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Short title: IPT3 is required for nodulation in *M. truncatula*

Full title: Spatiotemporal cytokinin signaling imaging reveals IPT3 function in nodule development in *Medicago truncatula*

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One-sentence summary: High-resolution spatiotemporal imaging of cytokinin signaling reveals IPT3 function during indeterminate nodule development in *Medicago truncatula*

AUTHOR CONTRIBUTIONS

PMT, MK, and DC designed the research; PMT, TBI, ZPK, SC, JMA, MK, and DC designed the methodology; PMT, HWS, and DC performed the experiments; TBI, ZPK, SC, CD, and JMA provided material and technical advice. KSM and JW supplied plant material. PMT, MK, and DC wrote the paper. TBI, HWS, SC, KB, WP, CD, and JMA supervised the data analyses and reviewed and edited the paper. MK and DC supervised the methodology, data analyses, and writing-editing the paper.

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ABSTRACT

Most legumes can establish a symbiotic association with soil rhizobia that triggers the development of root nodules. These nodules host the rhizobia and allow them to fix nitrogen efficiently. The perception of bacterial lipo-chitooligosaccharide (LCO) signal in the epidermis initiates a signaling cascade that allows rhizobial intracellular infection in the root and de-differentiation and activation of cell division that gives rise to the nodule. Nodule organogenesis and rhizobial infection need to be coupled in space and time for successful nodulation. The plant hormone cytokinin (CK) acts as an essential positive regulator of nodule organogenesis, and specific CK receptors are required for nodule formation. Temporal regulation of tissue-specific CK signaling and biosynthesis in response to LCOs or *Sinorhizobium meliloti* inoculation in *Medicago truncatula* remains poorly understood. In the present study, using a fluorescence-based CK sensor (*TCSn::nls:tGFP*), we performed a high-resolution tissue-specific temporal characterization of the CK response's sequential activation during root infection and nodule development in *M. truncatula* after inoculation with *S. meliloti*. Loss-of-function mutants of the CK-biosynthetic gene *ISOPENTENYL TRANSFERASE 3* (*IPT3*) showed impairment of nodulation, suggesting that IPT3 is required for nodule development in *M. truncatula*. Simultaneous live imaging of *pIPT3::tdTOMATO* and the CK sensor showed that *IPT3* induction in the root stele at the base of nodule primordium contributes to CK biosynthesis, which in turn promotes expression of positive regulators of nodule organogenesis in *M. truncatula*.

Keywords: Cytokinin, *IPT3*, *Medicago truncatula*, *Sinorhizobium meliloti,* nodule development, nodule organogenesis, cytokinin sensor, cytokinin signaling, cytokinin biosynthesis

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INTRODUCTION

Legume species acquired the capacity to interact symbiotically with rhizobium bacteria to fix atmospheric dinitrogen, allowing their growth without fertilizers on nitrogen-deprived soils. This interaction involves the development of specific organs, the root nodules, that host rhizobia and provide them with carbon sources and the microenvironment required for nitrogen fixation. In most rhizobia-legume associations, the perception of bacterial lipo-chitooligosaccharide (LCO) signals commonly known as Nod factors in the epidermis initiates a molecular cascade that is transmitted to the inner cell layers activating cell division, with simultaneous rhizobial infection of the host root. In *Medicago truncatula*, rhizobial infection is initiated within a curled root hair tip and the subsequent formation of a transcellular apoplastic compartment called the infection thread (IT). The IT traverses the epidermis, cortex and ramifies within the confines of the nodule primordium, developed by organized cell divisions in the root endodermis, cortex, and pericycle (Roy et al., 2020). These coordinated mechanisms of root infection and nodule organogenesis ensure that nodule maturation occurs in perfect coordination with nodule colonization by rhizobia (Xiao et al., 2014). *M. truncatula* produces indeterminate nodules that are characterized by a longitudinal gradient of differentiation with a persistent distal apical meristem and older proximal layers (Ferguson et al., 2010). Like other developmental processes, nodulation is modulated by phytohormones (Buhian and Bensmihen, 2018).

The plant hormone cytokinin (CK) is involved in various aspects of plant growth and development. CK signaling consists of a phosphorelay mediated by a two-component system, comprising a sensor and a response regulator. The site of CK binding is suggested to be the lumen of the endoplasmic reticulum. The CK-induced phosphorelay causes transcriptional changes in the nucleus mediated by type-B and type-A response regulators (RRs), which play positive, and negative roles in this regulation, respectively (Kieber and Schaller, 2018). Type-

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B RRs typically bind to target genes at the consensus sequence (A/G)GAT(T/C) enriched in their *cis*-regulatory regions, and synthetic CK sensors called Two-Component signaling Sensors (TCS) containing concatemeric versions of this sequence have been studied in plants (Zürcher et al., 2013). CK plays essential roles during nodule formation (Ferguson and Mathesius, 2014; Gamas et al., 2017). In *M. truncatula,* CK accumulates in the root susceptibility zone as early as 3 hours after LCO treatment (van Zeijl et al., 2015). The TCS is activated by rhizobium in the cortical cells in *M. truncatula* that forms indeterminate nodules and in *Lotus japonicus* that forms determinate ones (Held et al., 2014; Jardinaud et al., 2016). In *Glycine max*, a regulatory feedback loop involving auxin and cytokinin governs proper determinate nodule development (Turner et al., 2013). Rhizobia also induce the expression of CK biosynthetic and signaling genes in the epidermis, based on transcriptomic studies focused on the epidermal cells of *M. truncatula* (Liu et al., 2015; Damiani et al., 2016; Jardinaud et al., 2016). The *pMtRR9::GUS* transcriptional reporter, a cytokinin RR type-A (RRA), was rapidly detected in the root epidermis, in addition to other root tissues, in response to LCOs (Op den Camp et al., 2011). A more recently developed CK signaling sensor termed TCS new (TCSn) (Zürcher et al., 2013), driving GUS expression, enabled detection of the activation of a CK response in the *M. truncatula* root epidermis and the outer cortex 8 hours after the LCO treatment (Jardinaud et al., 2016). In *L. japonicus*, the *TCS* reporter was activated first in the cortex and only later in the epidermis by rhizobia (Held et al., 2014; Reid et al., 2017). The sequence of activation of CK responses during early symbiotic stages may differ between these two nodulating species, likely as a reflection of differences in the process of nodule development between them (Gamas et al., 2017).

CK plays an antagonistic role during root infection at the epidermis and nodule formation in the cortex (Gamas et al., 2017). The positive regulation of CK on nodule formation was first reported by physiological studies, which showed that exogenous CK

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103 induces the formation of nodule-like structures on the roots of several legumes (Heckmann et
104 al., 2011). Further evidence for the positive role of CK in nodule inception has come from the
105 analysis of nodulation-defective mutants altered in CK receptors, LOTUS HISTIDINE
106 KINASE 1 (LHK1) in *L. japonicus*, and CYTOKININ RESPONSE 1 (CRE1) in *M.*
107 *truncatula* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007; Plet et al.,
108 2011), and other CK receptors in both legumes (Held et al., 2014; Boivin et al., 2016).
109 Moreover, gain-of-function *LHK1* and *MtCRE1* lines generate spontaneous nodules in the
110 absence of the rhizobia (Tirichine et al., 2007; Madsen et al., 2010; Ovchinnikova et al.,
111 2011). Transcriptomic analyses in *M. truncatula* also enabled identifying symbiotic genes
112 that are rapidly induced by an exogenous CK on roots (Ariel et al., 2012), such as *NODULE*
113 *INCEPTION* (*NIN*). NIN and the CRE1-dependent pathways are connected by a positive
114 feedback loop, with NIN binding to the CRE1 promoter and activating its expression (Vernié
115 et al., 2015). Similarly, *CRE1* is required for cytokinin-induced *NIN* expression (Plet et al.,
116 2011). In *L. japonicus* roots, exogenous CK treatment also induces *NIN* specifically in root
117 cortical cells (Heckmann et al., 2011). Moreover, *NIN* ectopic expression leads to root
118 cortical cell divisions and nodule-like structures in both *L. japonicus* and *M. truncatula*
119 (Soyano et al., 2013; Vernié et al., 2015), through the activation of transcription factors, such
120 as Nuclear Factor Y subunit A1 (NFYA1) and Nuclear Factor Y subunit B1 (NFYB1)
121 (Soyano et al., 2013; Laloum et al., 2014; Hossain et al., 2016; Shrestha et al., 2021). In
122 contrast, in the epidermis of *M. truncatula,* CK negatively regulates root infection (Gamas et
123 al., 2017). The epidermal CK pool was depleted by expressing a CK
124 OXIDASE/DEHYDROGENASE (CKX) enzyme under an epidermis-specific promoter,
125 which lead to an increased number of ITs and nodules (Jardinaud et al., 2016). Additionally,
126 exogenous CK treatment inhibited the induction of the LCO response and pre-infection
127 marker *MtENOD11,* in a MtCRE1 dependent fashion (Jardinaud et al., 2016). Recently, a link

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between the epidermis-derived CK and cortical cell divisions was established (Jarzyniak et al., 2021). *M. truncatula* ATP-binding cassette (ABC) transporter 56 (MtABCG56), transports CK from the epidermal to cortical cells, activating the CRE-dependent CK responses, including the *RRA4* (Jarzyniak et al., 2021). These downstream responses trigger further CK biosynthesis required for nodule development (Mortier et al., 2014; van; Zeijl et al., 2015; Vernié et al., 2015).

Several CK biosynthesis genes, including *ISOPENTENYL TRANSFERASE 3* (*IPT3*) and *1* (*IPT1*), *CYP735A1*, *LONELY GUY 1* (*LOG1*), and *2* (*LOG2*), are upregulated in response to LCOs or during nodulation in *L. japonicus* and *M. truncatula* (Chen et al., 2014; Mortier et al., 2014; Azarakhsh et al., 2015; van Zeijl et al., 2015; Azarakhsh et al., 2018; Schiessl et al., 2019). The expression of *MtIPT3*, *MtLOG1* and *MtLOG2* transcriptional GUS reporters were also detected in the nodule primordium (Mortier et al., 2014; Azarakhsh et al., 2020). Decreasing *LOG1* expression leads to impaired nodulation in *M. truncatula*, being involved in the nodule primordium development (Mortier et al., 2014). All these studies highlight the importance of CK biosynthesis during root infection and nodule development. Transcriptional fusions using GUS gene reporter allowed identifying CK signaling at a tissue-specific level in *M. truncatula* roots during these biological processes. However, these studies have been limited in their temporal resolution. A detailed spatial and temporal characterization of the CK response in *M. truncatula* roots should clarify the role of this hormone in nodule induction and organogenesis.

In the current study, we present the spatiotemporal regulation of CK response in rhizobia-inoculated roots using a new fluorescence-based CK signaling sensor, *pTCSn::nls:tGFP*. To further explore the potential of this sensor, we employed it along with the transcriptional fusion of the CK biosynthetic gene *IPT3* during nodule development. Simultaneous monitoring of the *pIPT3::tdTOMATO* reporter and the CK sensor activities

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during nodule development suggested that *IPT3* induction at the base of nodule primordium contributes to CK biosynthesis, which in turn, promotes nodule organogenesis in *M. truncatula*. Furthermore, we analyzed the loss-of-function mutant of *ipt3* and found that it is required for nodule development in *M. truncatula*.

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RESULTS

A fluorescent protein-based cytokinin sensor is activated in root epidermal and cortical cells upon CK treatment in *Medicago truncatula*

CK responses have been studied in response to LCOs and *S. meliloti* in *M. truncatula* roots, using transcriptional reporters with *RRAs* or the synthetic *TCSn* promoters fused to the GUS gene (Op den Camp et al., 2011; Plet et al., 2011; Jardinaud et al., 2016; Fonouni-Farde et al., 2017). In soybean, fluorescent protein-based auxin and CK transcriptional reporters have been successfully used to monitor and determine their cellular level ratios in root and nodule tissues (Fisher et al., 2018). In *L. japonicus*, a tissue-specific time course experiment following the activity of *TCSn::nls:GFP* showed that CK response occurs in cortical cells before spreading to the epidermis (Reid et al., 2017).

In the present work, we designed a fluorescent protein-based CK transcriptional reporter that addresses the limitations of the GUS reporter system. This CK sensor consists of the *TCSn* promoter (Zürcher et al., 2013) driving the expression of the turbo green fluorescent protein (tGFP) fused to a nuclear localization signal peptide (*pTCSn::nls:tGFP*). This alternative approach to the GUS reporter system allows continuous, non-destructive monitoring of CK signaling throughout plant development by live imaging, and co-imaging with other fluorescence-based reporters.

Before evaluating the CK sensor activity, we characterized the timing of CK transcriptional responses in *M. truncatula* roots by analyzing the expression profiles of three *RRA* genes, *RRA3*, *RRA4,* and *RRA11* after 1, 8, 24, and 48 hours of 1 µM 6-benzylamino-purine (6-BAP) treatment. We found that *RRA*s reached their maximum gene expression after 24 hours of the 6-BAP treatment **(Fig. 1A)**. Based on the CK signaling activation timing, we assessed the tissue-specific CK response using the *pTCSn::nls:tGFP* CK sensor in *M.*

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truncatula transgenic roots after 24 hours of BAP treatment. In non-treated roots, tGFP was primarily detected in the columella, root apical meristem, and elongation zone of the root tip, as previously described for the *pTCSn::GUS* reporter (**Fig. 1B**; Jardinaud et al., 2016; Fonouni-Farde et al., 2017). Very few cells showed nuclei-localized fluorescence in the differentiation zone (DZ) of the root **(Fig. 1C)**, indicating the absence of CK response in the DZ in non-treated roots. In contrast, roots treated with 1 µM 6-BAP for 24 hours exhibited a strong induction of nuclei-localized fluorescence in the epidermis and cortex in the DZ and the differentiated zone of the root **(Fig. 1 D-E)**. DAPI counterstain confirmed that tGFP fluorescence was localized to the nuclei **(Fig. S1)**. These observations indicate that the *pTCSn::nls:tGFP* sensor constitutes a suitable molecular tool to investigate the CK response in *M. truncatula* and that CK signal transduction occurs in *M. truncatula* epidermal and cortical cells after the application of CK to the root.

Tissue-specific time-course of CK signaling during the early symbiotic interaction with *S. meliloti* in *M. truncatula* roots

To characterize the spatial-temporal regulation of CK response during indeterminate nodule development in *M. truncatula*, we analyzed the activity of *pTCSn::nls:tGFP* in a time-course experiment using transgenic roots after *S. meliloti* inoculation. Prior to inoculation, very low *pTCSn::nls:tGFP* activity was detected in the cell layers of the susceptibility zone (SZ; **Fig. 2A**). At 4 hours after inoculation (hai), *pTCSn::nls:tGFP* activity started in the epidermal cells of the SZ, indicating that rhizobia-induced CK response in the epidermis is a very early response in *M. truncatula* **(Fig. 2B)**. At 24 hai, nuclei-localized fluorescence was still observed in the epidermal cells but was also present in outer cortical cells of the SZ **(Fig. 2C)** and by 48 hai, a strong fluorescence was widespread in the outer and inner cortical cell layers

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of the SZ **(Fig. 2D)**. Thus, CK signaling is activated first in the epidermis, reaches the outer cortical cells within 24 hai and extends to the majority of cortical cell layers within 48 hai.

Tissue-specific time-course of the activation of CK signaling during the indeterminate nodule development in *M. truncatula*

Characterization of CK response during indeterminate nodule development remains limited to a few time points during the process by using *RRA* promoters fused to GUS reporters in *M. truncatula* (Op den Camp et al., 2011; Plet et al., 2011). To obtain a better spatio-temporal resolution of CK response during the indeterminate nodule initiation and development, we monitored the *pTCSn::nls:tGFP* activity throughout nodule development from the first cell divisions in the pericycle, the endodermis, and the cortex, until the mature nodule formation. Moreover, we associate the tissue-specific *pTCSn::nls:tGFP* activity time course with the sequential cell division program characterized previously during nodule formation (Xiao et al., 2014).

At 3 days after inoculation (dai), the *pTCSn::nls:tGFP* signal that was widely distributed across the cortical cell layers of the SZ (see previous section) disappears, giving rise to a robust and more localized signal at the pericycle and dividing cortical cell layers C3-C5 that are related with the nodule primordium initiation, at the developmental stages II and III (**Fig. 2E**; Xiao et al., 2014). This nodule primordium-specific pattern of the CK response allowed us to clearly distinguish a nodule primordium from a lateral root primordium (LRP) during their early developmental stages, where tGFP expression was weaker and limited to the vasculature and developing meristem (**Fig. S2**). At 4 dai, we found that the stage IV and V nodule primordia showed the CK signaling activation extending to most of the dividing cortical cell layers (**Fig. 2F**). At 5 dai, the nodule primordium emerges from the main root and becomes a true nodule when the meristem starts functioning (stage VI). At this point, the

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CK response was localized to the C3, and the C4-C5 derived cells that form the multi-layered nodule meristem (C3) and the non-meristem zone immediately below (C4-C5), respectively **(Fig. 2G)**. At 6 dai, the nodule is in an advanced developmental stage, with the vascular bundles starting to surround the nodule meristem. At this stage, the CK signaling is strongly activated in the central zone of the nodule, including the nodule meristem and the C4/5-derived cells that will be colonized by rhizobia (**Fig. 2H**).

Cytokinin biosynthesis by IPT3 is required for nodule development in *M. truncatula*

CK and its downstream responses are critical regulators of nodule initiation and development. However, the molecular mechanism of the local CK biosynthesis during nodule organogenesis remains poorly characterized in *M. truncatula.* It has been proposed that a KNOX3 controls the transcription of two CK biosynthesis genes, *IPT3* and *LOG2*, to promote CK biosynthesis during nodule organogenesis (Azarakhsh et al., 2015; Azarakhsh et al., 2020). However, the genetic characterization of CK biosynthesis genes in *M. truncatula* remains limited to *LOG1* (Mortier et al., 2014). Recently, it has been proposed that ABCG56 actively exports bioactive CKs from the epidermis to the cortex, driving the CRE1-dependent cortical CK response. This would lead to *de novo* CK biosynthesis in the cortex, required for nodule organogenesis (Jarzyniak et al., 2021).

It was previously shown that the *IPT3* expression is induced at 72 hai, reaching a maximum at 5 dai (Schiessl et al., 2019). Moreover, the *pIPT3*::*GUS* reporter showed that *IPT3* is expressed in the nodule primordium of *M. truncatula* (Azarakhsh et al., 2020). These observations indicate that IPT3 represents an excellent candidate to investigate the role of CK biosynthesis during nodule organogenesis.

We identified lines from the Noble Research Institute *Medicago truncatula Tnt1* collection showing an insertion in the single exon of *IPT3* (Tadege et al., 2008). These

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mutants were named *ipt3-1*; *ipt3-2,* and *ipt3-3* (**Fig. 3A**). After isolating homozygous individuals, *ipt3* knockout was confirmed by qRT-PCR analyses (**Fig. 3B**). Then, *ipt3* knockout mutants and wild-type plants were used to perform nodulation assays. At 14 dai, all the *ipt3* knockout mutants showed a significantly lower nodule number than the control (**Fig. 3C and D**). Moreover, between 40-55% of the *ipt3* mutants showed neither emerging nodule primordia nor nodules, while only 15% of wild-type plants lacked nodules (**Fig. 3E**). Root dry weight measurements revealed no significant differences between wild-type plants and *ipt3* mutants after 14 dai (**Fig. 3F**), while shoot dry weight significantly decreased only in *ipt3-1* (**Fig. 3G**). These results strongly suggest that the lower nodule number observed in *ipt3* mutants is a consequence of the loss of IPT3 function and not a pleiotropic effect caused by *Tnt1* insertions.

***IPT3* expression is induced in the stele at the base of the developing nodule primordium**

To get further insights into the role of *IPT3* during nodule organogenesis, we investigated *IPT3* expression by monitoring the activity of tdTOMATO fluorescent protein driven by *IPT3* promoter in a time-course experiment. This strategy allowed us to monitor *IPT3* expression and CK signaling simultaneously after the inoculation with *S. meliloti* through live imaging in *M. truncatula* transgenic roots. To achieve this goal, we cloned the *IPT3* promoter (2312 bp; see Method section) in frame with the tdTOMATO fluorescent protein fused to a nuclear localization signal peptide resulting in the *pIPT3::nls:tdTOMATO* construction. Before inoculation with rhizobia, *IPT3* expression and CK response overlapped at the root stele (**Fig. S3 A-C**). At 24 hours post *S. meliloti* inoculation, *IPT3* expression was similar to the control treatment and still localized in the vasculature (**Fig. S3 D-F**). In contrast, the CK response was observed in the epidermis and outer cortical cells of the SZ, as described above (**Fig. S3 G-I**). At 2 dai, CK response expanded to the majority of root cortical layers of the

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SZ, while *pIPT3::nls:tdTOMATO* activity was still mainly found in the stele with a similar expression level to that of the control (**Fig. 4A-C; Fig S3E and H**). This suggests that IPT3 is not involved in the CK signaling activation during the early symbiotic interaction described in the present research work (48 hai).

At 3 dai, nodule primordium was initiated (between stage II and III), and CK response was mainly localized to the dividing cortical cells and stele **(Fig 4D)**. *IPT3* expression was strongly induced in the stele at the base of dividing cortical cells of the nodule primordium **(Fig. 4E)**. This result is consistent with prior reports of the induction of the *IPT3* transcription 72 hours after *S. meliloti* inoculation (Schiessl et al., 2019; **Fig. S4**). The induction of the CK response and *IPT3* expression overlapped at the root vasculature and the base of the nodule primordium, likely involving pericycle **(Fig. 4F)**. At 4 dai, with the nodule primordium at advanced stages of development and CK response extended to more cortical cell layers **(Fig. 4G)**, *IPT3* expression levels remained high and localized in the stele below the dividing cortical cells **(Fig. 4H)**, showing lower overlapping with CK signaling in the stele **(Fig. 4I)**. At 6 dai, CK response was mainly localized at the central zone of the nodule **(Fig. 4J)**, while *IPT3* expression was strongly activated and started to propagate from the root stele to the nodule vasculature **(Fig. 4K)**. At this stage, the nodule meristem activates, and the overlap between the CK response and *IPT3* expression declined, with each one showing a specific spatial pattern **(Fig. 4L)**. At 7 dai, CK response was mainly localized to the nodule meristem **(Fig 4M)**. At the same time, *IPT3* expression was also detected in the developing nodule vasculature **(Fig. 4N)**, in line with *IPT3* expression in nodule vascular bundles recently reported using *pIPT3::GUS* (Azarakhsh et al., 2020). At this stage, CK response was restricted to the nodule meristem, and *IPT3* expression was localized at the base of the nodule and vascular bundles **(Fig. 4O)**. Together, these results suggest that the *IPT3* induction at the

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base of the nodule primordium contributes to the biosynthesis of CK, which in turn triggers CK signaling during nodule organogenesis.

IPT3 is required for the induction of symbiotic genes during nodule initiation

Rhizobia-induced nodule initiation is dependent on crucial nodule development regulators that promote cortical cell division, including the cytokinin receptor CRE1, the transcription factor NIN and its targets LBD16 and NFYA1 (Gonzalez-Rizzo et al., 2006; Plet et al., 2011; Laporte et al., 2014; Vernié et al., 2015; Schiessl et al., 2019). *ipt3* mutants show impairment of nodule development **(Fig. 3D)**, suggesting that the biosynthesis of CK precursors by IPT3 is required to activate CK-induced positive regulators of nodule development. To test this hypothesis, we performed an *in vitro* nodulation assay using wild-type and two *ipt3* mutant lines, *ipt3-2* and *ipt3-3,* to compare gene expression of these essential regulatory genes. *ipt3-1* was excluded due to the previously mentioned reduced shoot mass phenotype. 3-day-old *M. truncatula* plants were inoculated with *S. meliloti,* and the SZ of the root was harvested at 4 dai for gene expression analyses. We found that expression of *NIN*, *LBD16,* and *NFYA1* was upregulated in wild-type compared to non-inoculated control plants. In contrast, in the *ipt3* mutants, these genes were not significantly induced **(Fig. 5)**, indicating that the transcriptional activation of positive regulators of nodulation requires IPT3 at 4 dai. *CYCLINA3;A (CYC3;A)*, a CK-induced gene and a cell division marker during nodule initiation (Schiessl et al., 2019), did not show significant induction either in the wild-type or *ipt3* mutants with respect to the control treatment **(Fig. 5)**. Besides *CRE1*, we found significant transcriptional induction of CK signaling genes, such as *RRA3* and *RRA11*, which was affected in *ipt3* mutants compared to the control **(Fig. 5),** indicating IPT3 is required for the activation of CK signaling during nodule initiation. On the contrary, *RRA4* was not induced in wild-type and *ipt3* mutants by rhizobia compared to the control **(Fig. 5).** *IPT3* was

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also upregulated in wild-type plants at 4 dai with respect to the control **(Fig. 5)**, consistent with the rhizobia-induced expression pattern observed in previous work and with the visual reporter (**Fig. S4**; Schiessl et al., 2019; **Fig. 4H**). Together, these results suggested that rhizobia-induced *IPT3* expression contributes to CK biosynthesis. In turn, CK biosynthesis promotes transcriptional activation of positive nodule development regulators and CK signaling genes required for nodule development.

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DISCUSSION

In the present study we developed a novel tGFP-based *TCSn* reporter that allowed us to perform live imaging tissue-specific time course, with a high temporal resolution of the rhizobia-induced CK signaling. This reporter system allowed us to gain further insights into the cytokinin signaling induction during rhizobia perception and nodule formation in *M. truncatula*. Our data indicate that the CK signaling activation occurs in multiple, discrete stages, initially activating in the epidermis of the root SZ and expanding across the cortex during the first 48 hrs. Rhizobia trigger this first wave of CK signaling in the SZ of the *M. truncatula* root. After 48 hours, this widespread CK signaling activation disappears, giving way to the second wave of CK signaling activation in the cortex, localized in the specific zones where the cell divisions that will give rise to the nodule primordium. The characterization of *ipt3* mutants suggests that IPT3 contributes to the CK biosynthesis that triggers this second wave of signaling activation.

It has been proposed recently that the *MtABCG56* transporter, which is transcriptionally induced between 6 and 24 hours after the LCO treatment, exports bioactive CKs from the root epidermis to the cortex, promoting CRE1-dependent cortical CK response (Jarzyniak et al., 2021). In agreement with this model, we observed that CK signaling was activated at 24 hai in the outer cortical cells **(Fig. 2C)**, and it extends to the majority of cortical cell layers at 48 hai in the SZ **(Fig. 2D)**, possibly due to the amplification of CRE1-triggered CK response. These results are consistent with the similar CK activation patterns observed previously using *pTCSn:GUS*, showing GUS activity localized at the epidermis and outer cortical cells at 8 hai and the inner cortical cells at 72 hai, and *in situ* hybridization detected expression of *RRA4* mRNA widely in the root cortex at 48 hai (Vernié et al., 2015; Jardinaud et al., 2016). Here, a live imaging time course allowed us to precisely elucidate the timing of the CK signaling activation pattern observed during the early symbiosis interaction.

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365 Consistent with these observations, *in situ* hybridization showed that *RR4* mRNA levels
366 strongly accumulate in the different *M. truncatula* root cortex layers at 48 hai with *S. meliloti*
367 (Vernié et al., 2015). In contrast, in *L. japonicus*, CK signaling activation in cortical cells
368 precedes epidermal CK responses (Held et al., 2014; Reid et al., 2017). These results suggest
369 that the spatial-temporal CK signaling activation may differ between determinate and
370 indeterminate nodulating species. The difference in rhizobia-triggered CK signaling patterns
371 between these species highlights the need for high-resolution tissue-specific characterization
372 of the CK responses in other legumes.

373 The second wave of CK signaling activation in the cortex requires *de novo* CK
374 biosynthesis. It has been reported that CK biosynthesis genes, such as *LOG1*, *LOG2,* and
375 *IPT3,* are expressed in the nodule primordium of *M. truncatula* (Mortier et al., 2014;
376 Azarakhsh et al., 2020). It has been proposed that KNOX3 transcription factor directly
377 promotes the expression of cytokinin biosynthesis genes by *MtLOG1*, *MtLOG2*, and *MtIPT3*
378 which are co-expressed during nodule development in *M. truncatula* (Azarakhsh et al., 2020).
379 By monitoring the *pIPT3::nls:tdTOMATO* and the CK sensor simultaneously, we resolved
380 the spatial-temporal pattern of *IPT3* expression and its interaction with the CK signaling
381 during the indeterminate nodule development in *M. truncatula*. We found that *IPT3* is
382 induced in the stele, likely at the pericycle and adjacent cells to the first dividing cortical cells
383 at 3 dai **(Fig. 4E)**, overlapping with CK signaling activation at the base of the nodule
384 primordium **(Fig. 4F)**. This result disagrees with the *pIPT3::GUS* activity reported in the
385 whole nodule primordium after 3 days post inoculation (Azarakhsh et al., 2020). This
386 discrepancy may be explained by a higher sensitivity of the GUS reporter, which could reveal
387 the promoter activity even with very low expression levels. This outcome may also be
388 derived by the diffusion of the GUS reaction product to adjacent cells, especially when
389 extended incubation periods are required. However, similarly to what was reported with

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pIPT3::GUS, after 7 days post inoculation (Azarakhsh et al., 2020), we found that *IPT3* expression spreads from the stele at the base of the nodule to the nodule developing vascular bundles **(Fig. 4N)**.

IPT enzymes catalyze the formation of iP riboside 5'-diphosphate (iPRDP) or iP riboside 5'-triphosphate (iPRTP), which are precursors for bioactive cytokinin biosynthesis by LOG family enzymes (Sakakibara, 2006). The induction of *IPT3* at the base of the nodule primordium may provide the substrate for *LOG* genes, such as *LOG1* and *LOG2*, which catalyzes the production of the bioactive CKs (Kurakawa et al., 2007). Indeed, analyses of *LOG1* and *LOG2* promoters fused to GUS revealed that these genes are expressed in the central zone of developing nodule primordia, overlapping with our observations of the CK signaling activation (Mortier et al., 2014; Azarakhsh et al., 2020). Similarly, *LOG1* RNAi plants show lower nodules than control plants in *M. truncatula* (Mortier et al., 2014), and we found a similar result in our *ipt3* loss of function mutants. The same effect has been reported in *L. japonicus IPT3* RNAi plants, which produced fewer ITs and nodules than wild-type (Chen et al., 2014). However, it has been proposed that *LjIPT3* also participates in the generation of shoot-derived CK precursors, which participate in the autoinhibition of nodulation (AON) mechanism in *L. japonicus* (Sasaki et al., 2014). These findings suggested that IPT3-derived CKs may be involved in different mechanisms in shoots and roots. The different timing of *IPT3* activation explains its dual role during nodulation of *L. japonicus*. If *IPT3* participates in AON in *M. truncatula* remains an open question.

Rhizobia-dependent transcriptional activation of CK-induced positive regulators of nodule development, such as *NIN*, *CRE1*, *LBD16,* and *NFYA1,* was affected in *ipt3* mutants at 4 dai **(Fig. 5)**. Together, these results suggested that *de novo* CK precursor synthesis is required for the CK-mediated induction of key nodule development regulators, promoting CK downstream responses and cell divisions during nodule development.

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In conclusion, we propose a high-resolution model for the tissue-specific temporal activation pattern of CK signaling during indeterminate nodule development in *M. truncatula* **(Fig. 6)**. Furthermore, we show that *IPT3*, a cytokinin biosynthesis gene, contributes to *de novo* CK biosynthesis, which in turn promotes the expression of a critical positive regulator of nodule development, such as *NIN* and *CRE1*.

MATERIALS AND METHODS

Plant material and growth conditions

Medicago truncatula R108 is the wild-type background for the *Tnt1* mutant lines; Jemalong A17 was used for the rest. Seeds were scarified 8 min in sulfuric acid and sterilized for 4 min in bleach (12% [v/v] sodium hypochlorite). After rinsing with sterilized water, seeds were sown on 1% agar plates supplemented with 1 µM gibberellic acid and stored at 4°C for 3 days before incubating overnight at 24°C in the dark. Germinated seedlings were transferred to square plates (22.5x22.5 cm) containing modified Fahräeus medium (Boisson-Dernier et al., 2001) supplemented with 15 mM NH_4NO_3 and grown vertically at 24°C under long-day conditions in a growth chamber (16 h light/8 h dark; 150 µmol m^{-2} s^{-1} light intensity).

Cloning

The Golden Gate MoClo and MoClo Plant toolkits (New England Biolabs) were used to obtain all constructions described in this work (Engler et al., 2014). The cloning reaction consists of 25 cycles of 3 min at 37°C for digestion and 4 min at 16°C for ligation combining the restriction enzymes *Bsa*I-HF (New England BioLabs, Ipswich, UK) for level 1 reaction or *Bpi*I (Thermo Fisher Scientific, Waltham, USA) for level 2 reaction together with T4 DNA Ligase (New England BioLabs, Ipswich, UK). *TCSn* promoter DNA sequence (Zürcher et al.,

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2013) comprising overhangs was synthesized by Synbio Technologies and cloned in frame with a nuclear localization signal (Addgene, catalog number: 50294), turbo GFP (Addgene, catalog number: 50322), and *Solanum lycopersicum* ATPase terminator (Addgene, catalog number: 50344) in the level 1 vector pICH47811 (Addgene, catalog number: 48008). To amplify *IPT3* promoter (gene ID v4: Medtr1g072540; v5: MtrunA17Chr1g0185751) genomic DNA (gDNA) extraction was performed as previously described (Causevic et al., 2005) with the following modifications. 3-day-old *M. truncatula* A17 seedlings were frozen in liquid nitrogen and ground in 2 ml tubes using a homogenizer (MiniG 1600, Spex, Metuchen, NJ). 200 µL of pre-heated CTAB buffer containing 0.2% β-mercaptoethanol, 4% PVP, and 0.1 mg/ml Proteinase K (Invitrogen) was added to the ground tissue and digested for 1 h at 60 °C. After adding 1 volume of chloroform/isoamyl alcohol (24:1, v/v), samples were mixed and then centrifuged at 14,000xg for 10 min. The aqueous phase was transferred to a new tube and incubated with 1 µL of RNase A (Macherey-Nagel, 12mg/mL stock) for 30 min at 37 °C. The chloroform/ isoamyl alcohol cleaning step was repeated and the aqueous phase was transferred to a new tube and mixed with 1 volume of isopropanol and incubated for 30 min at -20 °C. The sample was centrifuged at 14,000xg for 15 min, and the pellet was washed with 70% and 95% ethanol by centrifugation for 5 min. The pellet was air-dried and resuspended in 50 µL of TE buffer (Invitrogen, catalog number: 12090015). A genomic fragment of 2312 bp upstream of the *IPT3* coding sequence including the 5' UTR was amplified using primers containing Golden Gate overhangs **(Table S1)** from *M. truncatula* A17 gDNA. 100 ng of gDNA were used to set up a PCR using Phusion® High-Fidelity DNA Polymerase (New England BioLabs). The PCR product was separated in a 1% agarose gel by electrophoresis. *IPT3* promoter DNA sequence was extracted and purified from the gel using Monarch DNA Gel Extraction Kit (New England Biolabs) and Sanger sequenced at Genewiz (South Plainfield, NJ). Also, tdTOMATO coding sequence (CDS) was amplified using

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primers containing Golden Gate overhangs **(Table S1)**. IPT3 promoter was cloned in frame with a nuclear localization signal (Addgene, catalog number: 50294), tdTOMATO CDS, and 35S terminator (Addgene, catalog number: 50337) in the level 1 vector pICH47802 (Addgene, catalog number: 48007). Level 1 pICH47811-*pTCSn::nls:tGFP::tATPase* (position 2) was cloned together with pICH47802-*p35S::ER:tdTOM::tNOS* selection marker (position 1) in the final plant expression vector pAGM4673 (Addgene, catalog number: 48014). For simultaneous live imaging, pICH47811-*pTCSn::nls:tGFP* (position 2) was cloned together with pICH47802-*pIPT3::nls:tdTOMATO::t35S* (position 1) in the final plant expression vector pAGM4673 (Addgene, catalog number: 48014).

Genotyping of *Tnt1* insertion lines

M. truncatula R108 *Tnt1* transposon insertion lines utilized in this research project, which are jointly owned by the Centre National De La Recherche Scientifique, were obtained from the Noble Research Institute, and were created through research funded, in part, by grants from the National Science Foundation, NSF #DBI-0703285, and NSF #IOS-1127155. Three different *Tnt1* transposon insertion lines, namely NF5762 (*ipt3-1*), NF3757 (*ipt3-2*), and NF4651 (*ipt3-3*), were genotyped by PCR using *Tnt1*-specific primers (Cheng et al., 2011) combined with *MtIPT3* gene-specific primers encompassing the insertions **(Table S1)**. The expression of *MtIPT3* in homozygous plants was tested by RT–qPCR to confirm they were knockout mutants for the *MtIPT3* gene **(Table S1)**.

***Agrobacterium rhizogenes*-mediated transformation and *in-vitro* nodulation assay**

The constructs described above were introduced into *Agrobacterium rhizogenes* MSU440 electrocompetent cells and used to generate transgenic roots in *M. truncatula* (Boisson-Dernier et al., 2001). The transgenic roots were selected based on the fluorescence emitted by

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pTCSn::nls:tGFP construct at the root tip under a fluorescence stereomicroscope. For *in-vitro* nodulation assay time-course experiment, 4-week-old transgenic roots were transferred to Buffered Nodulation Medium (BNM) (Ehrhardt et al., 1992) supplemented with 0.1 µM AVG (aminoethoxyvinyl glycine hydrochloride; Sigma-Aldrich) to reduce ethylene production. After 5 days of acclimation, transgenic roots were treated with a suspension of *S. meliloti* (OD_{600}=0.02) supplemented with 3 µM of luteolin to activate LCO production (Sigma-Aldrich) collecting transgenic roots at different timepoints.

In vitro Cytokinin treatment and _S. meliloti_ inoculation

3-day-old *M. truncatula* seedlings were transferred to Fahräeus medium supplemented with water (mock treatment) or 1 µM of 6-BAP (6-benzylaminopurine; Sigma-Aldrich) and maintained under the same growth conditions. Five roots from different seedlings were collected after 1, 8, 24, and 48 hours after CK treatment and immediately frozen in liquid nitrogen for RNA extraction. To analyze *pTCSn::nls:tGFP* activity after CK treatment, *M. truncatula* transgenic roots were submerged with a solution of 1 µM 6-BAP for 5 min and then the solution was poured off. After 24 hours, transgenic roots harboring *pTCSn::nls:tGFP* construct were harvested and used for microscopic analysis. For qRT-PCR studies of nodule development regulator and CK signaling genes in wild-type and *ipt3* mutants, germinated seedlings were transferred to nitrogen-free Fahräeus medium and grown under the same conditions described above. 3-day-old seedlings were inoculated alongside the root with 200 µL/root of *S. meliloti* (OD_{600}=0.02) resuspended in liquid nitrogen-free modified Fahräeus medium or with Fahräeus medium (mock treatment). Four days after inoculation, root segments from the susceptibility zone were harvested and pooled (10 plants per sample) for RNA extraction.

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Gene expression analysis

RNA extraction was performed using as previously described (Chang et al., 1993). RNA samples were digested with DNase I and purified using RNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses were performed using Luna Universal Probe One-Step qRT-PCR Kit (New England Biolabs) following the manufacturer's instructions, using 200 ng of total RNA. *EF1*α gene was used as a housekeeping gene and the average of two technical replicates was obtained to calculate relative gene expression (ΔΔCt method). Primers used for qRT-PCR experiments are listed in **Table S1**.

Microscopic live imaging

Before microscopic imaging, transgenic roots were placed in a staining solution with 0.1% Calcofluor white M2R (Sigma-Aldrich) in PBS 1X (Corning) for 10 minutes at room temperature and then rinsed with PBS 1X before imaging. A confocal microscope Leica TCS SP5 confocal 119 was used for live imaging. At least 10 different roots for treatment or time point were analyzed. The excitation wavelengths for tGFP, tdTOMATO, and Calcofluor white M2R were 488, 561, and 405 nm, respectively. Fluorescent signals were collected at 497–557 nm (tGFP), 570–643 nm (tdTOMATO), or 450–480 nm (Calcofluor white M2R). Fluorescent signals are presented with green (tGFP), red (tdTOMATO), and magenta (Calcofluor white M2R). For DNA staining, transgenic roots were incubated with 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS 1X (Corning) for 10 min at room temperature and then rinsed with PBS 1X before imaging. DAPI excitation wavelength was 405 nm, while emission was collected at 450–480 nm.

***In vivo* Nodulation assay and dry weight measurement**

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For *in vivo* nodulation assay, seedlings were germinated as described above. Then, plants were grown in pots (9x9x9 cm) containing pre-sterilized calcined clay, Turface® (Profile Products, Buffalo Grove, IL) and sand (2:2 v/v), where Turface® was placed at the bottom and on the top of a layer of sand. Plants were watered with modified Fahräeus medium supplemented with 0.5 mM of NH_4NO_3 and covered with a lid. After one week of acclimation, Fahräeus medium supplemented with 0.5 mM of NH_4NO_3 was removed entirely from the tray and replaced with a nitrogen-free modified Fahräeus medium. Plants were treated pouring 10 mL of *S. meliloti* 1021 suspension (OD_{600}=0.02) into each pot. Plants were watered using nitrogen-free modified Fahräeus medium every 2-3 days. After 2 weeks of inoculation, nodule number was assessed inspecting plant roots under a stereomicroscope. After counting nodules, roots and shoots for each individual were separated and dried in paper bags. After 48 hours of drying at 65°C, root and shoot dry weight were recorded.

CONFLICT OF INTEREST

None declared.

FUNDING

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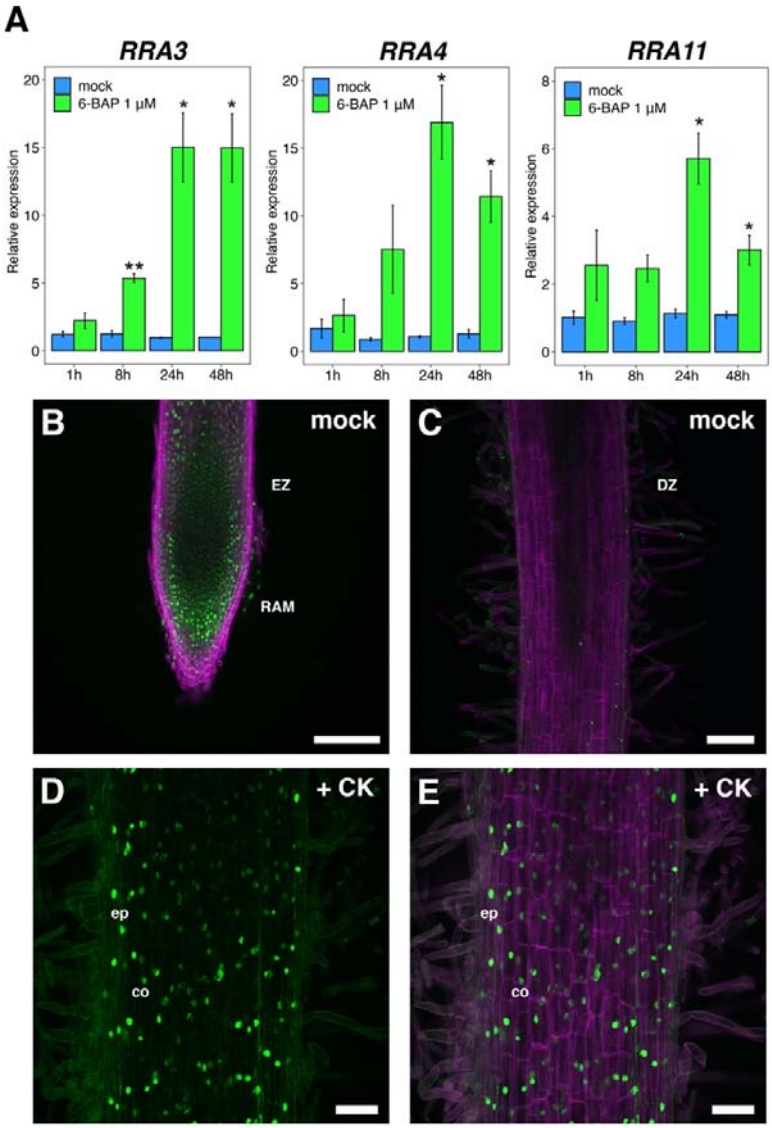
558 **FIGURES**



559

560 **Figure 1. A** reporter of CK signaling based on the *pTCSn::nls:tGFP* transcriptional fusion is
561 activated in the epidermal and cortical cells after CK treatment. (A) qRT-PCR analyses of
562 RRAs gene expression after 1, 8, 24, and 48 hours of 6-BAP or mock treatment. The
563 Student's *t*-test was performed, and asterisks represent statistically significant differences
564 between 6-BAP and mock treatments in each time point. **P*<0.05, ***P*<0.01. Values are the
565 means ± SE of two biological replicates (n=2). (B) *pTCSn::nls:tGFP* activity in the root tip of

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non-treated *M, truncatula* transgenic root. The tGFP signal from the nuclei of the root apical meristem (RAM) and elongation zone (EZ) is shown in green. In magenta, the signal emitted by cell wall polysaccharides bound to Calcofluor white M2R is shown. (C) *pTCSn::nls:tGFP* activity at the differentiation zone (DZ) of non-treated transgenic root and (D, E) in DZ of 1 µM 6-BAP treated transgenic root after 24 hours in the epidermis (epi) and cortex (co). Scale bar: 100 µm in B, C and 50 µm in D, E.

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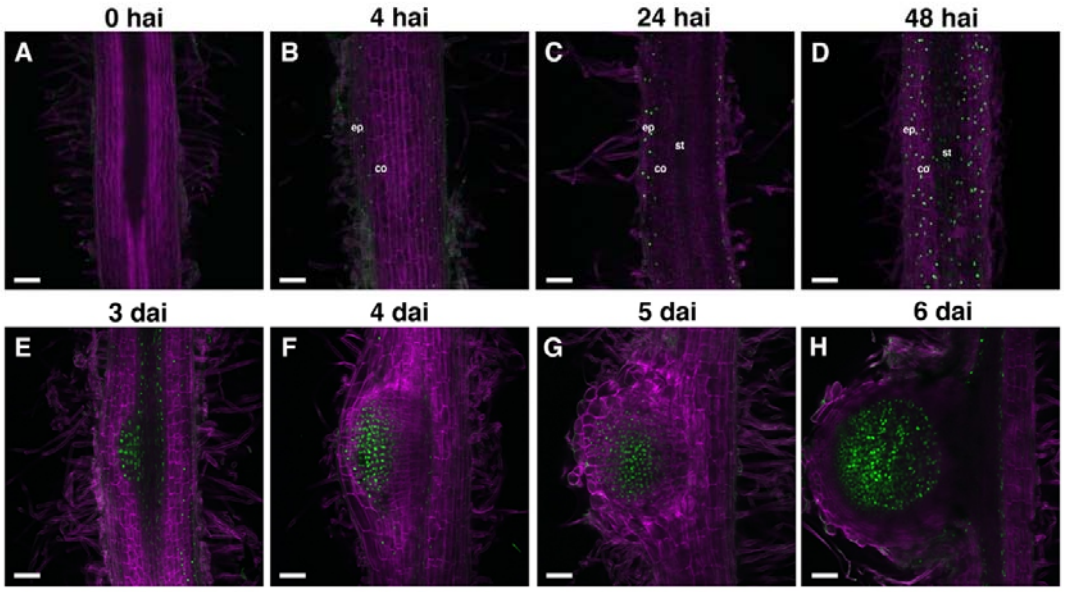

Figure 2. Spatio-temporal activation of cytokinin signaling during indeterminate nodule development in *M. truncatula*. (A-D) *pTCSn::nls:tGFP* activity (green) and cell walls (calcofluor white stained, magenta) in the susceptible zone of transgenic root after 0, 4, 24, and 48 hai with *S. meliloti*. Epidermis (ep), cortex (co) and stele (st). (E-H) *pTCSn::nls:tGFP* activity during nodule primordium development at (E) stages II/III, (F) stages IV/V, (G) stage VI and (H) mature nodule. Scale bar: 100 µm.

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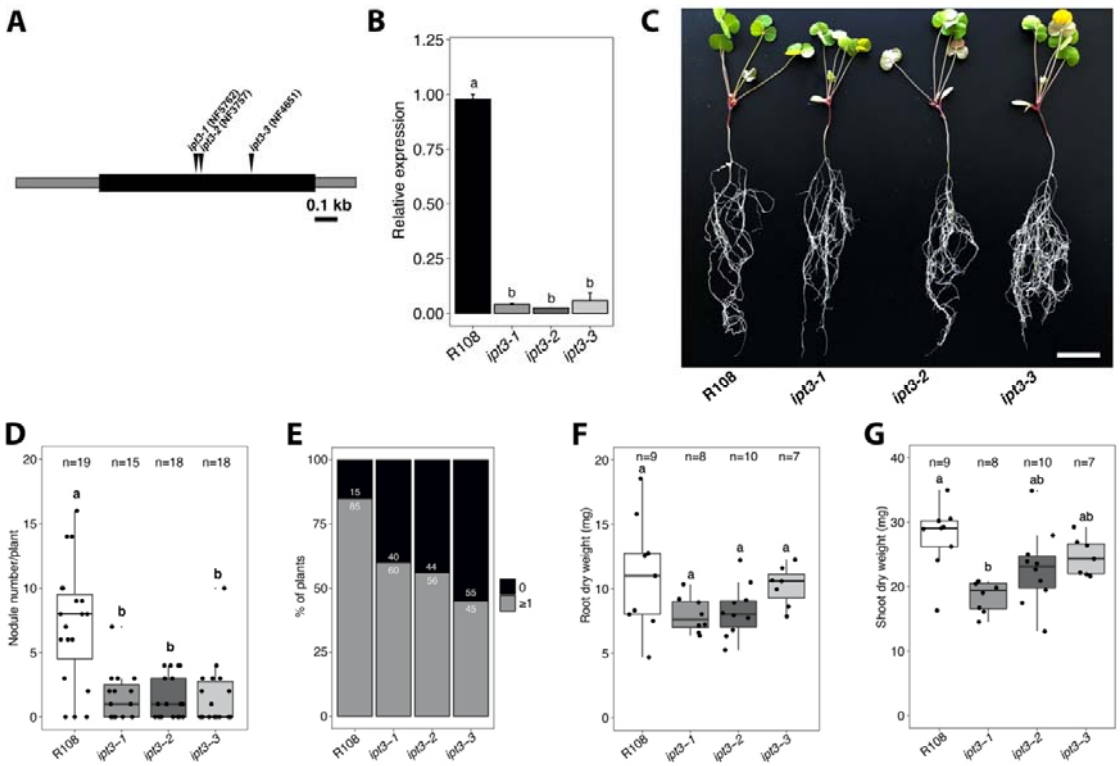

Figure 3. *IPT3* is required for nodule development in *M. truncatula*. (A) Schematic diagram showing genomic *IPT3* gene structure. NF5762, NF3757, and NF4651 *Tnt1* lines have one insertion in the unique exon region (black) flanked by 5'and 3' untranslated regions (gray) of the gene and were renamed *ipt3-1*, *ipt3-2,* and *ipt3-3*, respectively. Black bar, 100 bp. (B) qRT-PCR analysis of *IPT3* gene expression in R108 genotype and *ipt3* homozygous mutants. Values indicates means ± SE for three biological replicates (n=3). *P* values were calculated by ANOVA followed by Tukey's posthoc testing. Groups of different significance, at least $P<0.05$, are indicated with different letters. (C) Representative image of 3-week-old R108 and *ipt3* mutant plants after 14 dai of treatment with *S. meliloti* 1021. White bar represents 3 cm. (D) Nodule number in R108 and *ipt3* mutants after 14 dai. Statistical analysis was performed using ANOVA followed by Tukey's posthoc testing. Groups of different significance, at least $P<0.05$, are indicated with different letters. (E) Percentage of wild-type and *ipt3* mutant plants showing 1 or more nodules or none after 14 dai. (F) Root and (G)

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shoot dry weight measurements of wild-type and *ipt3* mutant plants after 14 dai. Statistical analysis was performed using ANOVA followed by Tukey's posthoc testing. Groups of different significance, at least $P<0.05$, are indicated with different letters.

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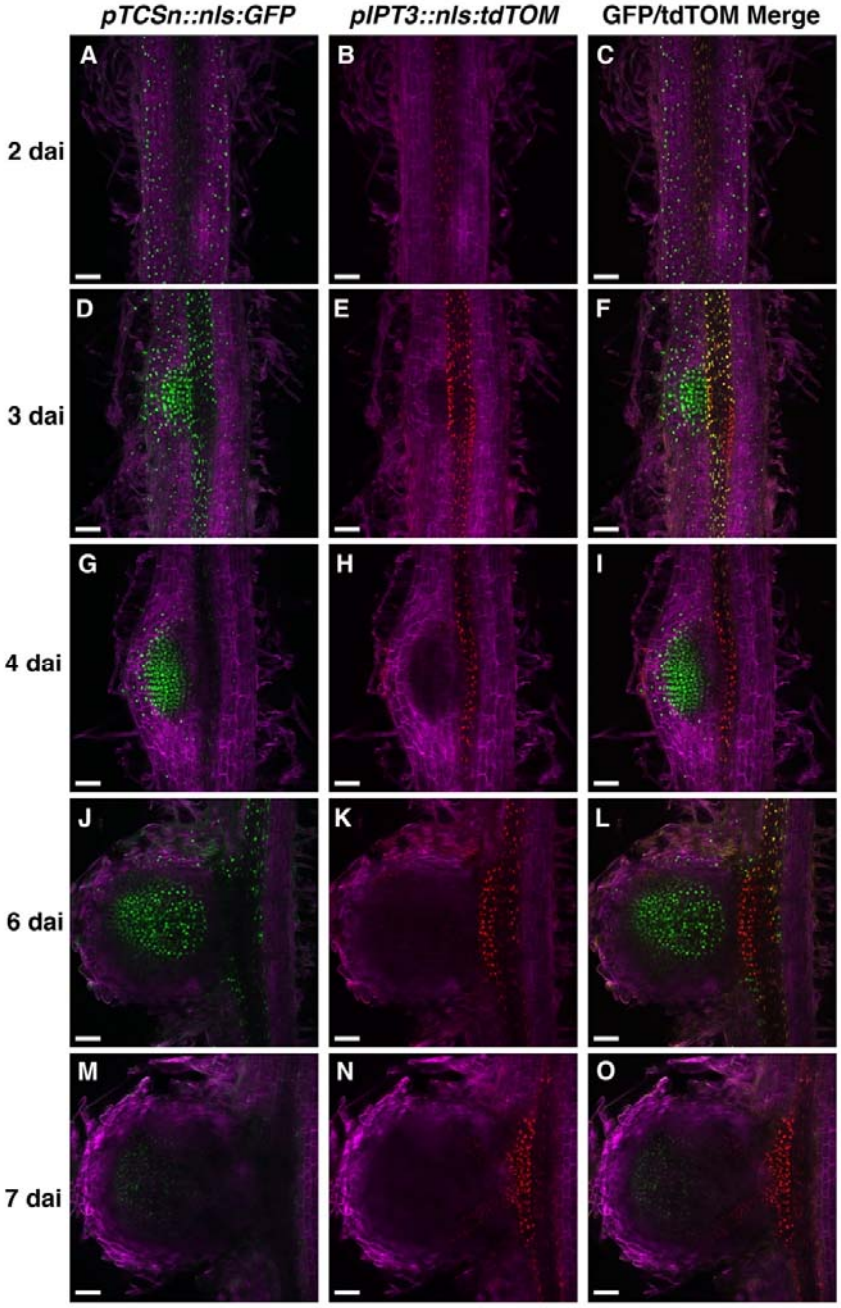

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599

600 **Figure 4.** *IPT3* expression is induced in the stele at the base of the nodule primordium during

601 the first cortical cell divisions. (A-C) *pTCSn::nls:tGFP* and *pIPT3::nls:tdTOMATO* activities

602 in the susceptible zone of transgenic root after 2 dai of *S. meliloti.* (D-I) *pTCSn::nls:tGFP*

603 and *pIPT3::nls:tdTOMATO* activities in different developmental stages of nodule

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primordium after 3 (D-F) and 4 dai (G-I). (J-O) *pTCSn::nls:tGFP* and *pIPT3::nls:tdTOMATO* activities in different developmental stages of mature nodules after 6 (J-L) and 7 dai (M-O). Green and red represent fluorescence signals emitted by tGFP and tdTOMATO, respectively. The white bar shows 100 μm.

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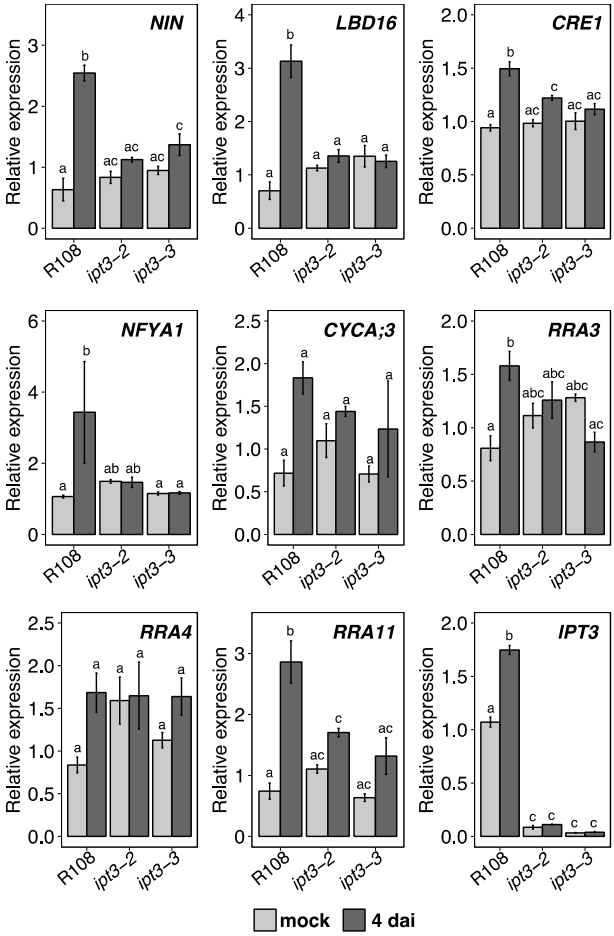

Figure 5. Rhizobia-dependent induction of nodulation regulators and CK signaling genes is impaired in *ipt3* loss-of-function mutants. qRT-PCR analyses of nodulation regulators (*NIN, LBD16, NFYA1*) cell cycle (*CYCA;3*) and CK signaling genes (*CRE1, RRA3, RRA4, RRA11*) after mock treatment or 4 dai in (A) wild-type, (B) *ipt3-2* and (C) *ipt3-3* mutants. Values are the mean of fold-changes of three biological replicates normalized to the untreated wild-type value (set as 1) for each gene for all genotypes. Values indicate means ± SE of three biological replicates (n=3). *P* values were calculated by ANOVA followed by Tukey's posthoc testing. Groups of different significance, at least $P<0.05$, are indicated with different letters.

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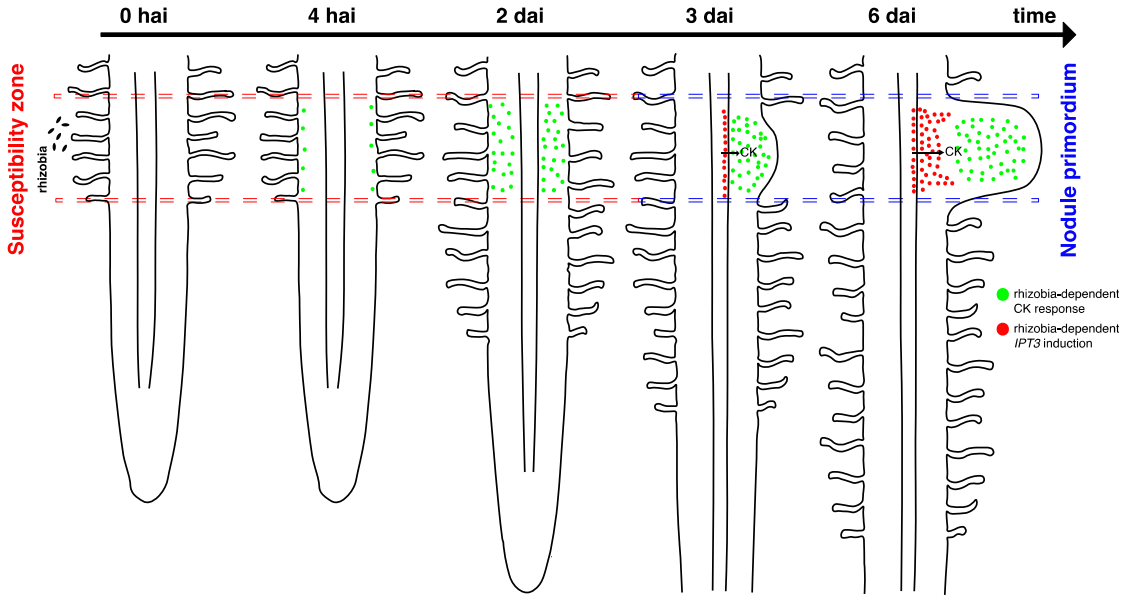

Figure 6. Schematic representation of the spatiotemporal regulation of CK response induced by rhizobia and the proposed function of IPT3 during indeterminate nodule development in *M. truncatula*. At 4 hai, CK signaling activation starts in epidermal cells, and progress to the majority of cortical cell layers within 48 hrs after rhizobia perception. At 3 dai, CK signaling is activated and localized in dividing cortical cells and *IPT3* expression is induced in the stele at the base of nodule primordium. At 6 dai, CK response is localized in the central zone of nodule primordium and *IPT3* is strongly activated and started to propagate from the root stele to the nodule vasculature.

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