, transgenic *LjCCD7*-silenced *L. japonicus* plants and *rms1* pea mutants, both defective in strigolactone biosynthesis, carry fewer nodules per gram weight than control plants (Foo and Davies, 2011; Liu *et al*. 2013). However, the negative effect of slightly higher GR24 concentrations was not observed in alfalfa or in pea (Soto *et al*., 2010; Foo and Davies, 2011). In these plants, addition of 10 μM of GR24, which is 2- to 5-fold the concentration used here, had no or a stimulating effect on nodule number. Also *rms4* pea mutants, affected in strigolactone responses, carried more nodules than the wild-type plants which was

opposite to the phenotype of the *rms1* biosynthesis mutant (Foo and Reid, 2013), but in agreement with the phenotypes observed here when GR24 concentrations higher than 0.1 μM were used. This discrepancy is difficult to explain, but might be resolved by studying *M. truncatula* mutants and by using natural strigolactones as exogenous compounds. Indeed, the strigolactone analogue GR24 used here and in most other studies is a racemic mixture in which one enantiomer mimics the natural strigolactones and the other is proposed to mimic karrikins, smoke-derived signals that also involve *MORE AXILLARY GROWTH2* (*MAX2*), the orthologue *RMS4* in pea, to provoke seed germination (Scaffidi *et al*., 2014). Hence, it is possible that through GR24 we visualize more than strigolactone effects. The use of natural strigolactones would undoubtedly help to shed light on these observations. Moreover, the effect of GR24 on nodulation might vary depending on the legume species or might be due to variability between different set-ups. Indeed, the inhibitory, but not the stimulating, effect was observed when *M. truncatula* was grown in perlite.

At which step nodulation is hampered by adding GR24 was investigated as well. Sinorhizobia enter through root hairs, of which the length can be affected positively or negatively by GR24 in *Arabidopsis* and tomato (Koltai *et al*., 2010; Kapulnik *et al*., 2011a). In *M. truncatula*, such an effect of GR24 could not be demonstrated, indicating that the observed negative effect of GR24 on nodulation is not caused by an influence on the root hair length.

Analysis of the transcriptional reporter line *pENOD11*:GUS, a marker for early NF signalling and infection events, revealed that GR24 affected nodulation at an early step of the process. Under control conditions, clearly stained foci of *ENOD11* expression, corresponding to successful infections (Journet *et al*., 2001), were observed. In contrast, when GR24 was added to the medium, the *ENOD11* expression was faint and few infection foci were seen. qRT-PCR analyses confirmed these results. Previously, *ENOD11* expression has been found to be controlled by two different regulatory regions in its promoter that are involved in NF signalling or in rhizobial infection (Cerri *et al*., 2012). In the future, it would be interesting to investigate via which regulatory element GR24 provokes its effect on *ENOD11* expression.

In agreement with our observation that GR24 affects *ENOD11* expression, the hypernodulation *skl* mutant, defective in ethylene signalling (Penmetsa and Cook, 1997; Penmetsa *et al*., 2008) was insensitive to GR24. Indeed, ethylene suppresses nodulation by influencing NF-induced calcium spiking, by blocking bacterial infection and by suppressing nodulin gene expression (Oldroyd *et al*., 2001). Hence, the negative effect of GR24 on the nodule number might be explained by the impact of ethylene on the early nodulation events. In *Arabidopsis*, ethylene and strigolactones have also been shown to operate via a common regulatory pathway, in which ethylene is epistatic to strigolactones (Kapulnik *et al*., 2011b). Strigolactones might induce ethylene biosynthesis, followed by the activation of ethylene signalling that, in turn, is necessary for the root hair response towards strigolactones (Kapulnik *et al*., 2011b; Koltai, 2011). A similar mechanism might be used to influence the main root length and nodulation in *M. truncatula*. However, further experiments, such as monitoring calcium spiking in response to GR24 and the use of ethylene biosynthesis inhibitors, are necessary to unravel this ethylene-strigolactone crosstalk in *M. truncatula* in more detail.

The number of nodules formed on legume roots is under the strict control of a systemic signalling pathway, the AON pathway (Mortier *et al*., 2012). In pea, no evidence was found that strigolactones play a role in the AON pathway to control nodulation (Foo *et al.,* 2014). Similarly, in *M. truncatula*, application of high (2 µM) concentrations of GR24 reduced the number of nodules in the supernodulating *sunn-4* mutant background to the same extent as in wild-type plants, indicating that the repression of nodulation by GR24 acts independently of the AON pathway.

In conclusion, this study provides the first insights into how GR24 shapes the root architecture in *M. truncatula*. Besides inhibiting both primary and lateral root development, GR24 can either stimulate or repress nodulation, depending on the hormone concentration. Moreover, given the importance of ethylene signalling and the observed transcriptional repression of the early nodulation marker *ENOD11* by GR24, strigolactones seem to influence the early signalling cascades prior to bacterial infection. Future studies will investigate the molecular network used by these hormones to fine-tune nodulation and will provide a valuable insight into the complex coordination of nodule number in *M. truncatula*.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Effect of GR24 on nodulation in perlite.

Supplementary Fig. S2. Effect of GR24 on the main root length of plants grown in SOLi medium supplemented or not with NH_4NO_3.

Supplementary Fig. S3. *ENOD11* expression in GR24-treated roots.

Supplementary Table S1. Primers used in the analysis

Acknowledgements

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Figure Legends

Fig. 1. Effect of GR24 on main root growth and lateral root development.

Average main root length (A), average lateral root number (B), and average lateral root density (C) of plants grown for 19 days in the presence of 0.1 μM or 2 μM GR24, compared to control mock-treated plants (n = 19-24). All experiments were repeated twice with comparable results and the total mean of both biological repeats is presented. Data and error bars represent means ± SE. Asterisks indicate statistically significant differences when compared to untreated control roots (**P < 0.01 and *** P < 0.001; Student's t-test).
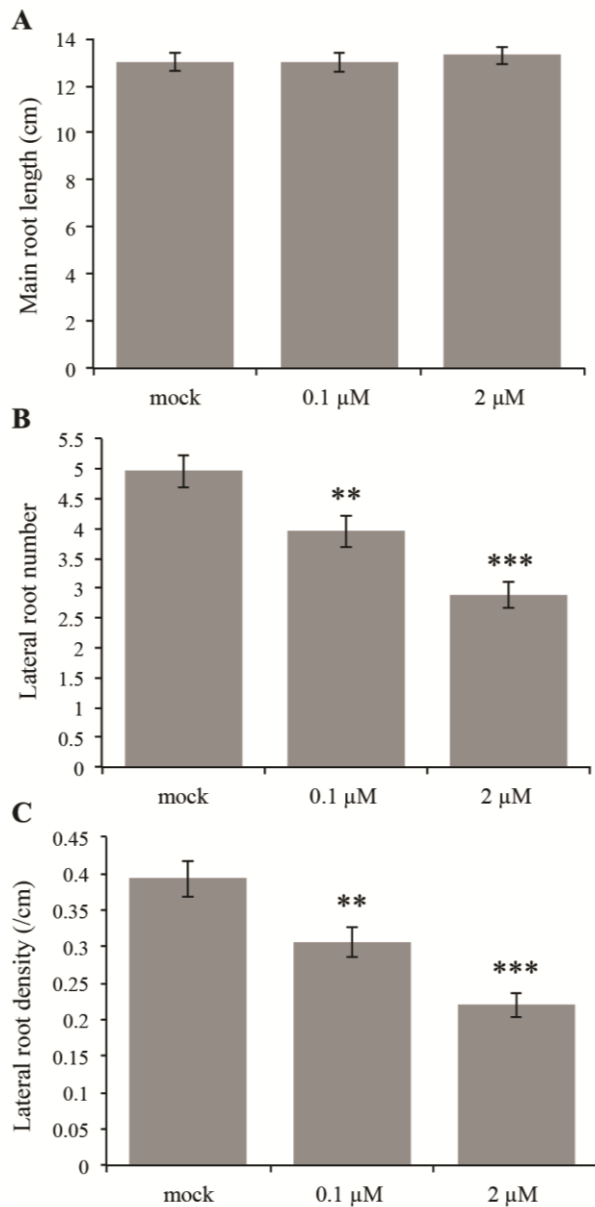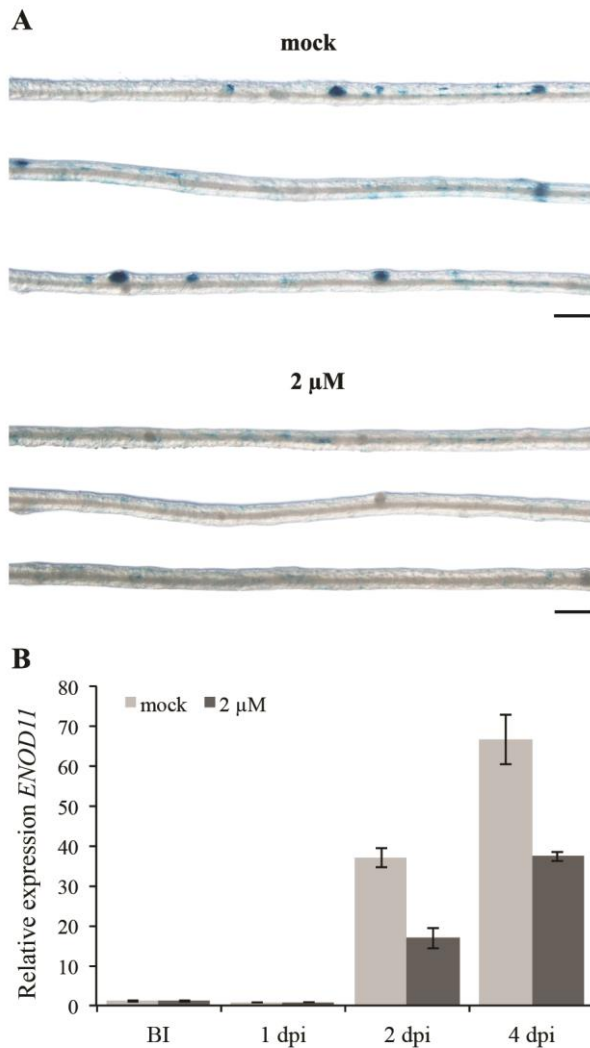
Fig. 2. Effect of GR24 on the development of inoculated roots.

Average nodule number and corresponding average main root length 14 days post infection with *S. meliloti 2011*. Plants were grown in the presence of 0.1 μM (A), 0.2 μM (B), 2 μM (C), or 5 μM (D) GR24, compared to control mock-treated plants (*n* = 22-24). All experiments were repeated at least three times with comparable results and the total mean of all biological repeats is presented. Data and error bars represent means ± SE. Asterisks indicate statistically significant differences in comparison to untreated control roots (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Student's *t*-test).
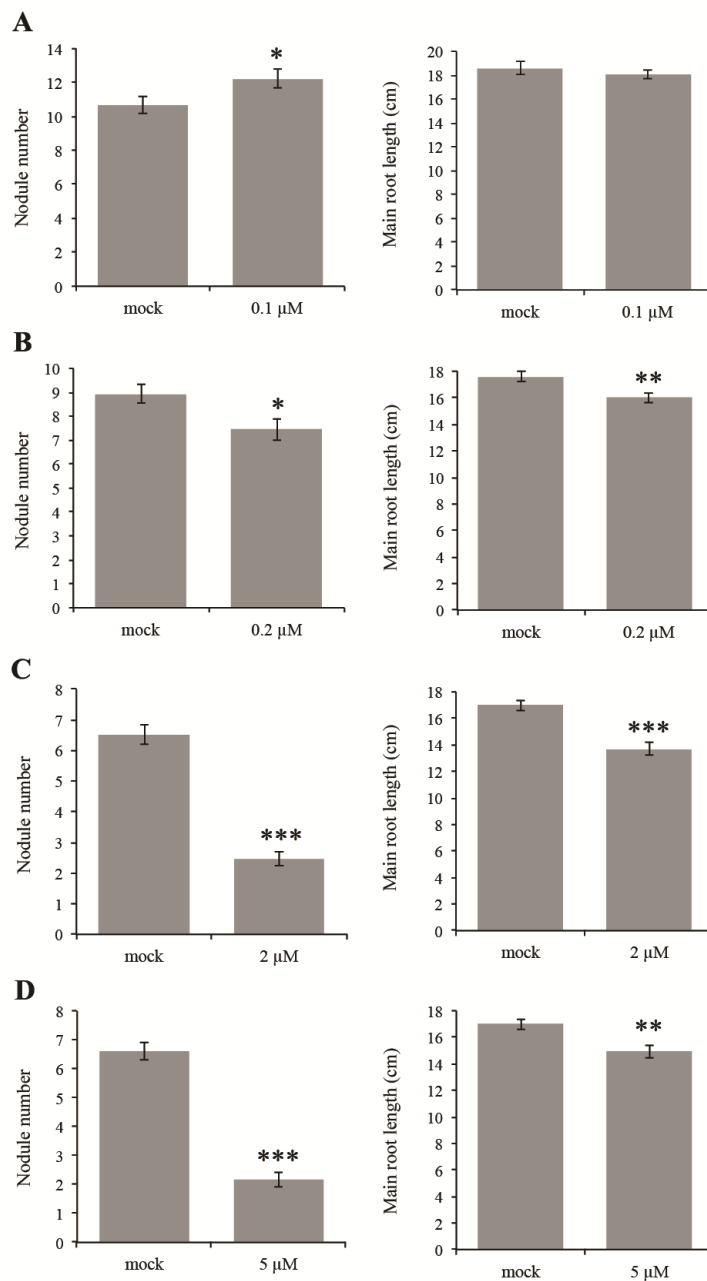
Fig. 3. Effect of GR24 on the length of the root hairs in the nodulation-susceptible zone**.**

Average length of the root hairs in the zone susceptible for nodulation of 5-day-old plants, before infection with *S. meliloti 2011*. Plants were grown in the presence of 0.1 µM or 2 µM GR24 and compared to control mock-treated plants (*n* = 17-20). All experiments were repeated twice with comparable results and the total mean of both biological repeats presented. Data and error bars represent means ± SE.
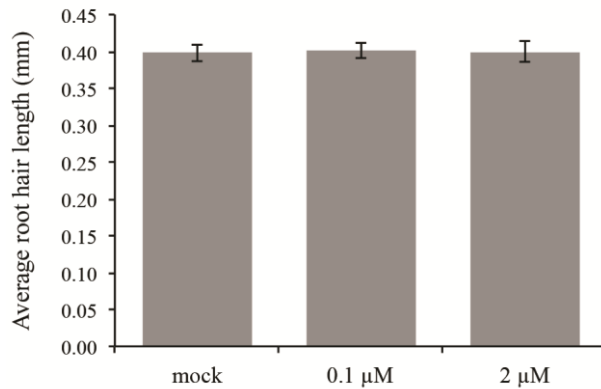
Fig. 4. Effect of GR24 on *ENOD11* expression in nodulated roots.

Bright-field pictures of the *pENOD11:GUS* expression in the nodulation-susceptible zone of roots treated with 0 μM (above) or 2 μM GR24 (below) (A). Pictures were taken 5 days after inoculation with *S. meliloti 2011* (*n* = 8-15). The experiment was repeated three times with comparable results and three representative pictures for both conditions are shown. Scale bars = 1 mm. Expression analysis of *ENOD11* by qRT-PCR in roots before or after inoculation with *S. meliloti 2011* and grown in the presence or absence of 2 μM GR24 (B). A cutoff was set at a Ct value ≥ 35. Data and error bars represent means ± SD. The infection-susceptible root zone was sampled before and after 1, 2, or 4 days of inoculation (*n* = 10-12). The experiment was repeated four times with comparable results and one representative repeat is shown.

Fig. 5. Effect of GR24 on root development and nodulation in *skl* mutants.

Average nodule number (A) and corresponding average main root length (B) at 14 days post inoculation with *S. meliloti 2011* on plants grown in the presence of 0.1 μM and 2 μM GR24 compared to the matching control mock-treated plants (*n* = 12-24). All experiments were repeated three times with comparable results and the total mean of all biological repeats is presented. Data and error bars represent means ± SE.
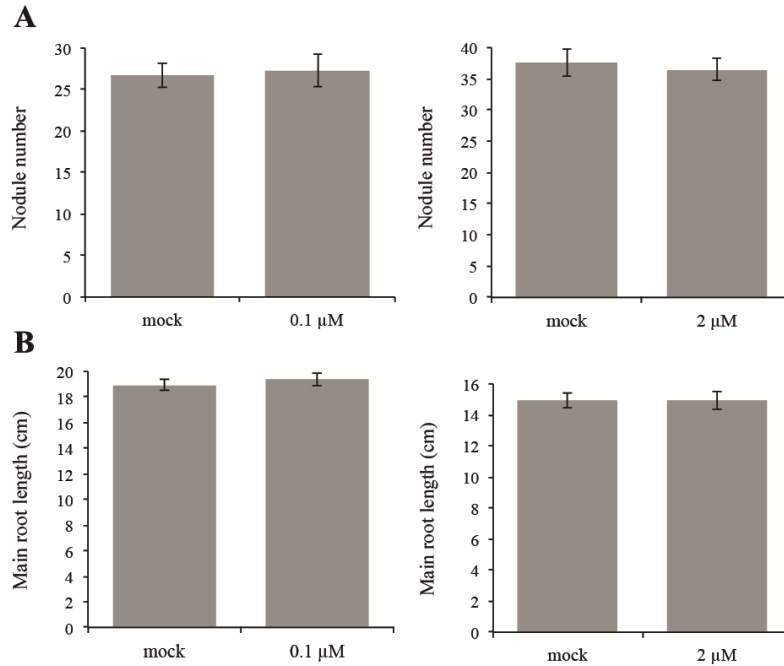
**Fig. 6.** Effect of GR24 on nodulation of *sunn-4* mutants.

Average nodule number at 14 days post inoculation with *S. meliloti 2011* on mutants grown in the presence of 0.1 μM and 2 μM GR24 compared to the control mock-treated plants (n = 22-24). All experiments were repeated three times with comparable results and the total mean of all biological repeats is presented. Data and error bars represent means ± SE. Asterisks indicate statistically significant differences in comparison to untreated control roots (***$P < 0.001$; Student's *t*-test).
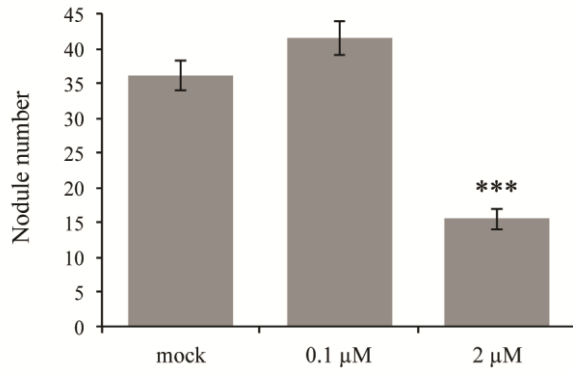
SUPPLEMENTAL INFORMATION

Fig. S1. Effect of GR24 on nodulation in perlite.

Average nodule number at 14 dpi with *S. meliloti 2011*. Plants were grown in presence of 0.1 μM (A), 0.2 μM (B), 2 μM (C), or 5 μM (D) GR24 compared to control mock-treated plants (*n* = 18-24). All experiments were repeated at least three times with comparable results and the total mean of all biological repeats is presented. Data and error bars represent means ± SE. Asterisks indicate statistically significant differences in comparison to untreated control roots (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001; Student's *t*-test).
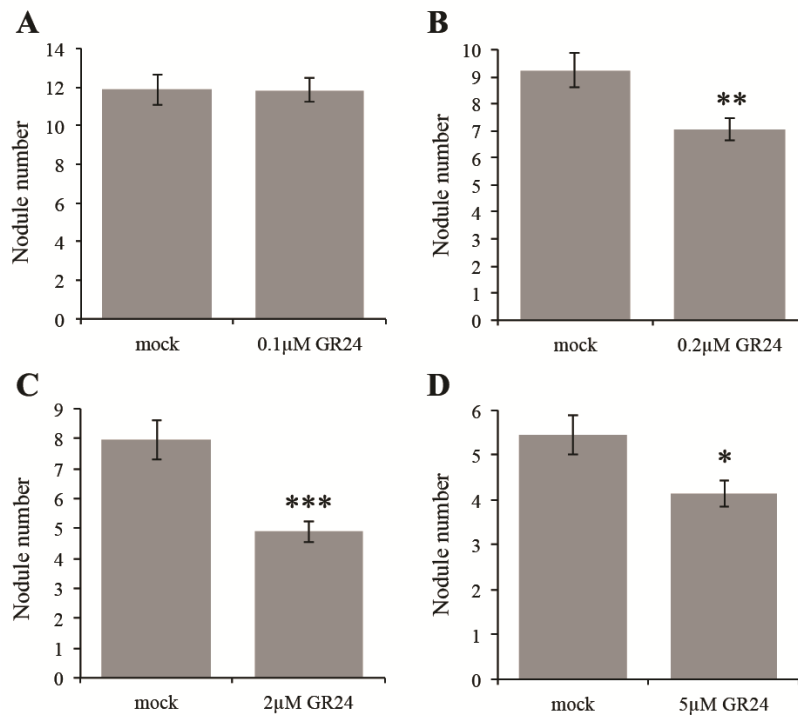
Fig. S2. Effect of GR24 on the main root length of plants grown in SOLi medium supplemented or not with NH_4NO_3.

Average main root length of plants grown for 16 days in the presence of 0.1 μM or 2 μM GR24, compared to control mock-treated plants in a medium without NH_4NO_3 (A) or supplemented with 1 mM NH_4NO_3 (B). All experiments were repeated three times and the total mean of both biological repeats is presented. Data and error bars represent means ± SE. Asterisks indicate statistically significant differences when compared to untreated control roots (**$P < 0.01$ and *** $P < 0.001$; Student's t-test).
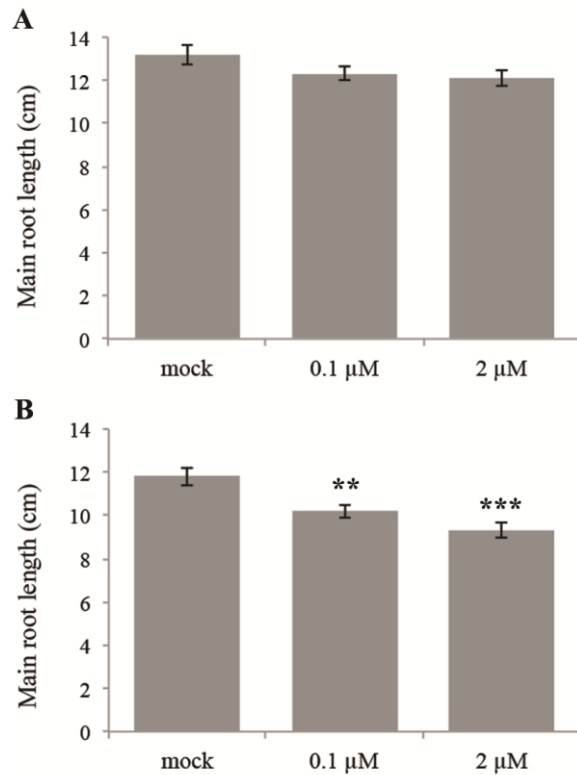
Fig. S3. *ENOD11* expression in GR24-treated roots.

Expression analysis of *ENOD11* by qRT-PCR in roots before or after inoculation and grown in the presence or absence of 2 μM GR24. A cutoff was set at a Ct value ≥ 35. Data and error bars represent means ± SD. The experiment was repeated four times, with comparable results. The three remaining repeats, not present in Figure 4B, are shown here.
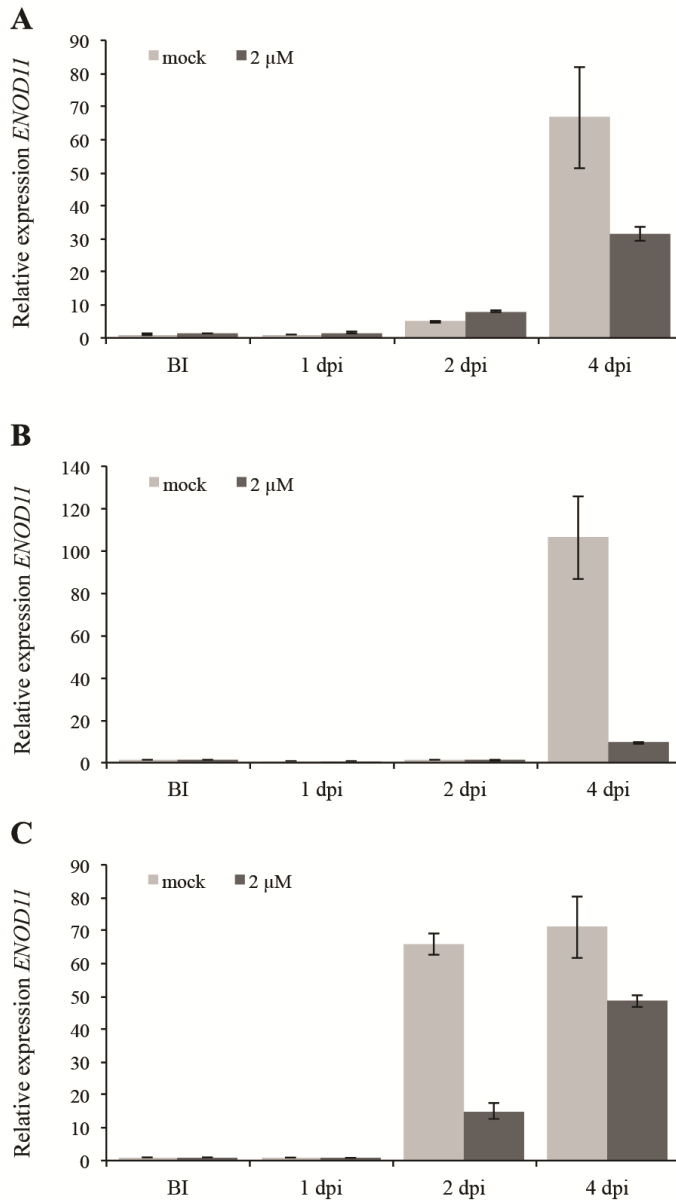
Table S1. Primers used in the analysis

Gene	Sense primer	Antisense primer
40S Ribos	GCCATTGTCCAAGTTTGATGCTG	TTTTCCTACCAACTTCAAAACACCG
ENOD11	ATCCCACAATATGCCTCCAA	AGGAAGTGGTGGCTTTAGCA