



The Role of the BELL1-2 Transcription Factor in the Development of Legume-rhizobial Symbiosis

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Abstract

Nodule development is a process that is tightly regulated by phytohormones, mainly gibberellins and cytokinins. During nodule development gibberellins and cytokinins play an important role in the infection development and organogenesis. However, the interaction between these phytohormones is not yet clear. In our research we first demonstrated that the BELL1-2 transcription factor can influence gibberellin and cytokinin biosynthesis genes during nodule development. It was also found that BELL1-2 can regulate SHY2-like gene which is specifically involved in the control of meristem maintenance and organogenesis. Localisation of the expression of the *pMtBELL1-2::GUS* promoter showed that the gene is expressed in the primordia, as well as in the infection zone and the nitrogen-fixing zone of mature nodules. Furthermore, we detected an increase in the infection zone in *M. truncatula* nodules with *BELL1-2* RNAi due to the modified growth of the infection threads. In summary, we conclude that BELL1-2 plays an important role in the control of infection and organogenesis in legume plants.

Keywords Legume-rhizobial symbiosis · BELL transcription factor · Gibberellin and cytokinin metabolism · *Medicago truncatula* · *Pisum sativum*

Introduction

Several classes of homeodomain-containing transcription factors are involved in the coordinated regulation of growth and development in multicellular organisms. Among them are members of the TALE superfamily which have an atypical homeodomain that contains three additional amino acids between helix 1 and helix 2 (Joo et al. 2018). In higher plants, the TALE superfamily includes two subgroups of proteins, BELL (BEL-like) and KNOX (Knotted1-like homeobox), which in most cases function in the form of heterodimers (Bürglin and Affolter 2016). Many studies show that TALE proteins play an indispensable role in the formation and maintenance of meristems, the regulation of

organogenesis, and are also responsible for the positioning of newly formed organs (Hamant and Pautot 2010b).

Members of the TALE protein superfamily have previously been shown to influence the regulation of genes that control the metabolism of phytohormones. The mechanism by which KNOX transcription factors support the development of the apical meristem and organogenesis has been studied quite well in *Arabidopsis* and is related to their ability to control the metabolism of phytohormones such as cytokinins and gibberellins (Jasinski et al. 2005). BELL transcription factors also play an important role in the regulation of cytokinin and gibberellin metabolism, as it was shown during the development of potato *Solanum tuberosum* (Chen et al. 2004a). Furthermore, BELL / KNOX heterodimerization was shown to be necessary for nuclear localization and binding to DNA target sites of this complex (Chen et al. 2004a).

The process of initiation and development of a symbiotic nodule in legume plants is a complex programme that requires the coordinated interaction of a network of transcription factors and a number of phytohormones (Oldroyd et al. 2011; Luo et al. 2023) During the initiation of the symbiosis, the perception of rhizobial signals, the Nod factors,

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stimulates induction of transcription regulators and hormonal changes in the plant epidermis, which are involved in the control of infection development (Lin et al. 2020). At the same time, plants must create a new ‘niche’ for the accommodation of rhizobia that leads to division of root cortical cells followed by penetration of rhizobia in these cells and the formation of mature nodules, where the nitrogen fixation process takes place (Crespi and Frugier 2008).

Phytohormones, primarily cytokinins, gibberellins, and auxins, play an important role in the process of infection development, nodule formation, and their further functioning (Ferguson and Mathesius 2014; Gamas et al. 2017; Lin et al. 2020). At the earliest stages of symbiosis development, auxins stimulate the infection thread growth in epidermis (Breakspear et al. 2014; Nadzieja et al. 2018; Liu et al. 2019a), while gibberellins and cytokinins regulate infection thread formation rather negatively (Held et al. 2014; McAdam et al. 2018). Interestingly, the effect of cytokinins and gibberellins is antagonistic in plants, which means that the interplay of these phytohormones should be strongly controlled by some regulators and feedback loops.

At the same time, another playground for phytohormone action during nodule development is the pericycle and endodermis, where cytokinins interact with their antagonists, auxins. It was shown that cytokinins and auxins both positively regulated the formation of the nodule primordium, while cytokinins suppressed lateral root development controversially to auxins (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007; Heckmann et al. 2011; Plet et al. 2011). Recently, some mobile factors have been shown to activate in response to epidermal events and may stimulate cytokinin (Liu et al. 2019b) and gibberellin biosynthesis (McAdam et al. 2018) in the remote pericycle and endodermis followed by local accumulation of auxins (Suzaki et al. 2012; Schiessl et al. 2019b), which is required for the initiation of nodule primordia in legume species. At subsequent stages, phytohormones cytokinins and gibberellins play an important role in the nodule development and functioning (Plet et al. 2011; Dolgikh et al. 2020a).

Due to the ability of homeodomain-containing transcription factors from the TALE superfamily to influence the metabolism of phytohormones, their study will allow us to understand how nodule morphogenesis and its functioning are controlled. However, the role of members of the TALE superfamily in the regulation of nodule development was studied only for members of the KNOX family. KNOX transcription factors have been shown to play an important role in nodule organogenesis and are already activated in the early stages of this process (Azarakhsh et al. 2015; Di Giacomo et al. 2017). Moreover, the main role in the control of nodulation apparently belongs to the class II of KNOX proteins (KNOX3, KNOX5, and KNOX9). However, despite the fact that the work of KNOX in tandem with BELL has

long been well known, in the context of the development of legume-rhizobial symbiosis, the role of BELL transcription factors has not been studied.

Thus, the search for BELL regulators that are involved in the switching of different phytohormonal programmes in symbiosis remains unexplored. In our previous work, we found that some BELL transcription factors are significantly activated in response to rhizobial inoculation and can interact with such a regulator of gibberellin metabolism as DELLA proteins (Dolgikh et al. 2020b). The essential role of DELLA proteins in the control of nodule development was previously shown (Fonouni-Farde et al. 2016; Jin et al. 2016). In this study we first report on the involvement of the BELL1-2 transcription factor in the regulation of gibberellin and cytokinin metabolism in legume plants such as *Medicago truncatula* Gaertn and *Pisum sativum* L. Furthermore, BELL1-2 can be important regulator that control extension of infection process and maintenance of organ identity during nodule development.

Materials and Methods

Bacterial Strains

The rhizobial strain *Sinorhizobium meliloti* 2011 was used for inoculation of *M. truncatula*. *S. meliloti* bacteria were cultivated on solid TY medium with the addition of tetracycline 10 µg/ml at 28 °C. To inoculate pea *P. sativum*, the strain *Rhizobium leguminosarum* bv. *viciae* 3841 was applied. *R. leguminosarum* bacteria were cultivated on solid TY medium supplemented with streptomycin 600 µg/ml at 28 °C.

For DNA cloning, *Escherichia coli* strains DH5α and TOP10 OneShot (Thermo Fisher Scientific, USA) were used, which were cultivated on solid LB medium with the addition of the necessary antibiotic at 37 °C. For the transformation of *M. truncatula* and *P. sativum*, the *Agrobacterium rhizogenes* Arqua1 strain was used and cultivated on solid TY medium with the addition of spectinomycin 50 µg/ml at 28 °C.

Plant Material and Growth Conditions

Medicago truncatula Gaertn line A17 and *Pisum sativum* L. cultivars Finale and SGE, and mutant pea lines in the *cochleate* gene. Seeds of parental plants of *M. truncatula* and *P. sativum* or mutant plants were sterilised with concentrated sulfuric acid for 10 min and then washed 3 times with sterile water. After this, the seeds were transferred to 1% agar and germinated for 4–5 days in the dark at room temperature until the seedlings appeared. The seedlings were transferred to pots with vermiculite watered

with Jensen medium and inoculated with a suspension of *R. leguminosarum* 3841 with an optical density of $OD_{600} = 0.8$. After being transferred to pots, the plants were grown in a phytotron at a temperature of 21 °C. Nodules were collected 2 weeks after inoculation (2 wai).

Creation of the *M. truncatula* Composite Plants

To obtain composite plants, *M. truncatula* plants of line A17 were used. Seeds were sterilised with concentrated sulfuric acid for 10–12 min, then washed 5 times with sterile water. After this, the seeds were kept in Clorax for 90 s, then washed 5 times with water and transferred to 0.8% agar. Plants were germinated for 1–2 days in the dark at +4, after which they were transferred to room temperature for 18–24 h until seedlings are appeared. The seedlings were then transferred to plates with Fahraeus agar medium. After the true leaf appeared, an agrobacterial transformation was carried out by cutting off the root and applying the *A. rhizogenes* Arqua1 strain suspension to the cut site. After transformation, the plants were kept on plates with Fahraeus medium for one week, after which they were placed on plates with Emergence medium with the antibiotic cefotaxime (300 µg/ml). After a week, the plants were transferred to pots containing vermiculite watered with Fahraeus liquid medium. For inoculation, we used a suspension of *S. meliloti* 2011 with optical density $OD_{600} = 0.6$.

Creation of Pea *P. sativum* Composite Plants

To obtain composite plants, *P. sativum* cultivar Finale plants were used. Seeds were sterilised with concentrated sulfuric acid for 10 min and then washed 3 times with sterile water. After this, the seeds were transferred to 1% agar and germinated for 4–5 days in the dark at room temperature. The seedlings were transferred to a dark plastic container (500 ml volume), in which the roots of the seedlings were placed in 1 × Jensen solution with 1.5 mM NH_4NO_3 with cotton wool. At the stage of one internode, the root of the seedlings was removed and the transformation was carried out by applying an *A. rhizogenes* Arqua1 suspension to the cut site. After transformation, the plants were transferred to a plastic vessel (volume 200 ml) with Jensen agar medium and left until callus appeared for 10–12 days. After this, the plants were placed to plastic vessels with Emergence medium with the antibiotic cefotaxime (150 µg/µl) and after 5 days the plants were transferred to pots with vermiculite watered with liquid Jensen medium. For inoculation, we used a suspension of *R. leguminosarum* 3841 with optical density $OD_{600} = 0.8$.

Creation of Constructs for Downregulation of the *MtBELL1-2* and *PsBELL1-2* Genes and their Introduction into *Agrobacterium Rhizogenes* Arqua 1

To create constructs for downregulation of the *MtBELL1-2* and *PsBELL1-2* genes, the target sequences (Table S2) were selected with help of pssRNAit web-tool with default parameters <https://www.zhaolab.org/pssRNAit/>. Complementary DNA (cDNA) synthesized on an RNA from *P. sativum* cv. Finale and *M. truncatula* A17 nodules (2 week after inoculation) was used as a template for amplification. PCR was carried out using primers flanking the target region and Phusion High-Fidelity DNA Polymerase (Termo Fisher Scientific, USA). The second stage of amplification was then carried out with primers containing *attB1* and *attB2* sequences at the ends and followed by subsequent cloning into the pDONR221 vector using BP clonase. After this, the fragment was sub-cloned into the pK7GWIWG2(II)-Red-Root vector using the LR-clonase enzyme (Termo Fisher Scientific, USA).

Isolation of Plant RNA

To assess gene expression in nodules, we collected 10 nodules per sample for both *M. truncatula* and *P. sativum*. The tissue was ground in a pre-cooled mortar using liquid nitrogen, then 1 ml of Purezol reagent was added, mixed thoroughly and allowed to thaw at room temperature. After final thawing, the sample was transferred to a 1.5 ml tube. The samples were mixed for 3 min (Vortex, Germany) and centrifuged for 20 min at 4 °C and 16000 rpm. The supernatant was transferred to a new 1.5 ml tube, 200 µl of chloroform was added, mixed for 3 min and incubated for 3 min at room temperature. Then centrifuged for 15 min at 4 °C and 16000 rpm. The supernatant was transferred to a new tube without capturing the interphase, an equal volume of isopropanol was added, mixed for 2 min, and incubated for 5 min in a chilled centrifuge. RNA was precipitated at 4 °C and 16000 rpm for 15 min. The precipitate was washed with 70% ethyl alcohol. After removing the alcohol, the precipitate was dried on ice and dissolved in 40 µl of deionised water. DNase treatment (2 U/1 µg) of RNA was carried out in a 37 °C water bath for 30 min. After treatment, the sample volume was adjusted to 200 µL with deionised water, then an equal volume of chloroform was added. Centrifuged for 10 min at 4 °C and 16000 rpm. 1/10 volume of 3 M Na-acetate (to a final concentration of 0.3 M) and 2.5 volume of 96% ethyl alcohol were added to the aqueous phase containing RNA. Incubated overnight at – 80 °C. Centrifuged for 30 min at 4 °C, 16000 rpm. The precipitate was washed with 500 µl of 70% ethyl alcohol, dried on ice, and dissolved in 10–30 µl of deionised water. RNA concentration

in the samples was measured using a spectrophotometer (Shimadzu, Japan).

Synthesis of Complementary DNA (cDNA) and Quantitative Reverse Transcription PCR (qPCR)

Complementary DNA was synthesised on an RNA template (2.5 µg). Synthesis was carried out for 1 h at 42 °C in a reaction mixture containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 5 mM MgCl₂, 1 mM deoxy-nucleotide triphosphates (dNTP), 100 pM oligo(dT) primer, 1 U ribonuclease inhibitor and 200 U reverse transcriptase RevertAidH (Thermo Fisher Scientific, USA). The volume of the reaction mixture was 20 µl. Before starting the reaction, the aqueous solution with RNA and oligo(dT) primer was heated at 65 °C for 5 min. To inactivate reverse transcriptase, the mixture was heated for 5 min at 95 °C. Samples were diluted 1:10 with deionised sterile water.

Real-time PCR was performed using a CFX96 Real-Time device and a ready-made mixture qPCRmix-SYBR (Evrogen, Russia). To carry out PCR, 2 µl of diluted cDNA was taken. The results of the gene expression evaluation were presented as the ratio of the expression of the gene under study to the expression of the *Ubiquitin* gene. All statistical tests were performed using R and ggpubr package (Warnes et al. 2022).

Creation of Constructs Containing the Promoter of the MtBELL1-2 and PsBELL1-2 Gene in the pB7WFS7.0 Vector (pMtBELL1-2::GUS/pPsBELL1-2::GUS), and their Introduction into A. rhizogenes Cells

To clone the promoters of the *MtBELL1-2* and *PsBELL1-2* genes, the corresponding sequence was amplified using primers flanking the 3000-bp region up to the ATG start codon. Genomic DNA from *M. truncatula* A17 was used as a template for *MtBELL1-2* and genomic DNA from *P. sativum* cv. Cameor was used for amplification of *PsBELL1-2* promoter region. PCR was carried out using primers flanking the region and Phusion High-Fidelity DNA Polymerase (Termo Fisher Scientific, USA). The amplified products were separated in a 1.5% agarose gel and purified using a commercial kit from Evrogen. The second stage of amplification was then carried out with primers containing *attB1* and *attB2* at the ends for subsequent cloning using BP clonase. The products were also separated in an agarose gel and purified using a commercial kit. Initially, using a mixture of BP clonase enzymes (Termo Fisher Scientific, USA), cloning was carried out on the pDONR221 vector, and from this vector the fragment was re-cloned on the pB7WFS7.0 vector using the LR-clonase enzyme (Termo Fisher Scientific, USA). After the reaction, the mixture was dialysed and then

transferred to electrocompetent *E. coli* TOP10 OneShot cells (Termo Fisher Scientific, USA). The detection of colonies carrying the vector with the insert was carried out by PCR. Target colonies were transferred to liquid LB medium with the appropriate antibiotic and incubated at 37 °C overnight, and plasmids were isolated and used to transform *A. rhizogenes* Arqua1 cells.

Localization of Expression of pMtBELL1-2::GUS by Colorimetric Staining with β-glucuronidase Substrate (GUS Staining)

For GUS staining, the material was fixed in a freshly prepared 4% paraformaldehyde solution with 0.1% Tween-20 in PBS buffer (pH 7.4) under vacuum. GUS staining was performed overnight at 37 °C. 60–70 µm sections of nodules were generated by microtome and imaged using an with ZEN 2009 software (Zeiss). ImageJ tool was used for ZII to ZIII ratio calculation.

Search for the BELL Binding Site on the Promoters of the M. truncatula and P. sativum Genes

To find possible targets of BELL1-2, we performed a whole genome search for previously reported BELL binding site TGACAGGT. We extracted promoter regions (3000 b.p. before ATG) for all genes in the *M. truncatula* genome v5 (Pecrix et al. 2018) and the *P. sativum* genome v1 (Kreplak et al. 2019). Extracting promoter regions on a whole-genome scale, a script was created, which is available at the Github repository https://github.com/Alexadol/Promoter_extraction. It takes a genome fasta file and a gene feature file (GFF) as input and creates a file containing the promoter regions of all genes defined in the GFF file. For these promoter regions, we performed a search for TGACAGGT site. Using ProteinOrtho tool (Lechner et al. 2011) we found pairs of orthologs in *M. truncatula* and *P. sativum*, which both have TGACAGGT in their promoter regions. Then we manually checked the list of such genes to find targets for further experiments.

Gene Numbers used in Study

PsBELL1-2 (Psat4g090560), *PsBELL1-3* (Psat0s189g0080), *PsBELL1-4* (Psat7g031320), *PsKNOX9* (Psat4g033160), *PsSHY2-like* (Psat6g113040), *PsGA20ox1* (Psat1g113960), *MtBELL1-2* (MtrunA17Chr8g0373801), *MtSHY2-like* (MtrunA17Chr11g0187961), *MtGA20ox1* (MtrunA17Chr6g0474391), *MtKNOX9* (MtrunA17Chr4g0065241), *MtIPT1* (MtrunA17Chr1g0209731).

Results

Localization of the BELL1-2 Promoter Activity in Inoculated Roots and Nodules

In our previous study, we have shown that *BELL1-2* has the highest expression level among all *BELL* genes in root nodules of *M. truncatula* and pea *P. sativum* (Dolgikh et al. 2020b). Therefore, here a more detailed analysis of the influence of the BELL1-2 transcription factor on nodulation was performed in both legumes that form indeterminate nodules.

To detect a more specific zone, where the *BELL1-2* expression can take place, we created composite plants of *M. truncatula*, in which transgenic roots carrying the *pMtBELL1-2::GUS* (3 kb) reporter fusion were obtained. GUS staining has already appeared in emerging nodule primordia (Fig. 1a,b,c), and reached the maximum in mature symbiotic nodules (2 weeks after inoculation, 2 wai), when the expression of *pMtBELL1-2::GUS* was more intensive. The staining was mainly related to the infection zone (ZII) and the nitrogen fixation zone (ZIII), but was not observed in the nodule meristem (ZI) (Fig. 1d,f). Interestingly, the most intensive staining was localized in the proximal part of ZII closer to the ZIII. Similarly, according to the

expression atlas for different nodule zones, the *MtBELL1-2* expression was the lowest in the meristem (ZI) and the highest in ZII and ZIII (Roux et al. 2014). Therefore, the role of BELL1-2 may be complex and related to regulation of the early stages of nodule development, as well as may be involved in control of infection zone development and regulation of later stages of nodule functioning.

To find out whether detected expression pattern is universal for *M. truncatula* and *P. sativum*, we have cloned the promoter region of the same size (3 kb) for *P. sativum* *BELL1-2* gene and checked the transgenic roots carrying the *pPsBELL1-2::GUS* reporter fusion in pea plants. However, the observed expression pattern differs from localization of *pMtBELL1-2::GUS* expression in *M. truncatula* nodules (Figure S1). To figure out the cause, we have also tested the staining distribution in pea nodules carrying the *pMtBELL1-2::GUS* construct (Fig. 2). Similarly to *M. truncatula* nodules, the localization of promoter activity was related to the infection zone (ZII) and the nitrogen fixation zone (ZIII), but was not observed in the nodule meristem (ZI) (Fig. 2). Therefore, the regulatory elements in the promoter of *BELL1-2* gene in both legumes seems to be universal, but they are differently located in *P. sativum* and *M. truncatula*. In accordance with this, the localization of *PsBELL1-2::GUS* in *M. truncatula* nodules was similar to that in pea nodules (Fig S1).

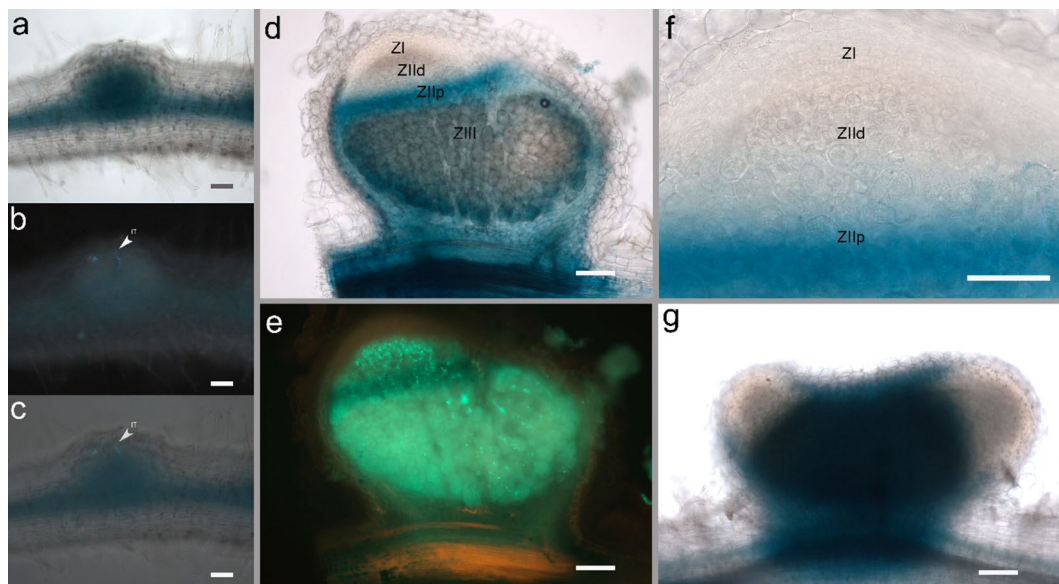
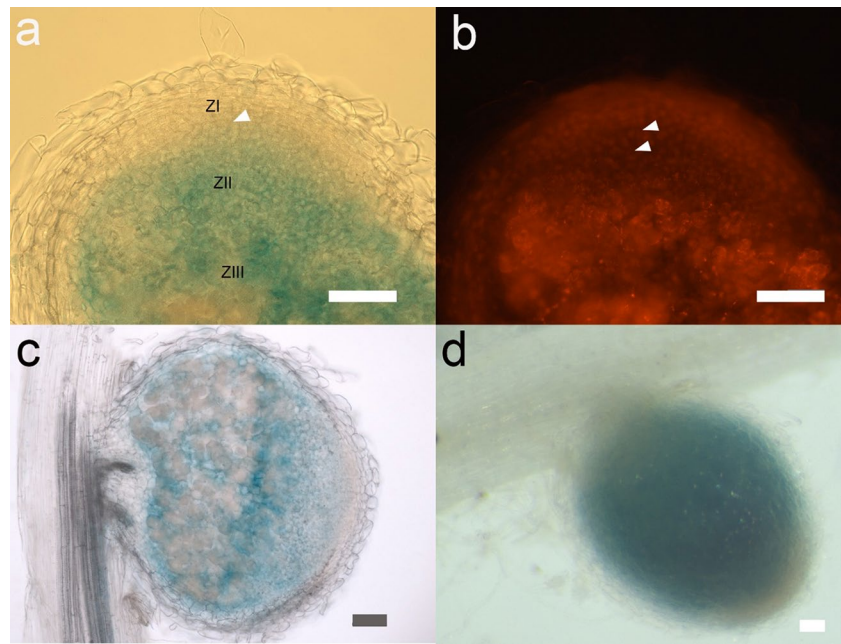


Fig. 1 Localization of *pMtBELL1-2::GUS* expression in *M. truncatula* nodule primordium (a–c) and in 2 weeks-old nodules (d–g). Scale bars = 200 μ m. Plants were inoculated with the GFP-labelled rhizobial strain *Sinorhizobium meliloti* 2011 and the blue channel was used for its visualization (b, e). White arrows indicate developing infec-

tion threads in primordium. ZI (Zone I)—bacteria free meristematic region; ZIIp (Zone IIp)—the proximal part of the infection zone; ZIIId (Zone IIId)—the distal part of the infection zone; ZIII (Zone III)—symbiosomes, which consist of differentiated bacteroids (d, f)

Fig. 2 Localization of *pMt-BELL1-2::GUS* expression in *P. sativum* 2 weeks-old nodules (a–d). Scale bars = 200 μ m. *P. sativum* cv. Cameor plants were inoculated with the mCherry-labelled rhizobial strain *Rhizobium leguminosarum* 3841. GUS staining was performed and nodule sections and whole nodules were analyzed using light (a, c, d) and fluorescent microscopy (b). White arrows indicate the position of GUS staining closed to the meristem (a) and infection thread location (b). ZI (Zone I)—meristematic region; ZII (Zone II)—the infection zone; ZIII (Zone III)—symbiosomes, which consist of differentiated bacteroids (a)



Effect of the BELL-2 Downregulation in *M. truncatula*

To study the functional role of BELL1-2, *M. truncatula* composite plants with decreased expression of *MtBELL1-2* (*BELL1-2-RNAi*) were created using the RNA interference approach. It was shown that the level of *MtBELL1-2* expression was reduced by approximately 50% in roots and nodules in *BELL1-2-RNAi* plants compared to control *beta-glucuronidase* overexpressing (*GUS-OE*) plants (Fig. 3). Downregulation of *MtBELL1-2* did not result in an essential decrease in the number of nodules (Fig. 3a,b,c), probably due to gene redundancy in the *BELL* multigenic family. However, we detected a slight, but statistically significant difference in the length of the lateral roots (Fig. 3f), demonstrating the possible role of BELL1-2 in the regulation of not only nodules but also lateral root development. In accordance with this, the *pMt-BELL1-2::GUS* expression was localized in lateral root tips (Figure S2).

At the same time, the microstructural analysis of *M. truncatula* nodules in *BELL1-2-RNAi* plants revealed an increase in the size of the infection zone (ZII) compared to those in the nodules of control *GUS-OE* plants (Fig. 4a,b). Furthermore, we observed some structural changes in infection threads (ITs) development in ZII (Fig. 4a) in the nodules of *BELL1-2 RNAi* plants. Therefore, the hypertrophied development of infection threads may be related to increasing size of the infection zone (ZII) in nodules in *BELL1-2 RNAi* plants. It was in line with the most intensive GUS staining in the infection zone (ZII) and the nitrogen fixation zone (ZIII) as localization of the *MtBELL1-2* promoter activity showed (Fig. 1).

In pea *P. sativum* downregulation of the *PsBELL1-2* gene using RNA interference approach resulted in 50–60% reduction of this gene expression (Fig. 5b). Similar to *M. truncatula* there was no significant decrease in the total number of nodules in *PsBELL1-2-RNAi* compared to control plants transformed with the *GUS* construct (*GUS-OE*) (data not shown). It suggests the similar effect of BELL1-2 on infection of nodules in both legumes.

Search for Possible Targets of the BELL1-2 Transcription Factor in Plants Based on Evaluation of Gene Expression in BELL1-2-RNAi Plants

To find possible targets of BELL1-2 in the process of nodule development, we checked the expression of some possible target genes in the nodules of *BELL1-2 RNAi* plants. It is known that BELL together with KNOX influence the expression of genes associated with gibberellin and cytokinin metabolism in model plants such as *Arabidopsis* (Jasinski et al. 2005). Therefore, the expression of genes involved in the regulation of gibberellin and cytokinin biosynthesis, as well as degradation, was estimated in *PsBELL1-2 RNAi* and *GUS-OE M. truncatula* and *P. sativum* plants (Fig. 5). We found that the expression of the *MtGA20ox1* gene was decreased in *M. truncatula* nodules with *MtBELL1-2 RNAi* (Fig. 5a). Similar data were observed in *P. sativum* nodules with *PsBELL1-2 RNAi* (Fig. 5b). Furthermore, we detected increase in *MtGA20ox1* expression level in *M. truncatula* nodules with *MtBELL1-2* overexpression (Fig. 5c). Therefore, BELL1-2 can directly or indirectly regulate the expression of this gene involved in the control of gibberellin biosynthesis in both plants. These data were consistent with

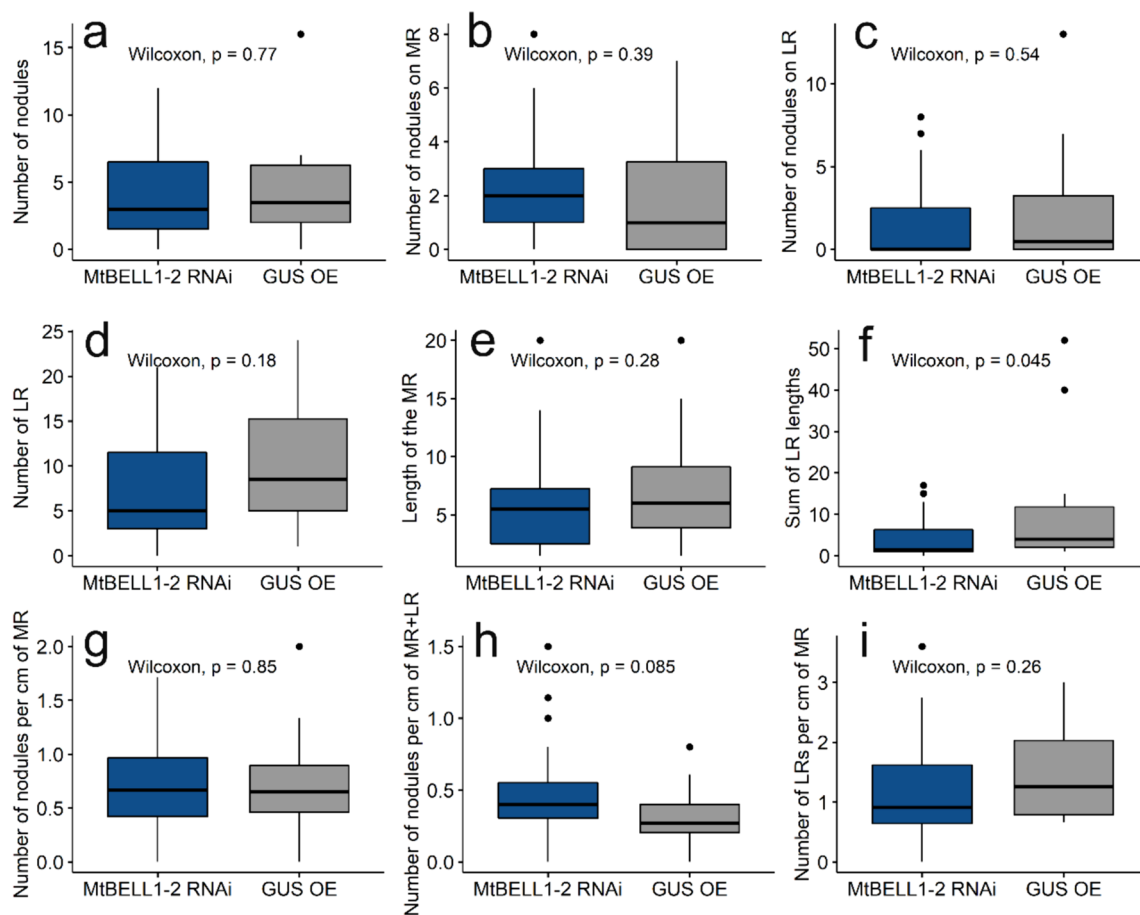


Fig. 3 Comparative analysis of the number of nodules, lateral roots and root lengths for control *GUS*-overexpressing plants (*GUS-OE*) and plants with RNAi of *MtBELL1-2* (*MtBELL1-2-RNAi*). Plants were harvested 2 weeks after inoculation and data for 25–30 trans-

genic roots from 12–15 plants from independent 3 biological experiments were analyzed. Each transgenic root was considered as replicate ($n=25-30$). Statistical analysis was performed in R using *ggpubr* package and Wilcoxon test was used. *MR* Main root, *LR* Lateral root

gibberellin immunolocalization in pea nodules. Previously, gibberellic acid 3 (GA_3) was detected in ZII and ZIII, but not in the meristem zone (ZI) using immunolocalization (Serova et al. 2019), which correlated with our data for the spatial localization of *pMtBELL1-2::GUS* expression (Fig. 1).

In our experiments, the expression of genes involved in cytokinin biosynthesis was also evaluated. We found a decrease in the expression level of the *MtIPT1* gene that regulates cytokinin biosynthesis in the nodules of *BELL1-2 RNAi* plants (Fig. 6). However, it showed only a tendency to statistically significant decrease (Fig. 6b). To check this effect in more detail we studied *MtIPT1* expression level in composite plants with *MtBELL1-2* overexpression in transgenic roots and nodules (*MtBELL1-2-OE* plants). In this case, a statistically significant increase in the expression level of *MtIPT1* was found in *MtBELL1-2-OE* plants (Fig. 6a). Therefore, the *BELL1-2* transcription factor is also involved in the cytokinin biosynthesis regulation. It seems like that the statistical variability in the decrease in

the *MtIPT1* gene in nodules of *BELL1-2 RNAi* plants may be related to its rather low expression level in plants.

Search for Target Genes for the *BELL1-2* Transcription Factor Based on the Analysis of Promoter Regions of Available Genes in the Genomes of *M. truncatula* and *P. sativum*.

BELL transcription factors have a common TGACAGGT binding site in the promoters of target genes (Viola and Gonzalez 2006). It was previously shown that in *A. thaliana*, *AtBLH2* / *AtBLH4* transcription factors that are orthologs of *MtBELL1-2* and *PsBELL1-2* had the same binding site in the promoters of some target genes (Xu et al. 2020a). To find possible targets of *MtBELL1-2* and *PsBELL1-2* transcription factors during nodule development, we examined the promoter regions for all genes in the genomes of *M. truncatula* and *P. sativum*. After detection of genes that have a TGACAGGT binding site in the

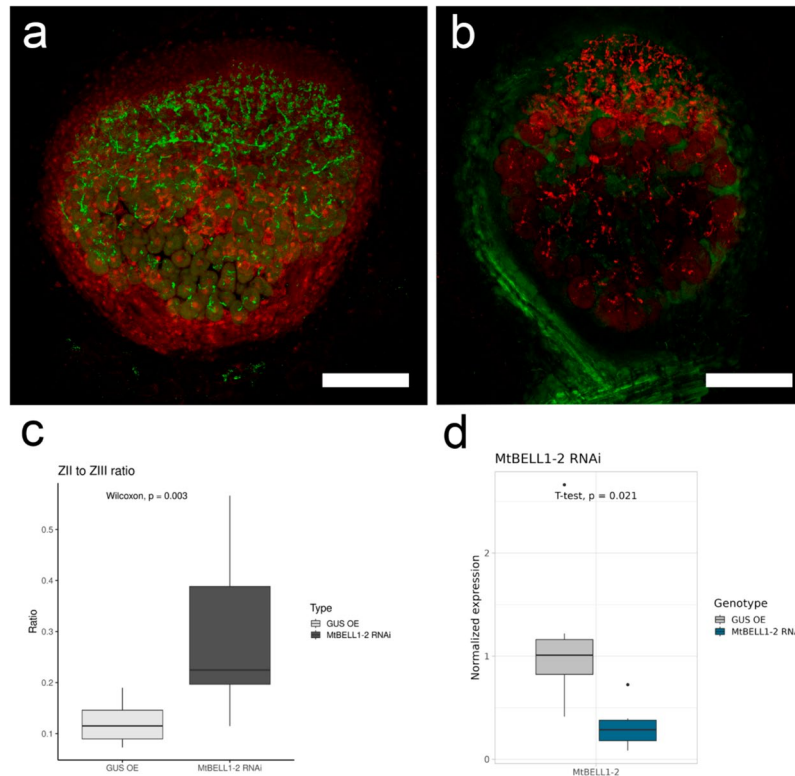


Fig. 4 Visualization of rhizobial infection in *M. truncatula* 2 weeks nodules with *MtBELL1-2* RNAi (a) and in control *GUS-OE* nodules (b). Scale bars = 200 μ m. Infection zone (ZII) area to fixation zone (ZIII) area ratio was calculated using ImageJ and statistically analysed with R in nodule sections from 10–15 nodules from 3 independent biological experiments were analyzed. Each nodule was considered as replicate ($n = 10$ –15). ZII was defined as nodule zone where infection threads, but not differentiated bacteroids are found. ZIII was

defined as nodule zone with differentiated bacteroids, respectively. (c). Plants with *MtBELL1-2* RNAi were inoculated with GFP-labelled *Sinorhizobium meliloti* 2011 (a), while control *GUS*-overexpressing plants were inoculated with mRFP-labelled *Sinorhizobium meliloti* 2011 (b). Level of *MtBELL1-2* expression in RNAi nodules and control *GUS*-OE nodules (d). Three biological replicates were analyzed included 8–10 samples (n) of transgenic nodules

3000 kb upstream region, we selected among them pairs of orthologs (Lechner et al. 2011). Among these genes, the *MtSHY2*-like and *PsSHY2*-like genes were found. Therefore, another possible target of *BELL1-2* may be the *SHY2*-like gene in both legumes. To check whether the presence of possible *BELL* binding site in the *SHY2*-like gene is a common feature in all legumes, we examined promoter regions of *SHY2*-like orthologs for legumes with indeterminate nodules (*P. sativum* cv. Cameor and cv. ZW6, *Lens culinaris*, *Trifolium pratense* and *Lupinus angustifolius*) and determinate nodules (*Phaseolus vulgaris*, *Glycine max* and *Lotus japonicus*). We found either exact matching to previously reported *BELL* binding site (TGACAGGT) (*P. sativum* cv. Cameor, *M. truncatula* A17, *Lens culinaris*), either target site with 1 substitution in 1st or 8th position which were previously shown as not significant for successful binding of *BELL* (Viola and Gonzalez 2006) (Fig. 7). We have also analyzed the promoter regions of *SHY2*-like orthologs in legumes with determinate nodules

such as *Phaseolus vulgaris*, *Glycine max* and *Lotus japonicus*. In these promoter regions we failed to find sequences that match to TGACAGGT binding site even with substitutions in 1st or 8th positions. Thus, we concluded that the presence of possible *BELL1-2* binding site in the promoter region can be distinct characteristic of legumes with indeterminate nodules.

After examination of *MtSHY2*-like expression in nodules with altered expression of *MtBELL1-2*, we found that it was changed in the opposite way to *MtBELL1-2* (Fig. 8). Indeed, in *M. truncatula* nodules with *MtBELL1-2* overexpression, we detected a decrease in the expression of the *MtSHY2*-like gene. Analysis of *MtSHY2*-like gene expression in *M. truncatula* nodules with *MtBELL1-2* RNAi showed that some increase in *MtSHY2*-like expression could be detected, but this was not statistically significant (Fig. S6). However, we detected an increase in the expression of *PsSHY2*-like gene expression in *PsBELL1-2* RNAi pea nodules, which was statistically significant.

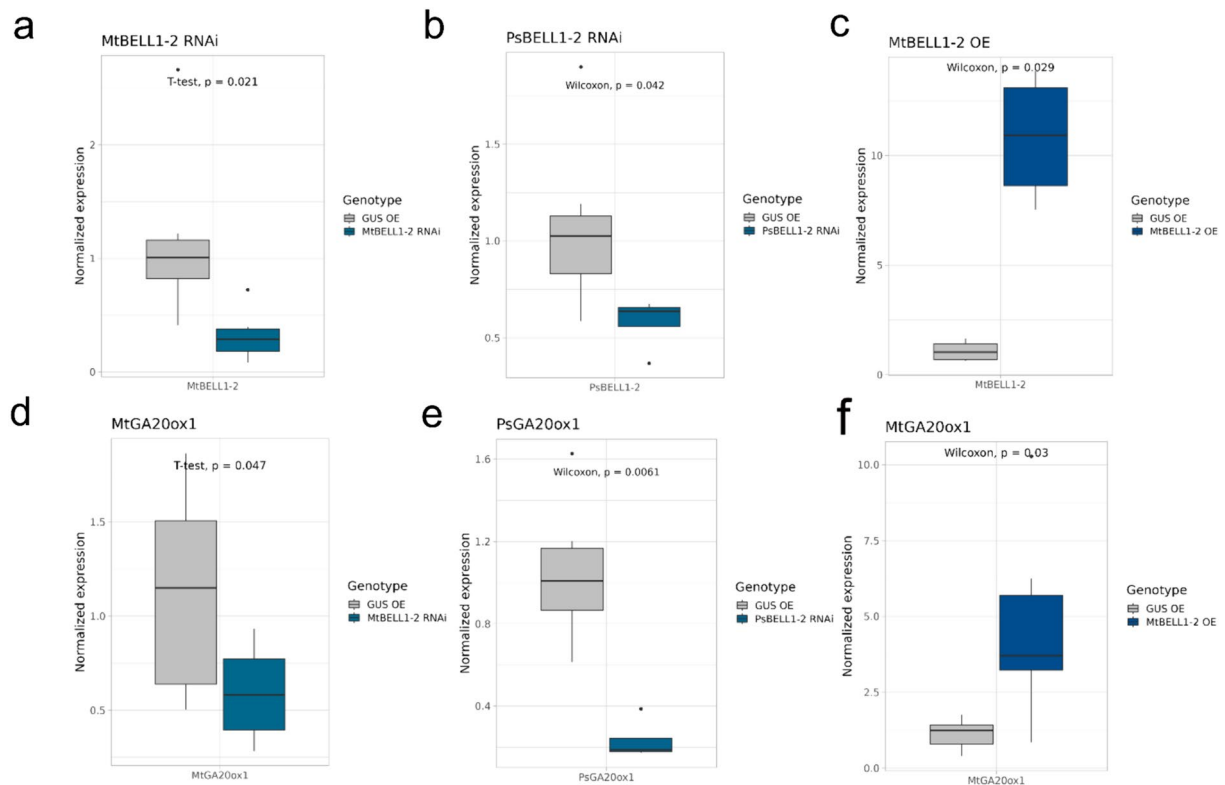


Fig. 5 RT-qPCR analysis of *MtGA20ox1* and *PsGA20ox1* gene expression in 2 weeks-old *M. truncatula* nodules with *MtBELL1-2* overexpression (**d**), knockdown of *MtBELL1-2-RNAi* (**e**) and in 2 weeks-old *P. sativum* nodules with knockdown of *PsBELL1-2-RNAi* (**f**). Levels of *MtBELL1-2* and *PsBELL1-2* expression in nodules with downregulation (**a**, **b**) and overexpression (**c**) of these genes are presented. The nodules of *M. truncatula* and *P. sativum* *GUS-OE* plants

were used as controls. Levels of mRNA were normalized against *Ubiquitin* gene and values were calculated as ratios relative to control (*GUS-OE*). For *M. truncatula* and *P. sativum*, 8–10 samples of transgenic nodules (each sample contained 10 nodules) of *BELL1-2-RNAi*, *BELL1-2-OE* and control *GUS-OE* plants from 3 independent biological experiments were analyzed ($n=8-10$). R and ggpubr package were used for statistical analysis and visualization

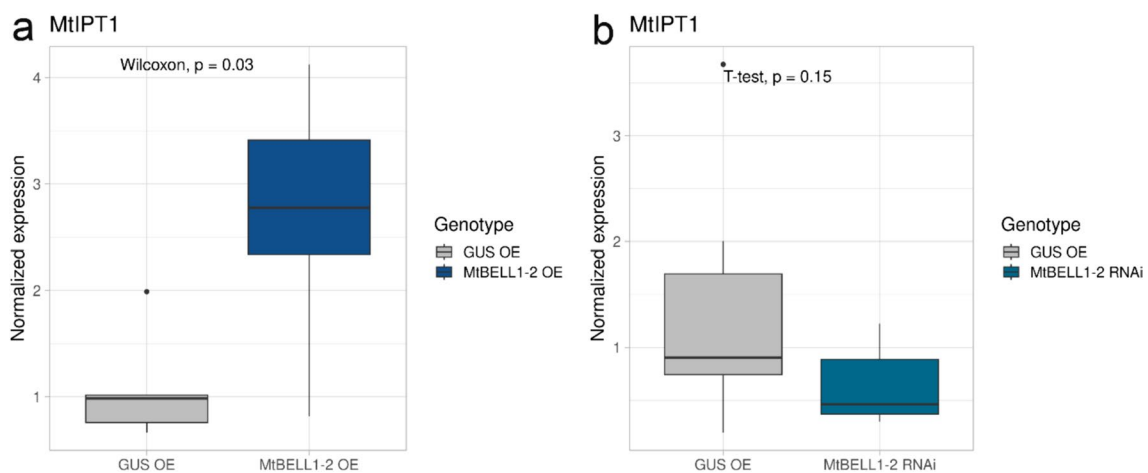


Fig. 6 RT-qPCR analysis of *MtIPT1* genes in *M. truncatula* nodules with *MtBELL1-2* overexpression (**a**), knockdown of *MtBELL1-2-RNAi* (**b**). The nodules of *M. truncatula* *GUS-OE* plants were used as a control. Levels of mRNA were normalized against *Ubiquitin* gene and values were calculated as ratios relative to control (*GUS-OE*).

The data for 7–8 samples of transgenic nodules (10 nodules per each sample) ($n=7-8$) from 3 independent biological experiments were analyzed. R and ggpubr package were used for statistical analysis and visualization

Fig. 7 Localization of possible BELL1-2 binding site in promoter regions of *SHY2*-like genes of legumes with indeterminate nodules (*P. sativum* cv. Cameor, *P. sativum* cv. ZW6, *M. truncatula* and *L. culinaris*, *T. pratense*, *L. angustifolius*) Promoter regions was defined as 3000 kb before ATG

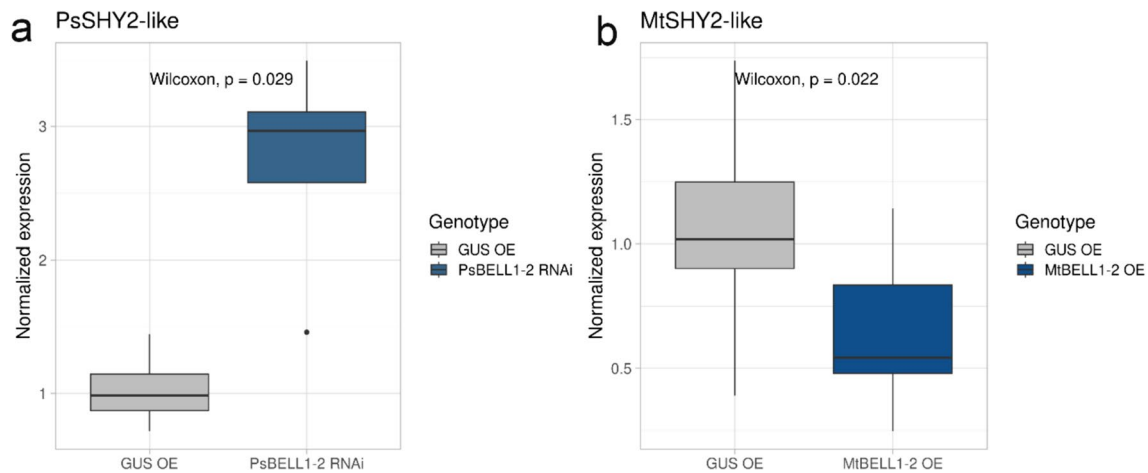
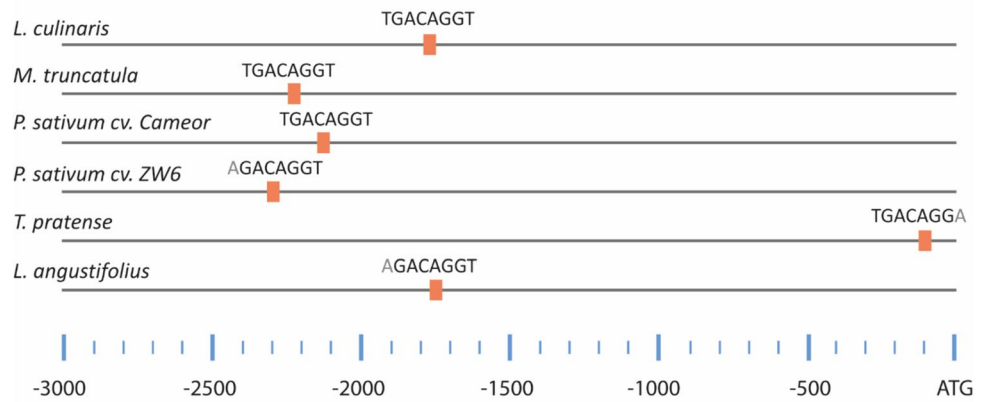


Fig. 8 RT-qPCR analysis of *PsSHY2*-like and *MtSHY2*-like genes in *P. sativum* and *M. truncatula* 2 weeks-old nodules with altered *MtBELL1-2* expression. The nodules of *M. truncatula* and *P. sativum* *GUS*-*OE* plants were used as controls. Levels of mRNA were normalized against *Ubiquitin* gene and values were calculated as ratios

relative to control (*GUS*-*OE*). The data for 6–8 samples of transgenic nodules (10 nodules per each sample) ($n=6-8$) from 3 independent biological experiments were analyzed. R and ggpubr package were used for statistical analysis and visualization

Master Regulators Responsible for the Activation of BELL1-2 During the Development of Legume-rhizobium Symbiosis. Analysis of BELL1-2 Expression in the Pea Cochleata Mutant

The involvement of the BELL1-2 transcription factor in the control of nodule formation and infection of this organ indicates that the expression of this gene should be finely regulated during the development of symbiosis. Therefore, it was important to find out which regulators can activate the expression of the *BELL1-2* gene during symbiosis. To answer this question, we previously used pea mutants affected in the *ipd3/cyclops* (*SGEFix*⁻⁵) and *nin* (*SGENod*⁻³) genes, encoding key transcription factors involved in the regulation

of symbiosis (Dolgikh et al. 2020b). Significant decrease in the level of *BELL1-2* expression in both mutants indicated the importance of IPD3/CYCLOPS and NIN regulators for activation of *BELL1-2* during symbiosis.

At the same time, our experiments showed that BELL1-2 may be required not only for the early stages but also for the later stages of nodule development. A suitable model to verify the role of the BELL1-2 transcription factor in the regulation of later stages of nodule morphogenesis may be the homeotic *cochleata* mutant (a homologue of the *NOOT1* gene in *M. truncatula*). This mutant is characterized by disturbance in nodule identity and appearance of newly formed roots from the apical nodule meristem (Couzigou et al. 2012).

The transcription factors of the TALE superfamily often play an important role in the control of meristem cell state and meristem-organ boundary maintenance (Hamant and Pautot 2010a). To find out whether BELL1-2 may perform a similar function in legume plants, the homeotic *cochleata* mutant was used in our experimental work. The expression of *PsBELL1-2* was found to be significantly decreased in the *cochleata* mutant nodules (Figure S3) compared to the wild-type nodules of *P. sativum* (parental cultivar SGE) (Fig. 9). Interestingly, we also detected downregulation of the expression level for *PsBELL1-3* in such nodules, but not for *PsBELL1-4*. It is important to note that the *PsBELL1-2* and *PsBELL1-4* genes have the highest homology level among all *BELL* genes in pea *P. sativum*. Furthermore, their orthologs BLH2 and BLH4 (SAW1/SAW2) in *A. thaliana* often have similar and redundant functions in the control of developmental processes (Kumar et al. 2007; Xu et al. 2020b). Apparently, the role of BELL1-2 and BELL1-4 in the development processes in legumes could be different and result in the specification of all their functions. Interestingly, we found that the expression level of *PsKNOX9* which was previously reported as a co-regulator of *PsBELL1-2* was also statistically significant decreased in *cochleata* mutant nodules.

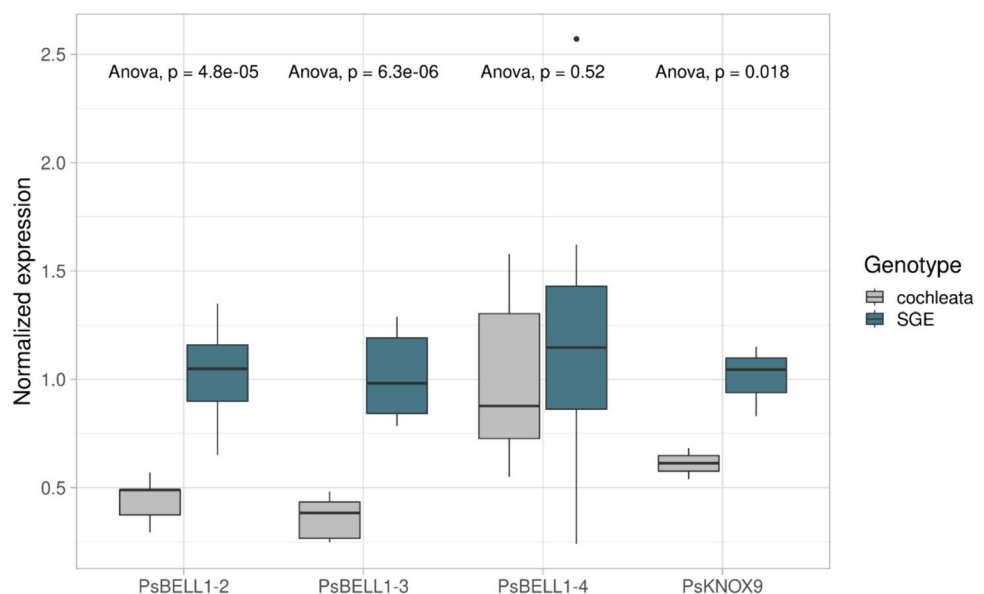
Analysis of the Interaction Between the BELL1-2 and KNOX Transcription Regulators During Nodulation

It is well known that BELL transcription factors can form functional heterodimers with members of the KNOX

family (Hamant and Pautot 2010b; Di Giacomo et al. 2017). Previously, in our experiments the ability of the BELL1-2 transcription factor to interact with the KNOX9 transcription factor of *M. truncatula* and *P. sativum* was demonstrated using a two-hybrid yeast system and coimmunoprecipitation (Dolgikh et al. 2020b). To investigate the influence of KNOX9 on nodulation and find possible crosslinking with BELL1-2, we used *ipd3/cyclops* mutants in which the expression of both genes was significantly decreased. The composite *M. truncatula ipd3/cyclops* plants carrying the construct for overexpression of *MtBELL1* (*MtBELL1-2-OE*) and overexpression of *MtKNOX9* (*MtKNOX9-OE*) in transgenic roots and nodules were created (Fig. 10). Analysis of changes in the number of nodules in *MtBELL1-2-OE* plants, as well as in *MtKNOX9-OE* plants, revealed a partial recovery of their amount per main root compared to the GUS-OE control (Fig. 10b). Here our findings showed that the transformation of *ipd3/cyclops* mutant with constructs for the overexpression of *BELL1-2* (*BELL1-2-OE*) and *KNOX9* (*KNOX9-OE*) resulted in a partial recovery of the number of nodules in both variants. On the basis of this data, we can suggest that BELL1-2 and KNOX9 may have an additive influence on nodule development.

At the same time, we failed to find difference in nodules number in wild-type *M. truncatula* A17 plants carrying the same constructs for overexpression of *MtBELL1* (*MtBELL1-2-OE*) and *MtKNOX9* (*MtKNOX9-OE*) (Figure S4). Probably, in contrast to *ipd3/cyclops* mutant in wild type the mechanisms of systemic regulation prevent such effect on nodule number.

Fig. 9 RT-qPCR analysis *PsBELL1-2*, *PsBELL1-3*, *PsBELL1-4* and *PsKNOX9* genes in *P. sativum cochleata* mutant 2 weeks-old nodules compared to the wild-type 2 weeks-old nodules of *P. sativum* (parental cultivar SGE). Levels of mRNA were normalized against *PsUbiquitin* gene and values were calculated as ratios relative to control (SGE). The data for 7–8 samples of transgenic nodules (10 nodules per each sample) ($n=7-8$) from 3 independent biological experiments were analyzed. R and ggpubr package were used for statistical analysis and visualization



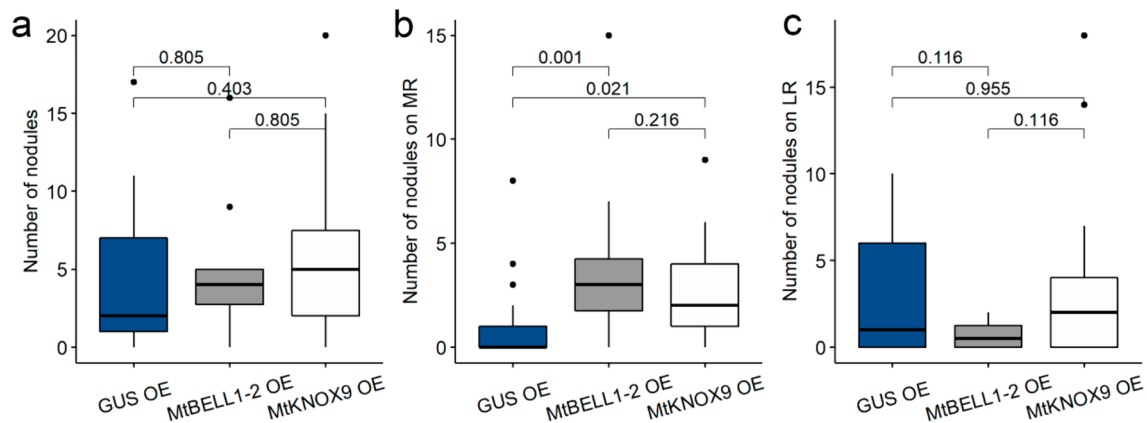


Fig. 10 Comparative analysis of the nodule number in control *GUS*-overexpressing plants (*GUS-OE*) and 2 weeks after inoculation plants with *MtBELL1-2* and *MtKNOX9* overexpression in *ipd3/cyclops M. truncatula* mutants. The total number of nodules (a), the number of nodules on main root (MR) (b) and on lateral roots (LR) (c). The data for 30–40 roots from 17–20 plants from 3 independent

biological experiments were analyzed. Each root was considered as replicate ($n=30-40$). Statistical analysis was performed in R using Kruskal–Wallis test with post-hoc Dunn’s test and ggpubr package for visualization and the numeric values indicated p-values received after Dunn’s test. *MR* Main root, *LR* Lateral root

Discussion

We have previously shown that *MtBELL1-2* expression was up-regulated already at 4–5 days after inoculation in *P. sativum* (Dolgikh et al. 2020b) as well as in *M. truncatula* (Schiessl et al. 2019a) and reached the highest level in nodules (Roux et al. 2014) based on quantitative reverse transcription PCR (qPCR) and transcriptomic analysis. However, in a recent single-cell transcriptomic atlas for *M. truncatula* roots inoculated by *S. meliloti*, *MtBELL1-2* expression was revealed in the epidermis already 48 h after inoculation (Cervantes-Pérez et al. 2022). It suggests that the expression of *BELL1-2* is activated quite early and probably related to the response upon inoculation in a small group of epidermal cells, as a more sensitive methodical approaches showed. This demonstrates that the *BELL1-2* may be also involved in the regulation of the early stages of root tissue infection along with the later stages of nodule development. Finally, our search in available transcriptomic databases showed that there was no difference in the expression level of *BELL1-2* between control and inoculated by arbuscular mycorrhizal fungi roots of *M. truncatula* (Karlo et al. 2020) and pea *P. sativum* (Afonin et al. 2020). It suggests that *BELL1-2* function is specific for rhizobial symbiosis.

In current experimental work, the functional role of the *BELL1-2* transcription factor was investigated using two species of legumes such as *M. truncatula* and *P. sativum* pea, both of which form nodules of indeterminate type. According to the previously published phylogenetic analysis (Dolgikh et al. 2020b) we found that *PsBELL1-2* gene (Psat4g090560) and *MtBELL1-2* (MtrunA17_Chr8g0373801) have the highest amino acid sequence similarity among all *BELL* genes found in *P. sativum* and *M.*

truncatula genomes. Besides, we also have shown that both of these genes had the highest expression level in nodules as well as activated at the early stages of symbiotic development. In addition, it was demonstrated in the same study that both *PsBELL1-2* and *MtBELL1-2* proteins are able to interact with *KNOX9* proteins, respectively. In current work we demonstrated that *MtBELL1-2* and *PsBELL1-2* influence on expression of *GA20ox* and *SHY2-like* genes was appeared in a similar manner. According to these data, we suggest that *PsBELL1-2* and *MtBELL1-2* can be considered as orthologs, but of course, further experiments such as mutant complementation analysis could be useful to confirm this idea.

At the first stage, we have aimed to find out what functional role the *BELL1-2* plays in nodulation, as well as the expression of which genes it directly or indirectly regulates, using localization of *pBELL1-2::GUS* promoter activity and the RNAi and overexpression approaches.

Analysis of qPCR data as well as available transcriptomes of both legumes indicated that the *BELL1-2* transcription factor may influence early infection events in epidermis and control initiation of nodule primordia. Furthermore, it is also involved in the regulation of later stages of nodule formation and functioning in *M. truncatula* and pea *P. sativum*. Indeed, here it was shown that hypertrophied development of infection threads may be related to an increase in the size of the infection zone (ZII) in nodules in *BELL1-2* RNAi plants of both legumes. These results were in line with the most intensive *GUS* staining in the infection zone (ZII) and the nitrogen fixation zone (ZIII), but low staining in the nodule meristem (ZI) as localization of the *MtBELL1-2* promoter activity showed. It should also be noted that the *GUS* staining pattern observed in *P. sativum* nodules with *pPsBELL1-2::GUS* expression was different from that of

pMtBELL1-2::GUS pattern in *M. truncatula*. However, the expression patterns of *pMtBELL1-2::GUS* in *M. truncatula* and *P. sativum* nodules were similar as well as the same is true for *pPsBELL1-2::GUS* in both legumes. Thus, the regulatory elements in the promoters of *BELL1-2* genes appear to be universal in both legumes, but are differently localized in the upstream regions of these genes in *P. sativum* and *M. truncatula*. The observed differences in GUS staining patterns may be explained by the limitations of such an approach to assess expression patterns, as it depends largely on potentially differently positioned regulatory elements in the *PsBELL1-2* and *MtBELL1-2* promoter regions of the similar size.

Previously, we found that *BELL1-2* expression was significantly decreased in inoculated roots and nodules of *ipd3/cyclops* mutant (Dolgikh et al. 2020b), which was characterized by arrested rhizobia release from infection threads (Tsyganov et al. 1998; Ovchinnikova et al. 2011). So, we can suggest that *ipd3/cyclops* mutant phenotype impaired in infection development can be partially explained by decrease of *BELL1-2* expression. These data also illustrate a possible role for the *BELL1-2* transcription factor in the regulation of infection development at later stages and probably bacterial release from infection threads. However, this suggestion requires future investigation.

Our data showed that the *BELL1-2* transcription factor can regulate *GA20ox* genes that control gibberellin metabolism. This is in agreement with the results of studies on potato *S. tuberosum*, which showed that *BELL* transcription factors are capable of influencing genes of the biosynthesis and degradation of active gibberellin forms (Chen et al. 2004b). In mature nodules of *M. truncatula* and pea *P. sativum*, *BELL1-2* may stimulate *GA20ox1* gene expression, as experiments with *BELL1-2*-RNAi showed. Previously, the stimulating effect of gibberellins on pea nodule functioning and prevention of nodule senescence was also shown (Serova et al. 2019). Furthermore, previously we showed the possibility of an interaction between *BELL1-2* and the regulator of gibberellin biosynthesis, the *DELLA1* protein (Dolgikh et al. 2020b), which also indicated the possibility of regulation of plant response to gibberellins using the *BELL1-2* transcription factor. This regulation of gibberellin level by *BELL1-2* may be directly related to control of infection development and the functioning of mature nodules.

In addition, the *BELL1-2* transcription factor can stimulate the *IPT1* gene that controls cytokinin metabolism. It is in the accordance with role of *KNOX* transcription factors, possible co-factors, which also play an important role in regulation of cytokinin metabolism and plant response to cytokinin (Azarakhsh et al. 2015; Di Giacomo et al. 2017).

The functional activity of the *BELL* transcription factor appears usually by means of its interaction with transcription factors from the *KNOX* family. To search for

the co-regulator of *PsBELL1-2*, we previously tested the possibility of interaction of this protein with the proteins *PsKNOX3*, *PsKNOX5* and *PsKNOX9*, since the activation of these members of the *KNOX* family after inoculation was previously shown (Azarakhsh et al. 2015; Di Giacomo et al. 2017). The possibility of interaction between the transcription factors *PsBELL1-2* and *PsKNOX9*, as well as their homologues *MtBELL1-2* and *MtKNOX9*, using the yeast two-hybrid system and coimmunoprecipitation was previously shown (Dolgikh et al. 2020b). Here, our findings showed that transformation of *ipd3/cyclops* mutant with constructs for overexpression of *BELL1-2* (*BELL1-2*-OE) and *KNOX9* (*KNOX9*-OE) resulted in partial recovery of nodule number in both variants. On the one hand, it confirms the joint effect of both transcription factors *BELL1-2*-*KNOX9* as a proposed complex. On the other hand, it demonstrates the stimulating effect of both transcription factors on nodule initiation, probably related to the regulation of this process by gibberellins or cytokinins.

At the next stage, we have also tried to answer the question which additional targets *BELL1-2* may regulate. As mentioned above, we observed a significant decrease in the expression of *PsBELL1-2* in *cochleata* mutant nodules, as well as the expression of *PsKNOX9*. Previously, decreased expression of the *MtKNOX9* gene was also found in *noot* *M. truncatula* mutant impaired in the homologous gene (Couzigou et al. 2012). The homeotic mutants *cochleata/noot* are defective in the specification of nodule meristem, because the mutation induces root development from nodule meristem in nodules of these legume plants. It suggests the importance of *BELL1-2* and *KNOX9* in the determination of nodule identity, as the mutant analysis showed.

Our findings showed that there was a putative *BELL1-2* binding site in the promoters of the *MtSHY2* and *PsSHY2* genes. Analysis of the promoter region of the *MtSHY2-like* and *PsSHY2-like* genes revealed the TGACAGGT motif, previously reported as the *BELL* binding site (Viola and Gonzalez 2006). We also found that *BELL* can act as a negative regulator of *SHY2*, because the expression of *MtSHY2* and *PsSHY2* was increased in *BELL1-2*-RNAi plants. It was shown previously that *SHY2* can act as a negative regulator of root meristem development, that stimulates the transition to subsequent stages of root development (Li et al. 2020). This function of *SHY2* may be directly related to its negative effect on the auxin signal pathway. It is known that *BELL* can regulate *SHY2* expression, which in its turn negatively regulates auxin signalling and positively regulates cytokinin biosynthesis (Tian et al. 2003). In legumes such as *M. truncatula*, *SHY2*-like is selectively expressed in the meristem zone of the nodule, where the balance between cell division and differentiation is tightly regulated (Roux et al. 2014). The auxin positively regulates nodule primordium initiation at the early

stages, but probably decreasing its level may stimulate the subsequent development of this organ. Therefore, down-regulation of *SHY2*-like expression may be related to its direct regulation by *BELL1-2* and the transition from the initiation of the nodule to the development of this organ in legumes forming an indeterminate type of nodules. Such a role of *BELL1-2* as putative morphogenetic regulator requires future investigation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00344-024-11487-5>.

Author Contributions DAV: Investigation, writing original draft preparation, methodology, imaging and data analysis. KES: Plant transformation, qPCR analysis. DAM (ER): Plant transformation, cloning. DEA: Conceptualization, writing and editing, and supervision. All authors have read and agreed to the manuscript.

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Data Availability The authors declare that all data supporting the findings of this study are available in this article and its Supplementary Information files.

Declarations

Competing Interests Authors declare no conflict of interest.

References

- Afonin AM, Leppyanen IV, Kulaeva OA, Shtark OY, Tikhonovich IA, Dolgikh EA et al (2020) A high coverage reference transcriptome assembly of pea (*Pisum sativum* L.) mycorrhizal roots. *Vavilov J Genet Breed* 24:331–339. <https://doi.org/10.18699/VJ20.625>
- Azarakhsh M, Kirienko AN, Zhukov VA, Lebedeva MA, Dolgikh EA, Lutova LA (2015) *KNOTTED1-LIKE HOMEODOMAIN 3*: a new regulator of symbiotic nodule development. *J Exp Bot* 66:7181–7195. <https://doi.org/10.1093/jxb/erv414>
- Breakspear A, Liu C, Roy S, Stacey N, Rogers C, Trick M et al (2014) The root hair “Infectome” of *Medicago truncatula* uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. *Plant Cell* 26:4680–4701. <https://doi.org/10.1105/tpc.114.133496>
- Bürglin TR, Affolter M (2016) Homeodomain proteins: an update. *Chromosoma* 125:497–521. <https://doi.org/10.1007/s00412-015-0543-8>
- Cervantes-Pérez SA, Thibivilliers S, Laffont C, Farmer AD, Frugier F, Libault M (2022) Cell-specific pathways recruited for symbiotic nodulation in the *Medicago truncatula* legume. *Mol Plant* 15(12):1868–1888. <https://doi.org/10.1016/j.molp.2022.10.021>
- Chen H, Banerjee AK, Hannapel DJ (2004) The tandem complex of *BEL* and *KNOX* partners is required for transcriptional repression of *ga20ox1*. *Plant J* 38:276–284. <https://doi.org/10.1111/j.1365-3113X.2004.02048.x>
- Couzigou J-M, Zhukov V, Mondy S, Abu el Heba G, Cosson V, Ellis THN et al (2012) Nodule root and cochleata maintain nodule development and are legume orthologs of *Arabidopsis* blade-on-petiole genes. *Plant Cell* 24:4498–4510. <https://doi.org/10.1105/tpc.112.103747>
- Crespi M, Frugier F (2008) De novo organ formation from differentiated cells: root nodule organogenesis. *Sci Signal*. <https://doi.org/10.1126/scisignal.149re11>
- Di Giacomo E, Laffont C, Sciarra F, Iannelli MA, Frugier F, Frugis G (2017) *KNAT3/4/5*-like class 2 *KNOX* transcription factors are involved in *Medicago truncatula* symbiotic nodule organ development. *New Phytol* 213:822–837. <https://doi.org/10.1111/nph.14146>
- Dolgikh EA, Kusakin PG, Kitaeva AB, Tsyganova AV, Kirienko AN, Leppyanen IV et al (2020a) Mutational analysis indicates that abnormalities in rhizobial infection and subsequent plant cell and bacteroid differentiation in pea (*Pisum sativum*) nodules coincide with abnormal cytokinin responses and localization. *Ann Bot* 125:905–923. <https://doi.org/10.1093/aob/mcaa022>
- Dolgikh AV, Rudaya ES, Dolgikh EA (2020b) Identification of bell transcription factors involved in nodule initiation and development in the legumes *Pisum sativum* and *Medicago truncatula*. *Plants* 9:1–13. <https://doi.org/10.3390/plants9121808>
- Ferguson BJ, Mathesius U (2014) phytohormone regulation of legume-rhizobia interactions. *J Chem Ecol* 40:770–790. <https://doi.org/10.1007/s10886-014-0472-7>
- Fonouni-Farde C, Tan S, Baudin M, Brault M, Wen J, Mysore KS et al (2016) *DELLA*-mediated gibberellin signalling regulates Nod factor signalling and rhizobial infection. *Nat Commun* 7:12636. <https://doi.org/10.1038/ncomms12636>
- Gamas P, Brault M, Jardinaud MF, Frugier F (2017) Cytokinins in symbiotic nodulation: when, where, what for? *Trends Plant Sci* 22:792–802. <https://doi.org/10.1016/j.tplants.2017.06.012>
- Gonzalez-Rizzo S, Crespi M, Frugier F (2006) The *Medicago truncatula* *CRE1* cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. *Plant Cell* 18:2680–2693. <https://doi.org/10.1105/tpc.106.043778>
- Hamant O, Pautot V (2010a) Plant development: a TALE story. *C R Biol* 333:371–381. <https://doi.org/10.1016/j.crv.2010.01.015>
- Heckmann AB, Sandal N, Bek AS, Madsen LH, Jurkiewicz A, Nielsen MW et al (2011) Cytokinin induction of root nodule primordia in *Lotus japonicus* is regulated by a mechanism operating in the root cortex. *Mol Plant-Microbe Interact* 24:1385–1395. <https://doi.org/10.1094/MPMI-05-11-0142>
- Held M, Hou H, Miri M, Huynh C, Ross L, Hossain MS et al (2014) *Lotus japonicus* cytokinin receptors work partially redundantly to mediate nodule formation. *Plant Cell* 26:678–694. <https://doi.org/10.1105/tpc.113.119362>
- Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I et al (2005) *KNOX* action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Curr Biol* 15:1560–1565. <https://doi.org/10.1016/j.cub.2005.07.023>
- Jin Y, Liu H, Luo D, Yu N, Dong W, Wang C et al (2016) *DELLA* proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. *Nat Commun* 7:12433. <https://doi.org/10.1038/ncomms12433>
- Joo S, Wang MH, Lui G, Lee J, Barnas A, Kim E et al (2018) Common ancestry of heterodimerizing TALE homeobox transcription factors across Metazoa and Archaeplastida. *BMC Biol*. <https://doi.org/10.1186/s12915-018-0605-5>
- Karlo M, Boschiero C, Landerslev KG, Blanco GS, Wen J, Mysore KS et al (2020) The *CLE53-SUNN* genetic pathway negatively regulates arbuscular mycorrhiza root colonization in *Medicago truncatula*. *J Exp Bot* 71:4972–4984. <https://doi.org/10.1093/jxb/eraa193>
- Kreplak J, Madoui M-A, Cápál P, Novák P, Labadie K, Aubert G et al (2019) A reference genome for pea provides insight into legume genome evolution. *Nat Genet* 51:1411–1422. <https://doi.org/10.1038/s41588-019-0480-1>
- Kumar R, Kushalappa K, Godt D, Pidkowich MS, Pastorelli S, Hepworth SR et al (2007) The *Arabidopsis* *BEL1-LIKE*

- HOMEODOMAIN proteins SAW1 and SAW2 act redundantly to regulate KNOX expression spatially in leaf margins. *Plant Cell* 19:2719–2735. <https://doi.org/10.1105/tpc.106.048769>
- Lechner M, Findeiß S, Steiner L, Marz M, Stadler PF, Prohaska SJ (2011) Proteinortho: detection of (Co-)orthologs in large-scale analysis. *BMC Bioinform* 12:124. <https://doi.org/10.1186/1471-2105-12-124>
- Li T, Kang X, Lei W, Yao X, Zou L, Zhang D et al (2020) SHY2 as a node in the regulation of root meristem development by auxin, brassinosteroids, and cytokinin. *J Integr Plant Biol* 62:1500–1517. <https://doi.org/10.1111/jipb.12931>
- Lin J, Frank M, Reid D (2020) No home without hormones: how plant hormones control legume nodule organogenesis. *Plant Commun* 1:100104. <https://doi.org/10.1016/j.xplc.2020.100104>
- Liu C-W, Breakspear A, Stacey N, Findlay K, Nakashima J, Ramakrishnan K et al (2019a) A protein complex required for polar growth of rhizobial infection threads. *Nat Commun* 10:2848. <https://doi.org/10.1038/s41467-019-10029-y>
- Liu J, Rutten L, Limpens E, van der Molen T, van Velzen R, Chen R et al (2019b) A remote cis-regulatory region is required for NIN expression in the pericycle to initiate nodule primordium formation in *Medicago truncatula*. *Plant Cell* 31:68–83. <https://doi.org/10.1105/tpc.18.00478>
- Luo Z, Liu H, Xie F (2023) Cellular and molecular basis of symbiotic nodule development. *Curr Opin Plant Biol* 76:102478. <https://doi.org/10.1016/j.cpb.2023.102478>
- McAdam EL, Reid JB, Foo E (2018) Gibberellins promote nodule organogenesis but inhibit the infection stages of nodulation. *J Exp Bot* 69:2117–2130. <https://doi.org/10.1093/jxb/ery046>
- Nadzieja M, Kelly S, Stougaard J, Reid D (2018) Epidermal auxin biosynthesis facilitates rhizobial infection in *Lotus japonicus*. *Plant J* 95:101–111. <https://doi.org/10.1111/tpj.13934>
- Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45:119–144. <https://doi.org/10.1146/annurev-genet-110410-132549>
- Ovchinnikova E, Journet E-P, Chabaud M, Cosson V, Ratet P, Duc G et al (2011) IPD3 controls the formation of Nitrogen-Fixing symbiosomes in pea and *Medicago* Spp. *Mol Plant-Microbe Interact* 24:1333–1344. <https://doi.org/10.1094/MPMI-01-11-0013>
- Pecrix Y, Staton SE, Sallet E, Lelandais-Brière C, Moreau S, Carrère S et al (2018) Whole-genome landscape of *Medicago truncatula* symbiotic genes. *Nat Plants* 4:1017–1025. <https://doi.org/10.1038/s41477-018-0286-7>
- Plet J, Wasson A, Ariel F, Le Signor C, Baker D, Mathesius U et al (2011) MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. *Plant J* 65:622–633. <https://doi.org/10.1111/j.1365-313X.2010.04447.x>
- Roux B, Rodde N, Jardinaud M, Timmers T, Sauviac L, Cottret L et al (2014) An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA-sequencing. *Plant J* 77:817–837. <https://doi.org/10.1111/tpj.12442>
- Schiessl K, Lilley JLS, Lee T, Tamvakis I, Kohlen W, Bailey PC et al (2019a) Nodule inception recruits the lateral root developmental program for symbiotic nodule organogenesis in *Medicago truncatula*. *Curr Biol* 29:3657–3668.e5. <https://doi.org/10.1016/j.cub.2019.09.005>
- Schiessl K, Lilley J, Lee T, Tamvakis I, Kohlen W, Bailey P et al (2019b) Nodule inception recruits the lateral root developmental program for symbiotic nodule organogenesis in *Medicago truncatula*. *Curr Biol*. <https://doi.org/10.1016/j.cub.2019.09.005>
- Serova TA, Tsyganova AV, Tikhonovich IA, Tsyganov VE (2019) Gibberellins inhibit nodule senescence and stimulate nodule Meristem Bifurcation in pea (*Pisum sativum* L.). *Front Plant Sci* 10:1–19. <https://doi.org/10.3389/fpls.2019.00285>
- Suzaki, T., Yano, K., Ito, M., Umehara, Y., and Sukanuma, N. (2012). Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. 4006, 3997–4006. <https://doi.org/10.1242/dev.084079>
- Tian Q, Nagpal P, Reed JW (2003) Regulation of Arabidopsis SHY2/IAA3 protein turnover. *Plant J* 36:643–651. <https://doi.org/10.1046/j.1365-313X.2003.01909.x>
- Tirichine L, Sandal N, Madsen LH, Radutoiu S, Albrektsen AS, Sato S et al (2007) A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science*. <https://doi.org/10.1126/science.1132397>
- Tsyganov VE, Morzhina EV, Stefanov SY, Borisov AY, Lebsky VK, Tikhonovich IA (1998) The pea (*Pisum sativum* L.) genes sym33 and sym40 control infection thread formation and root nodule function. *Mol Gen Genet* 259:491–503. <https://doi.org/10.1007/s004380050840>
- Viola IL, Gonzalez DH (2006) Interaction of the BELL-like protein ATH1 with DNA: role of homeodomain residue 54 in specifying the different binding properties of BELL and KNOX proteins. *Biol Chem* 387:31–40. <https://doi.org/10.1515/BC.2006.006>
- Warnes, G., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., et al. (2022). Various R Programming Tools for Plotting Data. Version 3.1.3. Available at: <https://github.com/talgalili/ggplots>.
- Xu Y, Wang Y, Wang X, Pei S, Kong Y, Hu R et al (2020a) Transcription factors BLH2 and BLH4 regulate demethylesterification of homogalacturonan in seed mucilage. *Plant Physiol*. <https://doi.org/10.1104/pp.20.00011>
- Xu Y, Wang Y, Wang X, Pei S, Kong Y, Hu R et al (2020b) Transcription factors BLH2 and BLH4 regulate Demethylesterification of Homogalacturonan in Seed Mucilage. *Plant Physiol* 183:96–111. <https://doi.org/10.1104/pp.20.00011>

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