Transcriptional regulators of legume-rhizobia symbiosis
Nuclear Factors Ys and GRAS are two for tango

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Transcription factors are DNA binding proteins that regulate gene expression. The nitrogen fixing symbiosis established between legume plants and soil bacteria is a complex interaction, in which plants need to integrate signals derived from the symbiont and the surrounding environment to initiate the developmental program of nodule organogenesis and the infection process. Several transcription factors that play critical roles in these processes have been reported in the past decade, including proteins of the GRAS and NF-Y families. Recently, we reported the characterization of a new GRAS domain containing-protein that interacts with a member of the C subunit of the NF-Y family, which plays an important role in nodule development and the progression of bacterial infection during the symbiotic interaction. The connection between transcription factors of these families highlights the significance of multimeric complexes in the fabulous capacity of plants to integrate and respond to multiple environmental stimuli.

The interaction between legumes and rhizobia has been the focus of intensive research over the last years. This symbiotic association is responsible for the majority of the nitrogen incorporation into biological systems and has the potential to reduce the chemical fertilization used in agricultural systems. In addition to the economical and social importance of the biological nitrogen fixation, this interaction between a eukaryotic and a prokaryotic organism is a fascinating biological system, in which mutual recognition leads to developmental changes in both symbionts. Low nitrogen availability in the soil, combined with the presence of compatible bacteria, triggers the development of a new root organ, the nodule, where nitrogen

fixation takes place. Rhizobia reach the nodule through a tubular structure called the infection thread, where bacteria progress toward the cortical cells. These two genetic programs -nodule organogenesis and infection- are independent but strongly coordinated.[1] Attempts to decipher the mechanisms underlying the mutualistic symbiosis between legume plants and rhizobia have resulted in the identification of a significant number of transcription factors that are required for nodulation, helping to understand the exquisite complexity of the plant response to nitrogen availability. In this work, we will briefly review the transcription factors already described in the genetic programs that are involved in the infection and nodule organogenesis, with emphasis in the recent discovery of a new GRAS regulator that participates in both, nitrogen-fixing symbiosis and lateral root growth.[2]

Transcriptional Regulators Involved in Symbiosis

Signal molecules secreted by rhizobia (like an oligo-polysaccharide called Nod Factor) can activate a signal transduction pathway that ultimately results in the activation of transcription factors in the nuclei of epidermal and cortical cells. These regulatory proteins recognize and bind to specific sequences of DNA located in the promoter of certain genes, controlling gene activity in response to environmental cues. Based on sequence similarity and the characteristics of their DNA-binding motifs, transcription factors have been classified into different families, which generally are conserved among eukaryotic organisms. Even though several transcription factors were identified by their capacity to bind to nodulin promoters, the most significant advances have aroused from genetic approaches by positional cloning of genes whose mutation altered infection or nodule development. The first transcription factor cloned in this way was *NODULE INCEPTION* (*NIN*).[3] Other genes, such as *Astray*,[4] *Ethylene Response Factor Required for Nodulation 1 (ERN1) and ERN2*,[5,6] *RR1*,[7] *CYCLOPS*,[8] *Nodulation Signaling Pathway 1* (*NSP1*) and *NSP2*,[9,10] *Nuclear Factor Y (NF-Y) A1* (formerly *MtHap2–1*)[11] and *NF-YC1*[12] were later added to the list (**Fig. 1**). The last ones belong to the GRAS
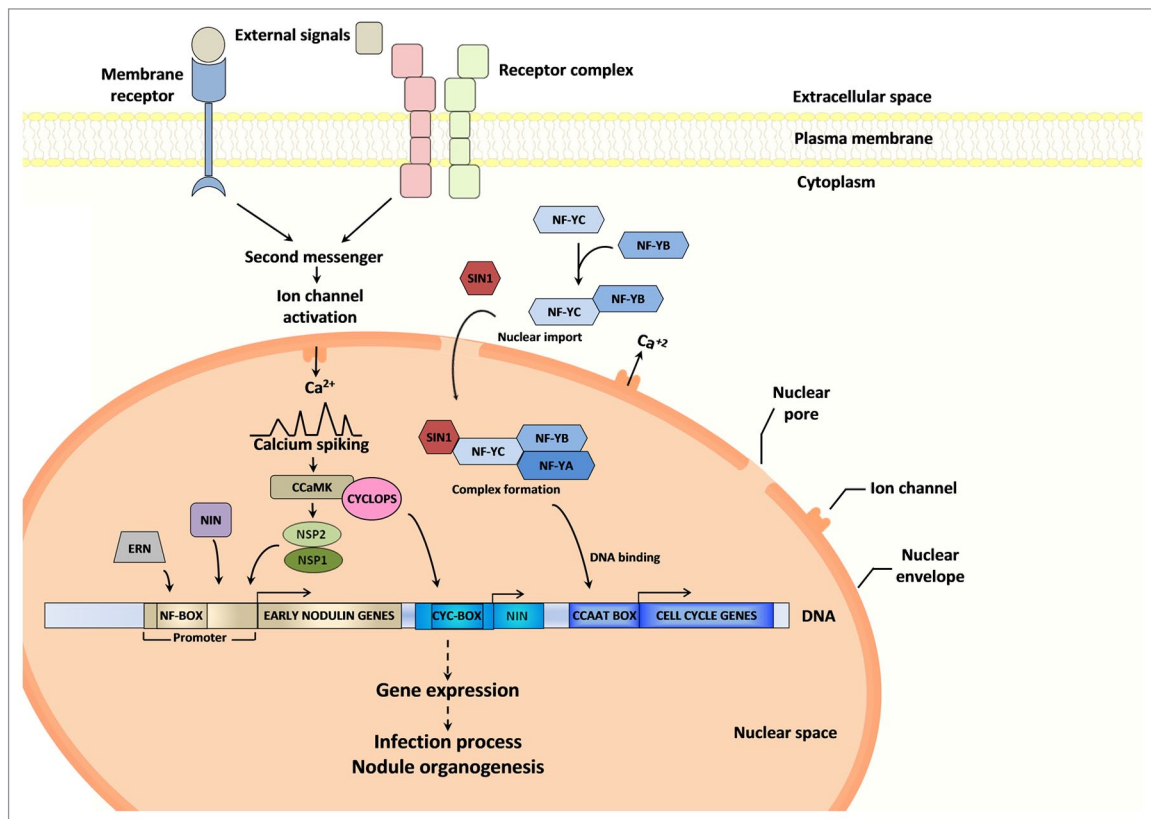
Figure 1. Transcription factors involved in nodulation. The nodulation signaling pathway is triggered when receptors located in the plasma membrane sense rhizobia derived signals. Upon receptor activation, oscillations of calcium concentration (calcium spiking) are produced in and around the nucleus. A nuclear located calcium and calmodulin dependent protein kinase (CCaMK) has been proposed as the protein that deciphers the calcium signal. Downstream from CCaMK, several transcriptional regulators are required for symbiosis establishment. CYCLOPS interacts and is phosphorylated by CCaMK, activating its capacity to recognize the *NIN* promoter. Nodulation signaling 1 (NSP1) and NSP2 both encode GRAS transcription factors that are essential for nodulation and activate the expression of early nodulation genes (nodulins). NIN and ERN are also involved in the transcriptional regulation of nodulins. Members of the NF-Y gene family are also required for nodulation. NF-YB would interact with NF-YC subunit in the cytoplasm prior to the nuclear import of the heterodimer. Once inside the nucleus, they interact with NF-YA to form the functional heterotrimer that binds with high affinity to CCAAT elements. SIN1 (a member of the GRAS family) physically interacts with the C subunit of the NF-Y transcription factor in the nucleus. This putative multimeric complex participates, directly or indirectly, in the transcriptional control of cell cycle genes.

(*NSP1* and *NSP2*) and NF-Y (*NF-YA* and *NF-YC*) families of transcriptional regulators.

NF-Y Transcriptional Regulators Involved in Symbiosis

NF-Ys are heterotrimeric complexes that bind to CCAAT boxes. They are composed by the NF-YA, NF-YB and NF-YC subunits. Whereas each subunit of NF-Y is encoded by one or two members in yeasts and mammals, these gene families have several members in plants. It is an open question whether this expansion has resulted in a functional diversification to cope with plant-specific responses. Individual NF-Y subunits have been shown to be involved in multiple developmental events and/or responses to environmental cues. In legumes, members of each subunit of NF-Y genes families have been implicated in the development of indeterminate (*MtNF-YA1 in Medicago truncatula*) and determinate nodules (*LjNF-YA1* in *Lotus japonicus* and *PvNF-YC1* in *Phaseolus vulgaris*). MtNF-YA1 was characterized as a symbiosis-specific transcription factor

that plays a key role during nodule development by controlling meristem persistence.[11] *MtNF-YA1* gene expression is restricted to the meristematic zone of indeterminate nodules by a regulatory mechanism of mRNA decay involving miRNA169 at early stages of symbiosis. More recently, it was shown that expression of *MtNF-YA1* is strongly upregulated during early stages of the symbiotic interaction, particularly in the root hairs of the infection zone. Moreover, mutant plants carrying a premature stop codon in the *MtNF-YA1* gene form aberrant (i.e., thick and branched) infection threads that fail to progress to the cortical cells.[13] On the other hand, Soyano et al. described that knockdown of *LjNF-YA1* inhibited root nodule organogenesis, but not the infection process in *L. japonicus*.[14] This report also showed that NIN is a direct regulator of the *LjNF-YA1* and *LjNF-YB1* genes. Interestingly, a recent report has shown that CYCLOPS is phosphorylated by the protein kinase CCaMK, exposing its DNA binding domain. In this conformation, CYCLOPS can recognize and bind to a specific region in the *NIN* promoter, initiating a transcriptional cascade that involves NF-YA1 and leads to nodule organogenesis.[8]

Reverse genetic studies performed in common bean (*P. vulgaris*) revealed that the NF-YC1 subunit is required for nodule organogenesis and rhizobial infection.[12,15] Overexpression of NF-YC1 was sufficient to improve nodulation efficiency of a relatively poor competitive rhizobium strain, possibly trough activation of the G2/M transition cell cycle genes.[12]

The number of genes that encodes for each subunit of the NF-Y complex in plants results in a wide number of potential trimeric complexes (1690 in *Arabidopsis*).[16] In addition, NF-Y complexes or individual subunits have been shown to associate with other transcriptional regulators, like SIN1 or transcription factors of the bZIP[17] or MAD box families,[18] further expanding the number of putative multimeric complexes that can be formed. This modular system provides an amazing versatility for the plant to integrate developmental programs with different environmental stimuli.

GRAS-Domain Transcription Factors and Their Roles in Symbiosis

The GRAS-type transcription factors constitute an important family of plant-specific proteins, whose initials come from the three members initially identified: Gibberellic-ACID INSENSITIVE (GAI), repressor of GAI (RGA) and SCARECROW (SCR).[19] They play different roles in the development of stem and root, in the gibberellic acid signaling and in the signal transduction pathways of phytochromes A and B.[20] In *M. truncatula* and *L. japonicus*, two proteins of this family, NSP1 and NSP2, were shown to be required for nodulation. They mediate different Nod Factor induced responses, such as root hair deformation, infection thread formation, cortical cell divisions and expression of nodulation genes known as nodulins.[9,10,21] Interestingly, NSP1 and NSP2 can form homo- and hetero- dimers and associate to promoters of early induced nodulins.[22] The complex formed by NSP1 and NSP2 can enhance the action of ERN on the transcriptional activity of the early nodulin 11 (*ENOD11*) during the progression of rhizobia infection.[6] Both proteins are also involved in mycorrhization, a symbiotic interaction with fungi that is present in the majority of land plants.[23] More recently, another GRAS protein, called RAM1 (Required for Arbuscular Mycorrhization 1), was shown to play a specific role in the formation of arbuscular mycorrhiza through its interaction with NSP2.[24]

A New Connection Between GRAS and NF-Ys

In a recent work, we identified a new GRAS-domain containing protein, named Scarecrow like 13 (SCL13) Involved in Nodulation (SIN1), which physically interacts with the C subunit of the NF-Y complex from common bean.[2] The GRAS family has been divided into eight subfamilies. Unlike NSP1 and NSP2, which belongs to the SHORT ROOT (SHR) and HAIRY MERISTEM (HAM) subfamilies, respectively, SIN1 is part of the PAT1 subfamily and highly similar to *Arabidopsis* SCL13. Both *in yeast* and *in planta* experiments have established that the NF-YC1 subunit physically interacts with SIN1. *SIN1* is expressed in aerial and root tissues, reaching higher levels in
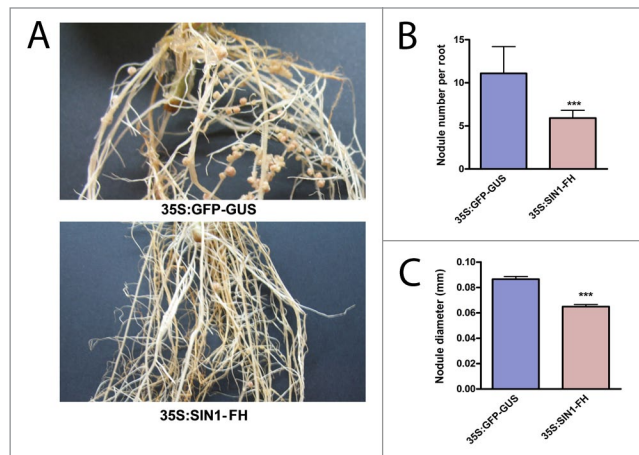
Figure 2. Overexpression of SIN1 resulted in the reduction of the number and size of root nodules. (**A**) Representative pictures illustrating the reduced nodule density and size found in 35S:SIN1-FH (FLAG-HIS) roots (lower panel) as compared with the control roots 35S:GFP-GUS (upper-panel) at 17 days post infection (dpi) with *R. etli*. The complete open reading frame of SIN1 was cloned in the p35S:FH vector.[28] Nodule number per root (**B**) and nodule size (**C**) recorded in 35S:GFP-GUS or 35S:SIN1-FH roots at 17 dpi with *R. etli*. Error bars represent the SEM. Asterisks indicate significant differences with the control in an unpaired two-tailed *t* test with $P < 0.001$.

roots and mature nodules. Post-transcriptional gene silencing of *SIN1* using RNA interference (RNAi) showed that the product of this gene plays a critical role in lateral root elongation and the establishment of successful symbiosis between *P. vulgaris* and *Rhizobium etli*.[2] The symbiotic phenotype was similar to that observed in *NF-YC1* silenced root, but the effect on nodule development and infection thread progression was comparatively milder.[12,15] Interestingly, *SIN1* silenced roots failed to upregulate mRNA levels of G2/M cell cycle genes and NF-YA1 in response to rhizobia infection. This suggests that SIN1 can act through its interaction with the NF-Y complex, but also might have a positive feedback on NF-Y gene regulation during nodule formation.

In addition to its role in nodulation, SIN1 showed to play a role during the elongation of lateral roots. This transcription factor with a dual role is an interesting connection between the developmental programs of two root organs, nodules and lateral roots. When *LjNF-YA1* was overexpressed, the tips of lateral roots showed malformations, an effect that was enhanced by the co-expression with *LjNF-YB1*.[14] This phenotype was accompanied by an increment of cell division in non-meristematic tissue, suggesting that the A1 and B1 subunits of LjNF-Y can modulate the activity of cell cycle genes. It would be interesting to explore the possible connections between NF-Y and GRAS transcription factors with the auxin/cytokinin balance, which are critical to initiate both lateral roots and nodules.[25,26,27]

Overexpression of SIN1 Affects Nodulation in Common Bean

As a complementary strategy to the post-transcriptional silencing, a fusion of *SIN1* with the FLAG and HIS tags was ectopically expressed under the control of the CaMV 35S promoter. The nodulation phenotype of transformed roots was compared with control plants transformed with the plasmid p35S:GFPGUS (**Fig. 2A**). Unexpectedly, plants overexpressing *SIN1* showed a reduced number of nodules per root as compared with control plants (**Fig. 2B**). Overexpression of *SIN1* also affected the diameter of the nodules (**Fig. 2C**), producing a growth impair similar to that observed in RNAi roots. These results support the role of SIN1 in the nodulation process, specifically in the nodule organogenesis, and suggest that a fine-tuned balance between individual components of the same transcriptional complex is critical to maintain its function.

Concluding Remarks and Perspectives

The fact that plants of *P. vulgaris* with reduced or ectopic expression of *SIN1* exhibited a significant reduction in the number and size of nodules formed by rhizobia raises the hypothesis that fine-tuned levels of SIN1 are required for an efficient performance of NF-YC1. NF-YC1 controls, directly or indirectly, the expression of G2/M transition cell cycle genes.[12] SIN1 is also required for upregulation of these cell cycle genes in response to rhizobia.[2] A key question is whether the complex formed by SIN1 and NF-YC1

can bind to promoters of these genes to regulate cell proliferation during nodule formation. Chromatin immunoprecipitation (ChIP) experiments followed by high throughput sequencing of isolated DNA fragments would certainly help to reveal downstream genes controlled by this complex during the interaction of legume roots with nitrogen fixing bacteria.

In addition to the role described in the plant response during symbiosis, SIN1 is also involved in lateral root elongation, suggesting that this is a shared component of two different root developmental programs. On the other hand, as previously mentioned, NF-Y subunits also have been implicated in these two developmental processes. It will be interesting to characterize at global scale the overlapping transcriptional networks governed by GRAS and NF-Y proteins during lateral root and nodule formation.

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