Legume nitrogen fixation under nitrate has "FUN" with zinc

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Legume root nodules are the main symbiotic nitrogen fixation (SNF) organs, which are deem for a sustainable nitrogen source that can greatly relieve the economic and environmental costs of chemical nitrogen fertilizers in agriculture.[1] However, legume nodulation is inhibited due to the excessive use of industrial nitrogen fertilizers in current agricultural system. High soil nitrate concentration reduces nodule formation, growth and function, and induces the senescence of active nodules. A series of studies showed that NIN-Like Proteins (NLPs), Nitrate Transporters (NRTs) and NAM-ATAF1/2-CUC2 (NAC) transcription factors drive core nitrate signal during these processes,[2] although it remains unclear how legume plants perceive soil nitrate and convert it into plant endogenous signals during nodulation.

A recent study from Aarhus University, Denmark, revealed that a transcriptional regulator named Fixation Under Nitrate (FUN), which acts as a novel type of zinc sensor, plays a crucial role in the nitrogen fixation process of legumes.[3] Researchers performed a forward genetic screening of approximately 150,000 mutants of *Lotus japonicus* under high nitrate conditions, under which the nodules of WT plants usually turn pale-green from pink, which indicates nodule senescence, whereas a high percentage of *fun* mutants nodules keeps pink, which typically indicates maintenance of the nitrogen-fixation function. Several *fun* alleles were isolated in that screening and FUN was identified as a Basic Leucine Zipper (bZIP)-class transcription factor which consists of a bZIP DNA-binding domain and a metal sensor

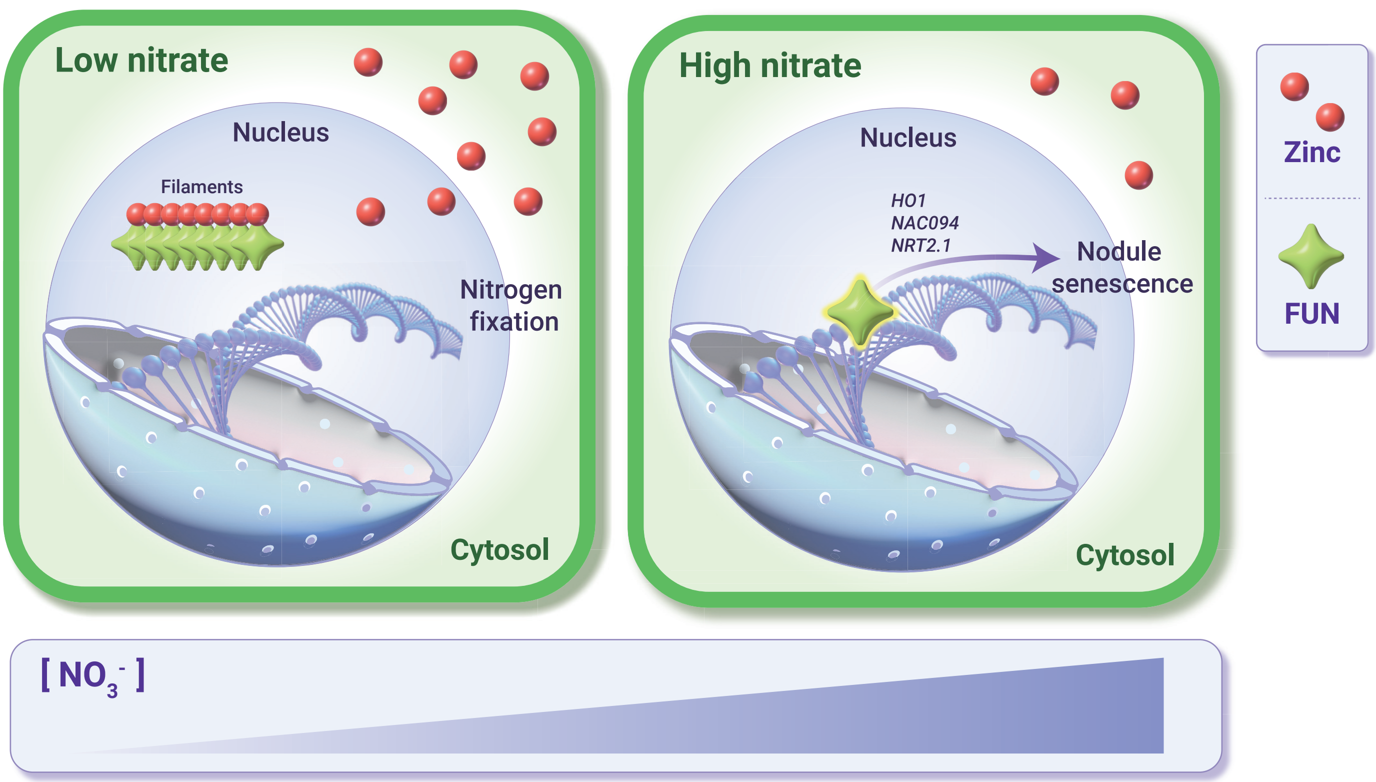

Figure 1. Mechanistic model on how FUN regulates nodule function Under conditions of low soil nitrate, zinc accumulates in cells, thereby sequestering FUN in a zinc-binding inactive filaments and facilitating the continuous process of nitrogen fixation. Conversely, under high soil nitrate level, cellular zinc concentrations decrease, releasing active FUN from the filamentous megastructure and upregulating target genes such as *NAC094*, *HO1*, and *NRT2.1* that promote nodule senescence.

domain. FUN is an important transcription factor that controls nodule breakdown when soil nitrate concentrations are high. RNA-seq, genetic and biochemical data convinced that the direct targets of FUN are *HO1*, *NAC094* and *NRT2.1* by which FUN regulates legume nitrogen fixation, nodule senescence and nitrate signaling, respectively, to control the SNF in the nodule.

As FUN has a potential metal binding domain and no transcriptional regulation of *FUN* has been observed in nodules, it is reasoned that the FUN activity could be regulated at the protein level. So, a binding affinity screening of FUN sensor towards common cellular metal ions and nitrogen compounds was performed by using thermostability and molecular size as indicators. Interestingly, only zinc ions at low physiological concentrations (3.9-7.8 μM) induced an increase in both thermostability and molecular size of the FUN sensor, suggesting its binding specificity to zinc. The negative-staining of electron microscopy of protein demonstrated that FUN sensor can form large filamentous structures after treatment with zinc ions and these filaments disassemble when zinc is removed using chelator EDTA. This result is consistent with the *in vitro* observation of size changes (Dynamic Light Scattering experiment, DLS) and the *in vivo* development of zinc-dependent nuclear condensates under different zinc concentrations. Further investigation confirmed that zinc controls the form transition of FUN between an inactive zinc-bound filamentous megastructure, which inhibits the target genes downstream of FUN, and an active transcriptional regulator.

Zinc is a vital micronutrient which is required for the function of over 300 enzymes and 1000 transcription factors. Zinc serves as an indispensable catalyst and structural component in numerous enzymes and regulatory proteins,[4] whereas it has rarely if ever been considered as a signal before. In this study, it is the remarkable discovery that zinc acts as a second messenger converting nitrogen status in soil to a zinc concentration signal in plants, which is subsequently decoded by its sensor FUN to regulate SNF in nodules. Two independent experiments utilizing a zinc-sensitive dye and micro-X-ray fluorescence microscopy both showed a substantial reduction in zinc levels within nodules following treatment with nitrate. On the other hand, zinc application relieves the suppression of nitrogen fixation in WT plants exposed to nitrate and this effect is *FUN*-dependent. Collectively, the working model of this fascinating study is: under low nitrate conditions, there is abundant free zinc in the nodules and FUN forms inactive filamentous megastructure, allowing nodules to fix nitrogen adequately; under high nitrate conditions, nodule free zinc concentration decreases and FUN dissociates from the protein filaments into an active form to activate the downstream target genes, including *HO1*, *NAC094* and *NRT2.1*, that regulate multiple pathways inhibiting SNF in nodules and promoting nodule senescence (Figure 1).

Recent research indicated the crucial roles of zinc in legume SNF process.[5] Yellow Stripe1-Like3 (YSL3) protein is essential for iron and zinc delivery from the phloem to nodules, and loss of this transporter leads to zinc retention in the vasculature. The plasma membrane-localized Zinc-Iron Permease6 (ZIP6) facilitates zinc uptake from apoplast into infected cells. ZIP1 protein has been reported to regulate zinc transport between cytosol and symbiosomes within infected cells. Thus, it is plausible that zinc acts as an intracellular second messenger that converts the extracellular signal changes, such as soil nitrate level, into intracellular signals, such as zinc concentration. Although how nitrate in soil governs zinc levels in the root nodules remains elucidated, the authors proposed an exciting new mechanism underlying metal ion sensing and transcription regulation in plants via filamentation of the FUN transcription factor. It is possible that soil nitrate concentration precisely regulates zinc allocation, uptake, and sequestration by modulating zinc transports through core transcript factors in nodulation pathway, such as NIN, NLP and NAC, and some components in nitrate signaling pathway. In the future, dissection of the mechanisms on how zinc signals are induced by soil nitrate and specifically decoded by FUN would help us to enhance nitrogen use efficiency, improve crop yields, and promote more sustainable agricultural practices. Legume nodules are sensitive to environmental factors including light, temperature, drought, salinity and high mineral nitrogen concentration in soil. This new understanding about intercellular zinc change based on extracellular factors makes it possible to generate more resilient plants. That means the plants could have increased tolerance to extreme weather and impoverished land. Mutation of *FUN* delays legume nodule senescence, which would keep more nitrogen by continued nitrogen fixation in the soil after legumes are grown. Maximizing the impact of enhanced nitrogen fixation reduces reliance on synthetic fertilizers and optimizes crop efficiency. Furthermore, as *fun* mutants do not have any adverse effects, the prospects for applying these research findings to leguminous crops such as soybeans (*Glycine max*), cowpeas (*Vigna unguiculata*), beans (*Vicia faba*), and chickpeas (*Cicer arietinum*) are highly promising.

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AUTHOR CONTRIBUTIONS

X. C. and B. R. wrote the manuscript. B. R. supervised the findings of this work.

DECLARATION OF INTERESTS

The authors declare no competing interests.