Trends in Plant Science

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Review

Cytokinins in Symbiotic Nodulation: When, Where, What For?

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Substantial progress has been made in the understanding of early stages of the symbiotic interaction between legume plants and rhizobium bacteria. Those include the specific recognition of symbiotic partners, the initiation of bacterial infection in root hair cells, and the inception of a specific organ in the root cortex, the nodule. Increasingly complex regulatory networks have been uncovered in which cytokinin (CK) phytohormones play essential roles in different aspects of early symbiotic stages. Intriguingly, these roles can be either positive or negative, cell autonomous or non-cell autonomous, and vary, depending on time, root tissues, and possibly legume species. Recent developments on CK symbiotic functions and interconnections with other signaling pathways during nodule initiation are the focus of this review.

Symbiotic Nitrogen-Fixing Nodulation in Legumes

During evolution, legume species have acquired the capacity to interact symbiotically with rhizobium bacteria able to fix atmospheric nitrogen (N), and thereby to grow efficiently without fertilizers on N-deprived soils. This interaction involves the production of specific organs, the root nodules, that provide rhizobium cells with carbon sources and a microoxic environment required for N fixation. In addition to its major interest regarding sustainable agriculture practices, this symbiosis represents a highly valuable model to decipher signaling pathways for both plant–microbe interaction and *de novo* postembryonic organogenetic programs. This organogenesis is indeed strictly induced upon symbiotic bacteria recognition and tightly regulated by the host plant, together with the rhizobium infection. These processes have been intensively studied in the past decades, and numerous molecular components of the underlying complex networks have been identified (see reviews [1–4]).

Legume–rhizobia symbiotic interactions rely on an exchange of diffusible signals. In most cases, specific flavonoids within root exudates induce compatible symbiotic bacteria to secrete Nod factors (NFs), which are perceived by root epidermis lysin motif receptor-like kinases (LYSM-RLKs) [1,2] (Box 1). Additional bacterial signals are required for rhizobial infection, notably exopolysaccharides, shown in *Lotus japonicus* to be also perceived by an LYSM-RLK [5,6]. On the plant side, several phytohormones play important roles in NF signaling, rhizobial infection, and nodule organogenesis (see [7] for a recent and thorough review of phytohormonal regulations involved in legume–rhizobia interactions). Among these plant signals, cytokinins (CKs) are major players, originally demonstrated to be key factors of nodule inception in both *L. japonicus* and *Medicago truncatula* model legume species, and more recently shown to be involved in the regulation of rhizobium infection and nodule number (Figure 1). Moreover,

Trends

In addition to the well-documented positive role of CK phytohormones in nitrogen-fixing nodule inception, additional functions linked to other aspects of this symbiotic interaction have emerged.

Negative CK functions in the root epidermis have been identified in relation with rhizobial infections and the NF signaling pathway mediating bacterial symbiont recognition and infection.

Potential non-cell autonomous functions of CK occur at short distance between root epidermal and cortical layers and at long distance between shoots and roots in the frame of the 'autoregulation of nodulation' systemic pathway.

Identifying specificities of CK pathways in the different root tissues involved in nodulation and their cross-talk with other hormonal pathways remains a challenge required for a detailed mechanistic understanding of the initial stages of symbiotic nodulation.

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Box 1. Recognition of Symbiotic Bacteria by Host Plant Roots: The NF Signaling Pathway

Initial NFs perception occurs in root hairs of the rhizobium-susceptible zone, above the root meristematic region. It is mediated by a complex of LYSM-RLKs, including NF perception (NFP) and LYSM receptor kinase 3 (LYK3) in *Medicago truncatula* and NF receptor 1 (NFR1) and NFR5 in *Lotus japonicus* [1,2] (Figure 2, NF signaling shown in black using *M. truncatula* gene names). This leads to the activation in root epidermal cells of a signaling pathway involving a leucine-rich repeat-RLK, called does not make infections 2 (DMI2) in *M. truncatula* and SYMbiosis RK (SYMRK) in *L. japonicus*. Secondary signals are then initiated, notably thanks to nuclear cation channels (encoded by Mt*DMI1*/Lj*CASTOR*-Lj*POLLUX*) and cyclic nucleotide–gated calcium (Mt*CNGC15*) channels [72]. The calcium spiking signature thus generated is decoded by a nuclear calcium-calmodulin-dependent protein kinase (CCaMK, or DMI3 in *M. truncatula*). CCaMK/DMI3 phosphorylates LjCYCLOPS/MtIPD3 (interacting protein of DMI3). LjCYCLOPS acts as a transcriptional regulator proposed in *L. japonicus* to directly activate the expression of two transcription factors: NIN and ERN1 [73], both essential for rhizobium infection, together with an *ERN1* paralogous gene in *M. truncatula*, *MtERN2* [23,38,74]. NIN and ERN1/ERN2 are also critical for nodule organogenesis [23,27,38,52–55]. NIN activates the NF-YA1 transcriptional regulator, itself contributing to *ERN1* upregulation in *M. truncatula* [33,34]. In addition, *M. truncatula* DELLA proteins promote the phosphorylation of IPD3 in response to rhizobia or NFs, and consequently its interaction with other transcriptional regulators such as NSP2, NSP1, and potentially NF-YA1 [30,75]. These transcriptional complexes ultimately directly regulate the expression of symbiotic infection markers in the root epidermis such as Mt*ENOD11* (*early nodulin 11*), depending on NSP1, NSP2, ERN1, or Mt*NPL1 (nodulation pectate lyase 1)* depending on NSP1, NSP2, and NIN [38,76]. In addition to the root infection pathway, NF-signaling genes are involved in nodule inception. Activation of DMI2/SYMRK, DMI3/CCAMK, and NIN in *L. japonicus* and *M. truncatula*, as well as NFR1–NFR5, CYCLOPS, and NF-YA1/B1 in *L. japonicus*, can initiate nodule organogenesis or at least cell divisions within the root cortex in the absence of rhizobia or of NF signals (Figure 2, in black) [33,73,77–79].

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interconnections of CKs with other phytohormonal pathways, including auxin, ethylene, and gibberellin, as well as certain plant peptide signals, have also been documented. This review describes how CKs impact different processes during nodulation and how CK functions may differ depending on plant tissues and symbiotic stages.

A Well-Established Positive Role for CKs in the Root Cortex to Initiate Symbiotic Nodule Organogenesis

The activation of the NF signaling pathway (Box 1) rapidly induces CK accumulation and response in the nodulation-susceptible root region (Figures 1 and 2). CK accumulation was detected shortly after an NF treatment, using ultraperformance liquid chromatography coupled to tandem mass spectrometry, on root fragments [8]. Accordingly, several CK biosynthesis genes [encoding the isopentenyl transferase 3 (IPT3) in *L. japonicus,* and IPT1, CYP735A1, LONELY GUY 1 (LOG1), and LOG2 in *M. truncatula*] are upregulated during nodulation [8–10] (Figure 2, CK pathways are indicated in blue). The expression of Mt*LOG1* and Mt*LOG2* transcriptional fusions is detected in rhizobium-induced nodule primordia within the root cortex. Meanwhile, the MtCRE1 CK receptor gene (for CK response 1) is induced by rhizobium [11] and CK-responsive genes are upregulated in *M. truncatula* and *L. japonicus*, notably type-A response regulators (RRs), which are CK primary response genes [12,13]. Thus, the *MtRR4* type-A RR and two CK-responsive reporters, the *Arabidopsis response regulator 5* (*ARR5*) gene and the *two component signaling sensor* (*TCS*), are upregulated by rhizobium in cortical cell divisions leading to nodule primordia [14–16]. This has led to the conclusion that the primary site of CK action is inner root tissues, and notably the cortex (Figure 2, in blue).

The positive role of CKs in the initiation of nodule organogenesis has been demonstrated by a large set of evidence. It was first discovered that overexpression of a CK synthesis gene in a non-nodulating (NF-defective) rhizobium strain allows nodule organogenesis to be induced in alfalfa [17]. Noteworthy, it is now known that rhizobia normally secrete bioactive CKs, but cannot rescue NF deficiency [18,19], and that in rhizobia that can nodulate independently of NFs, the production of bacterial CKs is not sufficient to initiate nodulation [18,19]. Exogenous CK applications mimic in various legumes the effects of NF treatments in the root cortex, including the transcriptional activation of 'early nodulin' markers (e.g., *ENOD12*, *ENOD40*), amyloplast accumulation, and sometimes cortical cell divisions or even 'pseudonodule'

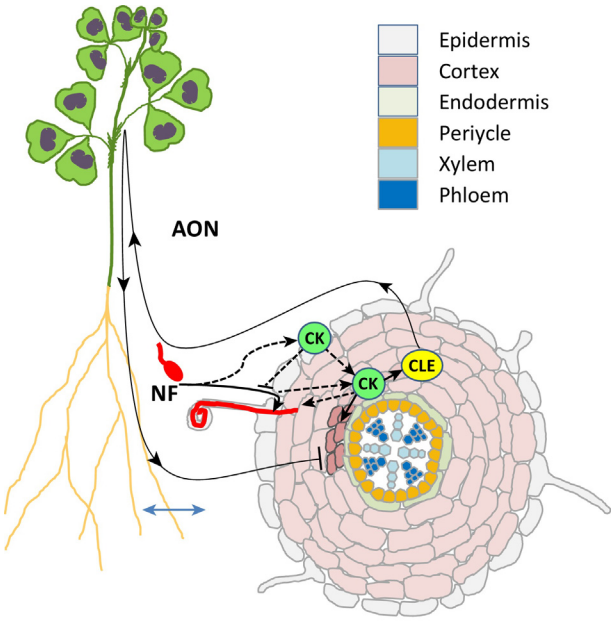
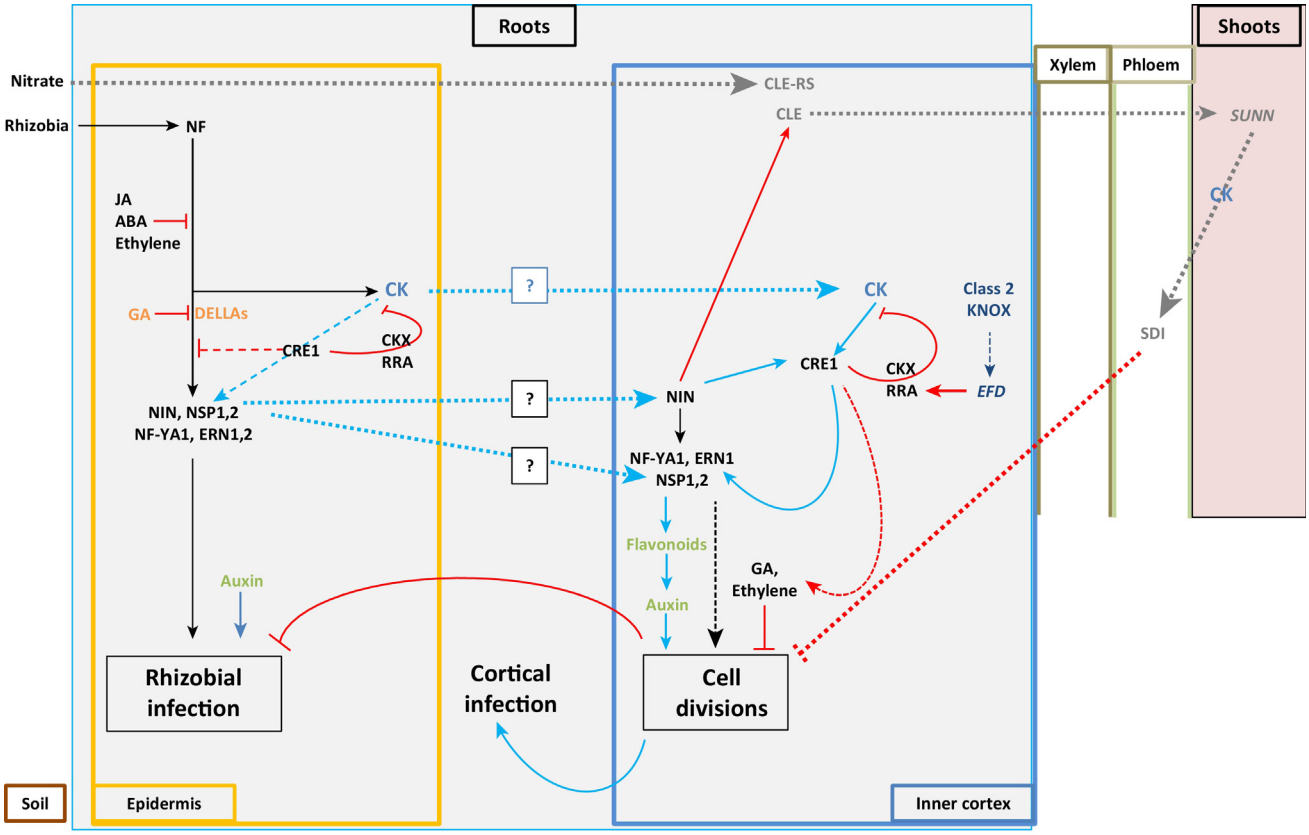


Figure 1. Overview of Proposed Positive and Negative Roles of Cytokinins during Nodulation in the Model Legume *Medicago truncatula*. The scheme represents, as an example, the indeterminate-type symbiotic nodulation occurring in *M. truncatula*. A transversal section of the root rhizobium susceptible zone (indicated on the plant root system by the blue double arrow) is represented. Dotted and solid lines, respectively, represent hypothetical and well-established relations discussed in this review: Nod factor-induced epidermal cytokinins (CKs) may play a negative role in epidermal rhizobium infection and a positive role in cortical infection. CK, either synthesized in the root cortex and/or generated in the root epidermis and transported to the cortex, induces cortical cell divisions. Cortical CK leads to the production of CLE peptides, involved in the systemic negative regulation of nodulation. Different root tissues are indicated by a color code. AON, autoregulation of nodulation; CLE, CLAVATA3-like peptides; NF, Nod factor.

formation [17,20–22]. In *L. japonicus*, the CK induction of nodule primordia is independent of the first components of the NF signaling pathway but shares several important features with rhizobium-induced nodules: (i) the same root tissue origin (the third root cortical cell layer) and patterning (e.g., peripheral vascular bundles); (ii) the requirement for key downstream symbiotic transcriptional regulators [nodule inception (NIN), nodulation signaling pathway1 (NSP1), nodulation signaling pathway2 (NSP2), and ERF required for nodulation1 (ERN1); Box 1]; and (iii) an inhibition by nitrate [20,23]. Conversely, depletion of the endogenous CK pool by overexpressing a CK oxidase/dehydrogenase (CKX) catabolic enzyme or decreasing *LOG1* expression leads to a decreased nodulation in *L. japonicus* and *M. truncatula*, respectively [10,14]. A convincing evidence for the positive role of CKs in nodule inception has come from the analysis of nodulation-defective mutants altered in CK receptors – *L. japonicus* lotus histidine kinase 1 (LHK1) and MtCRE1 [13,24,25] and more recently in additional CHK receptors as well in both legumes [15,26]; and from the 'spontaneous nodule formation' phenotype (i.e., in the absence of rhizobia) of gain-of-function (gof) *LHK1* and *MtCRE1* mutants [27–30]. The gof LHK1 mutant was named *spontaneous nodule formation 2 (snf2)* [28]. Genetic data indicate that *snf2* spontaneous nodule formation requires NIN and NSP2 in *L. japonicus*, as well as DELLA proteins in *M. truncatula*, therefore positioning CK upstream of these transcriptional regulators [28,30]. Another *L. japonicus* spontaneous nodulation mutant corresponding to a gof mutation in the CCamK calcium- and calmodulin-dependent protein kinase, *snf1*, requires LHK1, supporting that CKs act downstream of CCamK in the NF-signaling pathway during nodule inception ([27,28,31]; Figure 2).



Trends in Plant Science

Figure 2. A Model for Organ- and Tissue-Specific Positive and Negative Functions of Cytokinins in Symbiotic Nodulation in Relation with Other Phytohormones. The CK signaling pathway is shown in blue, the NF signaling in black, auxin/flavonoid pathways in green, the GA pathway in orange, and the CLE peptide systemic AON signaling in gray. Genes names correspond to the *Medicago truncatula* model for simplicity except for *CLE-RS* genes only identified in *Lotus japonicus*. Blue arrows indicate positive effects on nodulation or infection, red arrows negative effects. Solid arrows represent direct interactions, documented either in *L. japonicus*, *M. truncatula*, or both, and dotted lines are indirect interactions. Thick dotted arrows indicate the potential movement of a signaling component (question marks representing candidate non-cell autonomos signals, namely, CK, NIN, or NSP1). ABA, abscisic acid; AON, autoregulation of nodulation; CK, cytokinin; CKX, CK oxidase/dehydrogenase; CLE, CLAVATA3-like peptides; CRE1, CK response 1; EFD, ethylene response factor required for nodule differentiation; ERN1, ERF required for nodulation1; GA, gibberellic acid; JA, jasmonic acid; KNOX, knotted1-like homeobox; NF, Nod factor; NF-YA, nuclear factor Y, subunit A; NIN, nodule inception; NSP1, nodulation signaling pathway1; NSP2, nodulation signaling pathway2; SDI, shoot-derived inhibitor; SUNN, super numeric nodules.

CK-Regulated Genes Associated with Nodule Formation

A major role of the MtCRE1 CK receptor in NF signaling is suggested by the observation that ~73% of NF-induced transcriptional changes were found to be affected in the *cre1* mutant [8]. The NF treatment was, however, conducted in the presence of aminoethoxyvinylglycine, an inhibitor of ethylene synthesis, which releases a negative feedback on NF signaling (Box 2 and Figure 2) and consequently may overestimate the impact of CKs. Transcriptomic analyses in *M. truncatula* also enabled the identification of symbiotic genes that are rapidly induced by an exogenous CK treatment on roots [32], such as *NIN*, *ERN1,* and *NSP2*. NIN and the CRE1-dependent pathway are notably strongly interconnected in the cortex by a positive feedback loop, with NIN binding to the *CRE1* promoter and activating its expression [16]. In *L. japonicus* roots, an exogenous CK treatment also induces *NIN,* specifically in root cortical cells [20]. *NIN* ectopic expression leads to root cortical cell divisions and nodule primordium-like structures in both *L. japonicus* and *M. truncatula* [16,33], likely through the activation of transcription factors that play an important role in nodule development, namely, nuclear factor Y, subunit A1 [NF-YA1; as well as nuclear factor Y, subunit B1 (NF-YB1) in *L. japonicus*] [33–37] and ERN1 [23,38,39] (Box 1).

Box 2. Ethylene, Gibberellic Acid, and Cytokinins in Early Symbiotic Nodulation

Ethylene has been demonstrated in both *Medicago truncatula* and *Lotus japonicus* to be a negative regulator of NF signaling, rhizobium infection, and nodulation [51,80–83] (Figure 2). In *L. japonicus*, the spontaneous nodulation of *CCamK* and *LHK1* gof mutants (*spontaneous nodules formed 1* and *2*, *snf1* and *snf2*, respectively) is inhibited by a treatment with ethylene precursors [31]. Together with the observation in *M. truncatula* that *cre1* mutants are still sensitive to an ethylene inhibitory treatment (aminoethoxyvinylglycine), and that the *skl-cre1* double mutant has an intermediate phenotype compared with single mutants and with the wild type, this suggests that CKs and ethylene may act independently during nodule initiation [13]. *M. truncatula* genes involved in ethylene biosynthesis and signaling are however induced by NF and rhizobium, at least partly depending on the MtCRE1 CK pathway [8,51]. The cell/tissue(s) in which this activation of ethylene pathways takes place remains to be established. Intriguingly, no evidence for an NF induction of ethylene biosynthesis genes (such as *MtACS1*, *MtACS2*, *MtACS3* [8]) could be obtained in transcriptomes from laser-dissected root epidermis or isolated root hairs [47,48,58].

Among others, another intermediate signal between CK, NF signaling, rhizobial infection, and nodule organogenesis could be gibberellic acid (GA) (Figure 2, in orange). Indeed, several GA-related genes are strongly upregulated in response to NF in epidermal root hair cells [47,48] and some are activated by a short-term CK treatment [32]. GA has been reported to regulate symbiotic interactions either positively or negatively depending on plant species or approaches used [84]. In *L. japonicus*, an exogenous GA treatment negatively regulates rhizobium infection and nodule organogenesis, as well as spontaneous nodules of the *snf2* mutant, suggesting that GA inhibits nodule organogenesis downstream of LHK1. A GA treatment also inhibits the NF induction of *NSP2* and *NIN* and the CK induction of *NIN* [85]. Accordingly, the overexpression of a positive regulator of GA signaling, *SLEEPY1*, leads to an inhibition of nodulation [85]. In *M. truncatula*, a GA treatment inhibits nodulation and rhizobium infection [75]. Conversely, loss-of-function mutants affected in DELLA proteins negatively regulated by GA exhibit a reduced nodulation, rhizobial infection, and activation of the NF signaling pathway [75]. Further work is required to establish molecular mechanisms linking CK to ethylene and/or GA in the regulation of NF signaling, rhizobial infection, and nodule organogenesis.

Binding sites for a putative CK signaling transcription factor, the MtRRB1 type-B RR, were identified in the promoter of some CK-induced genes, such as Mt*RR4*, as expected for a CK primary response gene, but also in the Mt*NSP2* nodulation gene [32]. The mutation of these MtRRB binding sites abolishes the expression of a proMt*NSP2:GUS* fusion in nodule primordia. This supports a key role of the CK pathway to control Mt*NSP2* symbiotic expression in the root cortex. Mt*NSP2* response to an exogenous CK treatment is very dynamic, with a rapid induction followed by a repression. This repression may rely upon a microRNA (miR171h) reported to inhibit nodulation and whose expression is also dependent on CK and MtCRE1 [32,40]. Transcriptomic analyses also revealed a strong and significant enrichment for the CK induction of flavonoid biosynthesis genes [32]. Interestingly, flavonoids acting as auxin transport inhibitors can partially rescue the nodulation defects of the *cre1* mutant [41]. This is consistent with the CK pathway acting upstream of the local auxin accumulation in the *M. truncatula* inner cortex (Box 3 and Figure 2), itself triggering cortical cell divisions.

The CK pathway also activates genes that are typically involved in negative feedback responses, such as *RRA* and *CKX* genes, which affect CK signaling and bioactive pool, respectively ([32]; Figure 2). The mutation of one of these CK negative feedback genes expressed in nodule primordia, *LjCKX3*, increases CK accumulation in roots as expected, and reduces the nodule number [42]. Similarly, in *M. truncatula,* increasing the CK activity by the downregulation of type-A RR genes (*RR4*, *RR5*, *RR9*, *RR11*) by RNA interference decreases the number of nodules [10,12], suggesting that CK-mediated negative feedback occurs during nodule initiation. Interestingly, the MtEFD (ethylene response factor required for nodule differentiation) nodule primordium-associated transcription factor was shown to transcriptionally activate Mt*RR4* and therefore proposed to negatively impact CK responses [43]. Consistently, an *efd* null mutant exhibits increased nodulation. On the same line, the Mt*EFD/RR4* regulatory module was recently shown to be positively regulated by three functionally redundant knotted1-like homeobox (KNOX) homeodomain transcription factors, which are expressed in the nodule primordia and regulate nodule initiation ([44,45], Figure 2).

Box 3. Auxin and Cytokinin Interactions in Early Symbiotic Stages

An activation of auxin responses at the site of cortical cell divisions leading to nodule initiation was identified in *Lotus japonicus*, *Medicago truncatula*, and in soybean, based on the use of the DR5 or GH3 auxin-sensitive reporters [13,41,58,86–90]. In *L. japonicus*, an activation of auxin responses is observed in *snf2* (*spontaneous nodules formed 2*) spontaneous nodules, indicating that this induction is independent of rhizobial infection and occurs downstream of the LHK1 CK pathway [86]. In *M. truncatula*, a local inhibition of polar auxin transport by rhizobia is associated with nodule primordia formation, correlating with the activation of cortical cell divisions mostly in the inner cortex (vs. the outer cortex in *L. japonicus*) [13,41,86–88,91]. Mathematical modeling revealed that the formation of such primordium-associated auxin maximum most likely results from a reduction in auxin transport [92]. The auxin response, polar transport, and accumulation of PIN-formed (PIN) auxin efflux carriers are reduced in the *Mtcre1* mutant, indicating that auxin pathways linked to nodule organogenesis are activated downstream of the MtCRE1 CK pathway [13]. In addition, an MtCRE1-dependent CK induction of flavonoid biosynthetic genes was detected, and accordingly specific flavonoids acting as polar auxin transport inhibitors (e.g., naringenin, isoliquiritigenin) rapidly accumulate in the nodulation competent zone in response to rhizobium inoculation [32,41]. Importantly, these specific flavonoids are able to partially rescue nodulation defects of the *cre1* mutant [41]. CKs and auxins therefore both play positive roles in nodule initiation, respectively, downstream of the NF-signaling pathway and of the MtCRE1 pathway (Figure 2, in blue and green, respectively). Recent transcriptomic studies focused in root hairs/epidermis additionally highlighted that flavonoid and auxin metabolisms are also rapidly regulated by NF and/or rhizobium (Figure 2, in green; [47,58]). Interestingly, the *M. truncatula* auxin response factor 16a (ARF16a) promotes rhizobial infection [58]. As the MtCRE1 CK pathway regulates flavonoid metabolism and auxin response during nodule inception, it is tempting to speculate that a related crosstalk may exist in the epidermis to control NF signaling and/or rhizobial infection. However, in contrast to the root cortex, the expression of the auxin-responsive DR5:GUS reporter is still activated in *cre1* root epidermal cells [41]. This suggests that at least part of the auxin pathway is independent of CRE1 in the root epidermis, even though it cannot be excluded that a functional redundancy exists with other CHK receptors. In soybean, the role of auxin in determinate nodulation is conflicting depending on studies. Whereas the GmTIR1/AFB3 auxin receptor was recently shown to positively regulate soybean nodulation and its postranscriptional regulation by the miR393 accordingly to negatively regulates nodulation [93], previous studies focused on the miRNA regulation of two different ARF transcription factors subsets suggested a negative role of auxin in soybean nodulation. Indeed, the miRNA miR167 repressing the *ARF8a* and *ARF8b* genes acts as a positive regulator of nodulation [94] and the miR160 targeting a subset of proposed repressor ARFs reduced nodule formation [95]. These later roots were hyposensitive to cytokinin and had a reduced expression of CK-regulated nodulation-associated transcription factors such as NIN, which may explain the reduced nodule organogenesis [89]. Accordingly, exogenous CKs restore nodule formation in miR160 overexpressing roots [94].

An Emerging Negative Role for CKs in Regulating NF Signaling and Rhizobial Infection in the Root Epidermis

More recent transcriptomic studies focused on the epidermal or root hair cells of *M. truncatula* [46–48]. These analyses highlighted that NFs and rhizobia rapidly induce the expression of CK biosynthesis and signaling genes in this tissue. Those include *MtCRE1* and several *RRA* genes (Mt*RR2*, Mt*RR8,* Mt*RR9,* and Mt*RR10*), but not Mt*RR4* that is only expressed in the cortex under symbiotic conditions [16]. Accordingly, an Mt*RR9* transcriptional fusion is rapidly detected in the root epidermis, in addition to other root tissues, in response to NFs [12]. Moreover, a CK signaling sensor termed *TCSn* (for *TCS new* [49]) enabled to detect an initial activation of the CK response in the *M. truncatula* root epidermis and outer cortical cells, before the one associated with nodule primordia [47]. In *L. japonicus*, a different pattern was reported for a previous version of the *TCS* reporter [15], with a rhizobium-induced expression detected first in the cortex and only later in the epidermis. The sequence of activation of CK responses during early symbiotic stages may therefore differ between the two species. It cannot be ruled out, however, that the discrepancies between these data sets merely result from different experimental systems and the use of different CK response reporters (*TCS* vs. *TCSn*). On the same line, the *Arabidopsis ARR5* gene, also used as a CK-reporter in *L. japonicus*, revealed an early induction in root hairs in response to rhizobium [14].

What could be the role of the root epidermal activation of CK pathways in response to rhizobium? In *L. japonicus,* both single *lhk1* and triple *lhk1 lhk1a-1 lhk3* mutants show a rhizobium hyperinfection epidermal phenotype. This indicates that CKs are not required for the

formation of epidermal infection threads (ITs) and may actually negatively regulate epidermal infections [15,25,50]. By contrast, the *lhk1 lhk1a-1 lhk3* triple mutant abolishes the formation of ITs in the cortex, thus implying a positive role of CKs in cortical infections [50]. CKs would therefore have positive and negative impacts depending on root tissues (cortex vs. epidermis) and symbiotic processes. In *M. truncatula,* the *cre1* mutant is also defective for inner cortical infections [13]. In contrast to *lhk1,* a normal number of epidermal infections is however observed in *cre1*, associated with sometimes exaggerated IT growth with numerous ramifications and saclike structures detected in outer root cell layers [13]. Although several interpretations are possible, the simplest one would be an inability of ITs to enter inner cortical cells, possibly because of missing cortical cell divisions.

The functional relevance of the epidermal activation of CK pathways in response to NFs and rhizobia was recently more directly explored in *M. truncatula*. The epidermal CK pool was depleted by expressing a CKX enzyme from an epidermis-specific promoter (pEPI:*CKX* construct) [47]. This led to an increased nodulation, correlated with a trend to form an increased number of ITs. An exogenous CK treatment was in addition found to inhibit, through MtCRE1, the NF induction of the root epidermal Mt*ENOD11* (pre)infection marker [47]. Altogether, these observations are consistent with a predominantly negative regulatory role of the epidermal CK pathway, as proposed in *L. japonicus* [50]. This may involve various secondary signals, such as ethylene [8,50,51] or gibberellic acid [32] (Box 2), or even the NIN transcription factor. Indeed, MtCRE1 is required for the NF induction of *NIN* [8], and while a *nin* mutant is defective for rhizobium infection [52,53], it also exhibits a broader Mt*ENOD11* expression domain in the root epidermis [54]. This suggests a possible dual role of NIN in the control of infection, positive for the formation of ITs and negative for the number of infections (Figure 2). While the negative role of NIN could be indirect, that is, due to missing signals from the nodule primordia [55] (as described below), it might also be direct. It was indeed recently shown that MtNIN binds the *ENOD11* promoter and negatively regulates its expression, perhaps through competition with MtERN1 [16].

Another Layer of Non-Cell Autonomous CK Effects

Molecular mechanisms linking NF signaling in the epidermis to the activation of cell divisions in the cortex, and vice versa, remain unclear. A key issue is the nature of signals triggering CK responses in the root cortex, and consequently cell divisions, at a stage where rhizobia and NF production are confined to the root epidermis. CKs have been hypothesized to be possible signals moving from epidermal to cortical cells [21,31], but this was then considered unlikely notably based on the *TCS* and *MtRR4* expression patterns, which suggest the absence of CK in outer root tissues at early symbiotic stages [15,16,50]. In *M. truncatula*, the fact that other CK responsive as well as CK biosynthesis genes and *MtCRE1* are quickly activated in epidermal/root hair cells in response to NF or rhizobium [12,14,46–48] suggests, however, that CK might have a non-cell autonomous function to activate the CRE1 pathway in the cortex and consequently trigger nodule inception in the cortex [8,47,56] (Figure 2). This response would be amplified by a positive feedback loop depending on NIN [16]. Noteworthy, several genes encoding candidate CK transporters [57] are upregulated in NF-treated epidermal cells ([47]; Figure 2). Moreover, the rhizobium-induced cortical expression of Mt*LOG1* and Mt*LOG2* is abolished in the *cre1* mutant [10], suggesting that the cortical CK biosynthesis is dependent on a previous MtCRE1 activation.

If CKs are moving signals triggering nodule inception, how to explain the increased nodulation phenotype observed in *M. truncatula* roots expressing the *pEPI:CKX* construct [47]? One can hypothesize that the CK transport from the epidermis to the cortex is faster than the CK degradation by the CKX; and/or that inhibitory effects of CK in the epidermis, through secondary signals as proposed above, could be slower and therefore predominantly affected

by *pEPI:CKX*. In addition, other epidermis-to-cortex mobile signals may exist, for example, other hormones or peptides predicted to be NF induced in the epidermis based on transcriptome data [47,58], or transcription factors such as NIN or NSP1 (closely related to SHORT ROOT, which acts as a mobile signal in *Arabidopsis* roots [59]) (Figure 2).

Conversely, a cortex-to-epidermis CK-dependent signaling may also exist. Thus, the hyperinfection phenotype of the *hit-1* and *daphne* mutants, affecting LHK1 and LjNIN, respectively [25], could be due to the defective nodule primordium formation and consequently the absence of a cortex-derived signal negatively regulating rhizobium infections. Indeed, the *daphne* mutant allele of Lj*NIN,* which is defective for CK-triggered cortical expression of *NIN* and nodulation, is hyperinfected in the epidermis [55]. Conversely, a mutant in the cortical Lj*CKX3* gene exhibits a reduced rhizobial infection correlating with CK accumulation [42]. The nodule primordium may therefore, on the one hand, condition cortical cells to enable cortical infections [50], and on the other hand, negatively regulate, non-cell autonomously, epidermal infections (Figure 2). Noteworthy, the latter could involve rhizobium itself, shown to control the infection number in response to an unknown plant signal during nodule development [60].

The negative feedback of nodule primordia on rhizobium infection may be local and/or rely on a long-distance systemic pathway involving root–shoot communication, called the autoregulation of nodulation (AON) [2]. The AON pathway involves CLAVATA3-like (CLE) peptides as well as a CLAVATA1 receptor-like kinase, termed MtSUNN (super numeric nodules) or LjHAR1 (hypernodulation and aberrant root 1). *MtCLE13* expression is associated with nodule primordia, and dependent on MtCRE1 and MtNIN [61,62] (Figures 1 and 2), while similarly in *L. japonicus*, *CLE-RS1* (CLE-root signal 1) and *CLE-RS2* expression is upregulated by CKs and directly activated by NIN [63,64]. The overexpression of these CLE peptides in roots leads to a systemic downregulation of nodulation, through MtSUNN/LjHAR1 activity in shoots [61,63,65,66] (Figure 2 in gray). In addition, CK biosynthesis in shoots may participate in *L. japonicus* to the AON downstream of the CLE/HAR1 regulatory module ([67]; Figure 2, in gray). A shoot-to-root movement of CK through the phloem was even proposed, although other mobile signals may be involved. CKs may thus trigger two steps of the AON: the production of the root-to-shoot and of the shoot-to-root signals. Finally, downstream of the CLE/SUNN–HAR1 systemic pathway, an F-box protein identified in *L. japonicus*, Too Much Love (TML), likely regulates nodule organogenesis by affecting yet unknown targets ([2,67,68]).

Concluding Remarks and Future Directions

Thanks to substantial progresses achieved by a combination of genetic, molecular, and genomic approaches, the vision of early symbiotic signaling has profoundly evolved in the past years from a simple linear pathway to a much more complex pathway. CKs, and more generally phytohormones, are key factors to regulate both rhizobial infection and nodule organogenesis. Recent advances suggest that CKs play both positive and negative roles in a tight interplay with several transcriptional regulators. This is certainly essential for the homeostasis of symbiotic responses depending on endogenous and environmental factors.

An intriguing issue relates to possible differences between *M. truncatula* and *L. japonicus.* Although those might be related to the type of nodules formed (determinate vs. indeterminate), it should be pointed out that significant variations in response to CK treatments were already observed within *L. japonicus* ecotypes [20]. Additional experiments would be valuable to clarify the kinetics of CK pathway activation, for example, tissue-specific transcriptome analyses of both epidermal and cortical cells at different time points in parallel in both legume species. It would also be essential to determine in *L. japonicus* whether the *TCSn* reporter validates the *TCS* pattern previously reported and to independently corroborate this result by demonstrating that no *RRA* gene is activated in epidermal cells before the activation of cortical responses.

Outstanding Questions

Which signaling pathways and transcription factors activate CK biosynthesis, degradation, and potentially transport in the epidermis and/or the cortex in response to NF and rhizobium?

What molecular mechanisms explain the ability of CKs to inhibit NF signaling and/or rhizobial infection?

Are the candidate (ENT and ABCG) transporters that are NF upregulated in the *M. truncatula* epidermis involved in CK mobility between the epidermis and the cortex during nodule initiation?

How does the activation of CK signaling lead to the reactivation of the cell cycle in the root cortex at the onset of nodule inception? Is this mediated only by the regulation of auxin responses or are there additional target pathways? How different are a nodule and a lateral root primordium with regard to CK- and auxin-mediated regulations?

What is the nature of cortex to epidermis non-cell autonomous signals generated downstream of CRE1/LHK1 CK pathways by nodule primordia to regulate rhizobial infection and progression? What is the respective relevance of plant and bacterial cells to perceive those signals regulating infection progression?

How does the systemic autoregulation of nodulation mechanistically relate to CK action?

Are CK pathways and their interconnections with other signals determining some of the developmental differences characterizing determinate versus indeterminate nodulation types?

What is the relevance of CK pathways at later stages of nodule development?

Requirements of known NF-signaling genes for the CK epidermal versus cortical action should also be compared in parallel in both species, for example, by introducing constructs affecting the CK pool specifically in the root epidermis or cortex in NF-signaling mutants. In addition, the recently reported function of CK in the root epidermis should be better defined, to identify which process (es) is (are) controlled by this signal: IT initiation and progression within the root hair and/or in the basal part of the epidermal cell toward the outer cortex. To this end, a detailed phenotyping of the infection process should be monitored in the various CK-related mutants that are available.

It will also be particularly interesting to investigate the interconnection between CK and other regulatory signals revealed by recent transcriptomic studies (see Outstanding Questions). Indeed, in addition to interactions with ethylene, auxin, and possibly gibberellic acid, other hormones such as abscisic acid, jasmonic acid, and peptides already known to affect NF signaling, rhizobial infection, and/or nodule organogenesis [69–71] (Figure 2) may act depending or independently of CKs on these different nodulation steps.

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