ARTICLE ADDENDUM

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Auxin distribution and lenticel formation in determinate nodule of *Lotus japonicus*

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Legumes can establish a symbiosis with rhizobia and form root nodules that function as an apparatus for nitrogen fixation. Nodule development is regulated by several phytohormones including auxin. Although accumulation of auxin is necessary to initiate the nodulation of indeterminate nodules, the functions of auxin on the nodulation of determinate nodules have been less characterized. In this study, the functions of auxin in nodule development in *Lotus japonicus* have been demonstrated using an auxin responsive promoter and auxin inhibitors. We found that the lenticel formation on the nodule surface was sensitive to the auxin defect. Further analysis indicated that failure in the development of the vascular bundle of the determinate nodule, which was regulated by auxin, was the cause of the disappearance of lenticels.

Legumes (Fabaceae) constitute the third largest plant family with around 700 genera and 20,000 species.[1] Legume plants form root nodules through symbiosis with a soil microbe called rhizobia. This plant-microbe symbiosis in nodules mediates an harmonized exchange of chemical signals between host plants and rhizobia.[2] Nodules are biologically divided into two different groups, i.e., indeterminate nodules and determinate nodules. Indeterminate nodules, represented by *Trifolium repens* (white clover) and *Medicago truncatula*, are initiated from the inner cortex to form a persistent nodule meristem, which allows continuous growth, and leads to the formation of elongated nodules, whereas in determinate legumes, nodules are mostly developed from outer cortical cells and form spherical nodules.[3]

Auxin is one of the most important regulators for nodule development. Since the possible involvement of auxin in nodule formation was first reported by Thimann,[4] auxin distribution during nodulation has been studied in particular with indeterminate nodules.[5] However, little is known about auxin involvement in determinate nodule formation. To evaluate auxin functions in the determinate nodulation of legume plants, we performed an auxin-responsive promoter analysis in detail. Using GH3:GUS transformed *Lotus japonicus* (a kind gift from Dr. Herman P. Spaink, Leiden State University, Netherlands),[6] we detected auxin signals throughout the nodulation process, e.g., at the basal and front part of the nodule primordia, circumjacent to the infection zone of the young developing nodules (**Fig. 1**), and at the nodule vascular bundle in mature nodules. We also investigated the effect of several auxin inhibitors, including newly synthesized auxin antagonist PEO-IAA (kindly provided by Dr. Hayashi, Okayama University of Science, Japan),[7] on the nodulation of *L. japonicus*, and revealed that auxin was required for forming a nodule vascular bundle and lenticels (**Fig. 2**).[8]

In indeterminate legumes, auxin is accumulated at the site of rhizobia inoculation.[9] This is caused by the inhibition of polar auxin transport by accumulation of flavonoids around the infection site, which are known as regulators of auxin transport. When flavonoid biosynthesis is reduced by the gene silencing of chalcone synthase, which catalyzes the first step of flavonoid synthesis, *M. truncatula* was

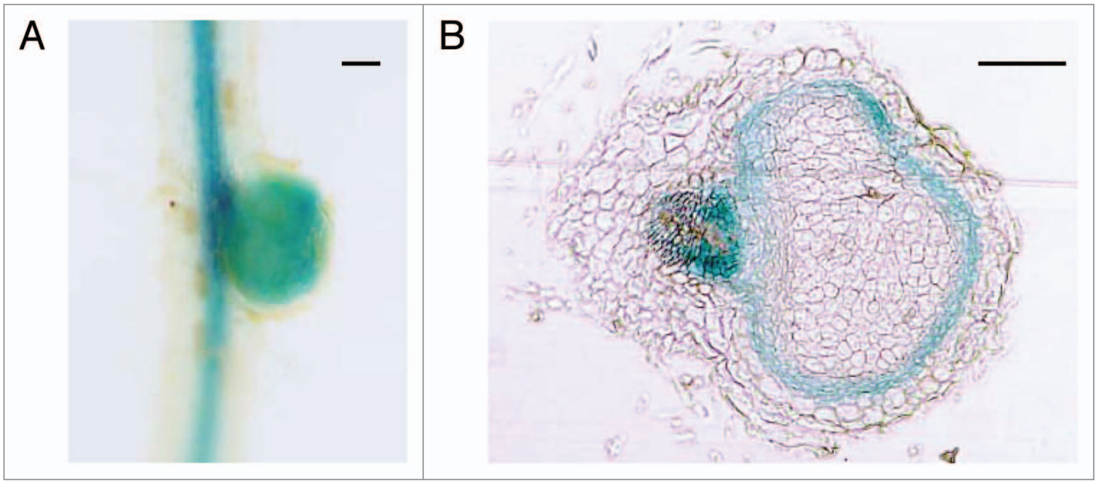

Figure 1. GH3:GUS expression in determinate nodule at 6 dpi. (A) GUS staining was observed in the central cylinder of the root vascular bundle and in the nodule. (B) Cross section of (A). GUS expression was observed around the infection zone of the nodule. Bars = 100 μm.

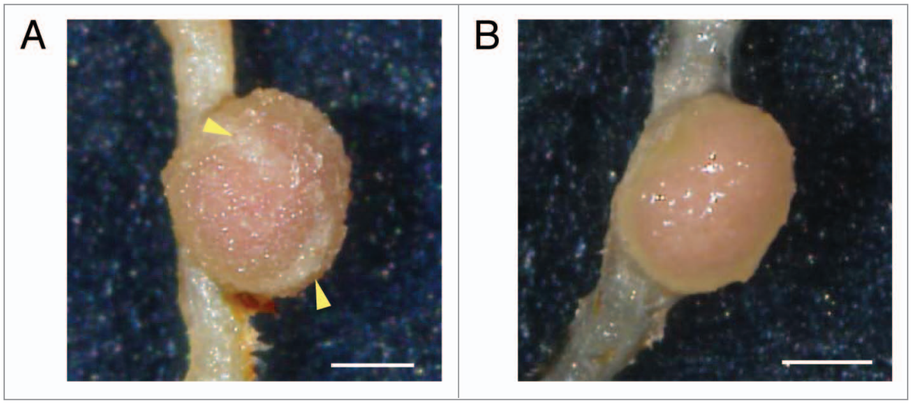

Figure 2. The effect of auxin inhibitor on nodule surface. (A) Typical mature nodule of *L. japonicus* at 21 dpi. Lenticels are pointed out by yellow arrowheads. (B) The treatment of auxin inhibitor (NPA 100 μM) inhibited lenticel formation on the nodule surface. Bars = 500 μm.

unable to inhibit polar auxin transport and resulted in reduced nodule number.[10,11] A similar phenotype was observed when the auxin transporter gene was silenced.[12] In addition, treatment of polar auxin transport inhibitors such as NPA and TIBA induce pseudonodule formation,[9] suggesting that auxin accumulation is required for nodulation of indeterminate legumes. In contrast, the treatment of polar auxin transport inhibitors in determinate nodules did not induce a nodule-like structure, suggesting a different function of auxin between indeterminate and determinate nodules. It is, however, of interest to investigate the involvement of flavonoids in determinate nodule formation, because several genes in the flavonoid biosynthesis pathway are upregulated at 2 dpi (days post inoculation) in *L. japonicus*.[13]

Lenticels regulate gas permeability of nodules.[14] Under low oxygen or waterlogged conditions, they develop more extensively, whereas they collapse, or develop very little during insufficient water conditions, or under high oxygen pressure.[14,15] Because lenticel development on the nodule surface is accompanied with the nodule vascular bundle, growth regulators supplied from the vascular system likely facilitate lenticel development.[15] Our data suggests that auxin is necessary to form the nodule vascular bundle, and in fact, auxin itself is one of the candidates of growth substances that control lenticel formation. It is necessary to analyze mutants, which lack in lenticel formation, but can form a nodule vascular bundle, for clarification of further mechanisms of lenticel development.

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