

Induction of localized auxin response during spontaneous nodule development in *Lotus japonicus*

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In leguminous plants, rhizobial infection of the epidermis triggers proliferation of cortical cells to form a nodule primordium. Recent studies have demonstrated that two classic phytohormones, cytokinin and auxin, have important functions in nodulation. The identification of these functions in *Lotus japonicus* was facilitated by use of the *spontaneous nodule formation 2 (snf2)* mutation of the putative cytokinin receptor LOTUS HISTIDINE KINASE 1 (LHK1). Analyses using *snf2* demonstrated that constitutive activation of cytokinin signaling causes formation of spontaneous nodule-like structures in the absence of rhizobia and that auxin responses are induced in proliferating cortical cells during such spontaneous nodule development. Thus, cytokinin signaling positively regulates the auxin response. In the present study, we further investigated the induction of the auxin response using a gain-of-function mutation of Ca²⁺/calmodulin-dependent protein kinase (CCaMK) that causes spontaneous nodule formation. We demonstrate that CCaMK^{T265D}-mediated spontaneous nodule development is accompanied by a localized auxin response. Thus, a localized auxin response at the site of an incipient nodule primordium is essential for nodule organogenesis.

Leguminous species have the ability to form nodular structures on their roots in a symbiotic relationship with soil bacteria (termed rhizobia). The formation of nodules is initiated by rhizobial infection of the plant root epidermis, which induces an infection signaling cascade that ultimately activates a Ca²⁺/calmodulin-dependent protein kinase (CCaMK).^{1,2} This kinase is believed to be a key regulator for decoding Ca²⁺ signals. Loss-of-function mutations of *CCaMK* cause a nodulation-deficient phenotype in plants.³ By contrast, spontaneous formation of nodule-like structures in the absence of rhizobia occurs in plants with a gain-of-function mutation that confers a constitutively active CcaMK,³⁻⁷ e.g., *spontaneous nodule formation 1 (snf1)* or CCaMK^{T265D}.

In *Lotus japonicus*, spontaneous nodule formation is also mediated by *snf2*, a gain-of-function mutation of LOTUS HISTIDINE KINASE 1 (LHK1), which encodes a putative cytokinin receptor.^{8,9} Phenotypic analysis of *snf2* mutant plants indicated that cytokinin signaling is constitutively activated. Confirmation of the important role of cytokinin comes from the observation that spontaneous nodule formation is induced by exogenous application of the phytohormone in *L. japonicus*.¹⁰ Moreover, some cytokinin response regulators are reported to be involved in nodulation.¹¹ The findings from these studies suggest that activation of cytokinin signaling is crucial for the initiation of nodule organogenesis.

Although there is now strong evidence of the role of cytokinin in nodule formation, much less is known about how a second phytohormone, such as auxin, functions to regulate nodule development. We recently investigated auxin reporter lines in *L. japonicus* to determine auxin response patterns and the interaction between auxin response and key factors in nodule development.¹² These analyses showed that induction of auxin response predominantly occurs during cortical cell proliferation. In addition, auxin response appears to occur in a downstream part of the cytokinin signaling pathway since it is induced during *snf2*-dependent spontaneous nodule formation.

In this study, we sought to elucidate the potential interaction between auxin and CCaMK by analyzing auxin response patterns during spontaneous nodule development resulting from the introduction of the CCaMK^{T265D} mutation into *DR5::GFP-NLS* *L. japonicus* plants. To identify transgenic hairy roots in these plants, the GFP of the original binary vector containing the CCaMK^{T265D} construct was replaced by mKO2.^{6,12} We used the *Agrobacterium rhizogenes* mediated hairy root transformation method to introduce the recombinant plasmid into the *DR5::GFP-NLS* plants. In the CCaMK^{T265D} /*DR5::GFP-NLS* plants, GFP expression was observed in cortical cells that were proliferating to form the primordia of spontaneous nodules

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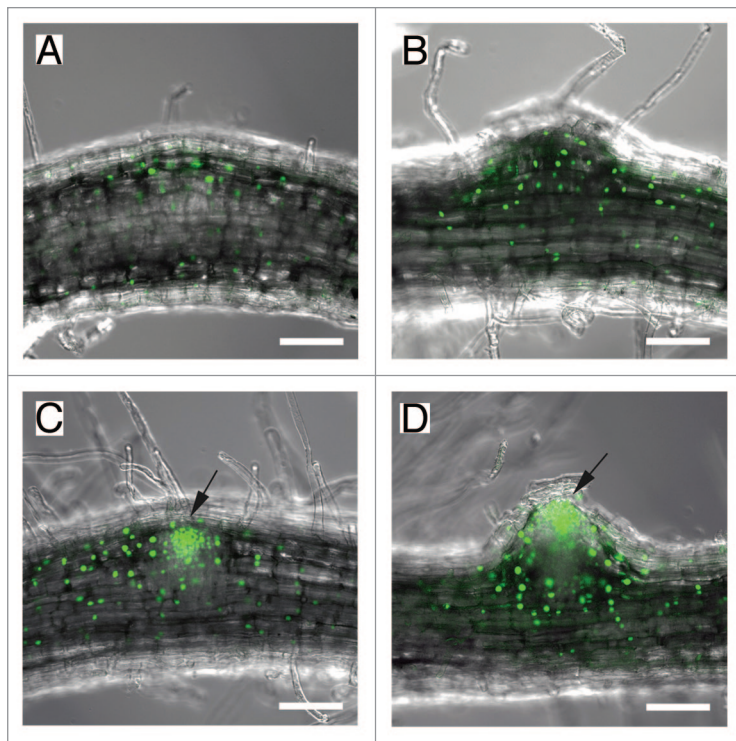


Figure 1. Auxin response patterns during spontaneous nodule formation and lateral root development in *Lotus japonicus*. Auxin responses were monitored indirectly by GFP expression patterns during spontaneous nodule formation (A and B) and lateral root development (C and D) in *DR5::GFP-NLS* stable transgenic plants; these plants have transgenic hairy roots in which CCaMK^{T265D} and mKO2 are constitutively expressed. Transgenic hairy roots were identified by the mKO2 signal (data not shown). Arrows indicate the strongly localized GFP expression, which is observed specifically during lateral root development. Scale bars = 100 μ m.

(Fig. 1A). GFP continued to be expressed after the proliferating cortical cells had formed an obvious protuberance at the site of a spontaneous nodule (Fig. 1B). At this early stage of spontaneous nodule development following the initial rounds of cell division, the morphology of the spontaneous nodule primordium resembled that of a developing lateral root; this similarity made it difficult to identify spontaneous nodules on the basis of their physical appearance. However, differences in GFP expression patterns enabled us to unambiguously distinguish these two types of

structure: the developing lateral root had a more strongly localized GFP signal, corresponding to the position of the future root apex (Fig. 1C and D) than the spontaneous nodule. The induced GFP expression during CCaMK^{T265D}-dependent spontaneous nodule formation indicates that an auxin response takes place in a downstream part of the calcium signaling pathway mediated by CCaMK. This conclusion is consistent with the suggestion that activation of cytokinin signaling occurs in a downstream part of the CCaMK signaling pathway as the *ccamk* mutation does not affect *snf2*-dependent spontaneous nodulation.⁸

The present study demonstrates that an auxin response is induced during CCaMK^{T265D}-dependent spontaneous nodule development. This result provides further support for the hypothesis that a localized auxin response at the site of an incipient nodule primordium is essential for nodule organogenesis. Interestingly, in an examination of normal nodule development, we showed previously that after colonization by rhizobia the auxin response was restricted to the regions surrounding the sites of rhizobial colonization.¹² This observation suggests that there might be a mechanism to exclude auxin from sites of rhizobial colonization. Since nodulation requires a collaborative interaction between rhizobia and the plant host, it is possible that rhizobial factors are also involved in the regulation of auxin exclusion as well as those of the host. Further investigation of the detailed auxin response patterns, such as through use of host and rhizobial mutants that are deficient in rhizobial colonization in nodules should aid elucidation of the genetic factors involved in nodulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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