

A C Subunit of the Plant Nuclear Factor NF-Y Required for Rhizobial Infection and Nodule Development Affects Partner Selection in the Common Bean–*Rhizobium etli* Symbiosis

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Legume plants are able to interact symbiotically with soil bacteria to form nitrogen-fixing root nodules. Although specific recognition between rhizobia and legume species has been extensively characterized, plant molecular determinants that govern the preferential colonization by different strains within a single rhizobium species have received little attention. We found that the C subunit of the heterotrimeric nuclear factor NF-Y from common bean (*Phaseolus vulgaris*) *NF-YC1* plays a key role in the improved nodulation seen by more efficient strains of rhizobia. Reduction of *NF-YC1* transcript levels by RNA interference (RNAi) in *Agrobacterium rhizogenes*-induced hairy roots leads to the arrest of nodule development and defects in the infection process with either high or low efficiency strains. Induction of three G2/M transition cell cycle genes in response to rhizobia was impaired or attenuated in *NF-YC1* RNAi roots, suggesting that this transcription factor might promote nodule development by activating cortical cell divisions. Furthermore, overexpression of this gene has a positive impact on nodulation efficiency and selection of *Rhizobium etli* strains that are naturally less efficient and bad competitors. Our findings suggest that this transcription factor might be part of a mechanism that links nodule organogenesis with an early molecular dialogue that selectively discriminates between high- and low-quality symbiotic partners, which holds important implications for optimizing legume performance.

INTRODUCTION


Legume plants have the capacity to establish a symbiotic association with nitrogen-fixing soil bacteria known as rhizobia. This symbiosis involves the formation of a unique and specialized root organ, the nodule, where differentiated rhizobia convert atmospheric nitrogen into a reduced form readily assimilable by the plant. In return, plants supply the bacteria with a protected niche and photosynthates. The formation of nitrogen-fixing nodules requires the simultaneous activation of two coordinated developmental processes: the reinitiation of cell division in the root cortex to form the nodule primordium and the infection by the bacteria that will colonize the nodule (Madsen et al., 2010). Rhizobial infection of many legumes is initiated near the tip of the root hair, which responds with swelling and curling that entrap the bacteria into infection foci. Infection is established by invagination of the plant plasma membrane and cell wall, leading to the formation of a tubular structure called the infection thread (IT). Within the IT, bacteria divide and progress toward the root cortex, where they are released into the plant cells via an


endocytosis-like event, surrounded by a symbiosome membrane and finally differentiate into nitrogen-fixing bacteroids (Gage, 2004). Initiation and progression of ITs are perhaps the most critical steps of infection, which is reflected by the complex recognition of the required bacterial signals (Ardourel et al., 1994; Cheng and Walker, 1998; Walker and Downie, 2000; Gibson et al., 2008; Downie, 2010). It has been proposed that strict specificity is required to avoid penetration by unwanted bacteria, from pathogens to ineffective rhizobia that would use host resources without a benefit for the plant. Nod factor receptors play a key role in recognition and signaling processes that determine host range in legume-rhizobia symbiosis. It was demonstrated that the expression of *Lotus japonicus* Nfr1 and Nfr5 Nod factor receptors in *Medicago truncatula* and *Lotus filicaulis* extended their host range to include *Mesorhizobium loti* and *Rhizobium leguminosarum* species, which normally infect *L. japonicus* (Radutoiu et al., 2007). Seminal evidence of host range specificity came from the studies of the *sym2^A* allele of the wild pea (*Pisum sativum*) variety Afghanistan, which allows nodulation only by specific strains of *R. leguminosarum* that carry the host specificity *nodX* gene (Firmin et al., 1993; Geurts et al., 1997). A more recent report showed that plant resistance genes of the Toll-interleukin receptor/nucleotide binding site/leucine-rich repeat family are also involved in determining host specificity in the soybean-rhizobium symbiosis (Yang et al., 2010)

At the molecular level, perception of rhizobia initiates a signal transduction cascade responsible for morphological and molecular changes in both epidermal and cortical tissues of the host root (Oldroyd and Downie, 2008). This cascade activates a

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number of transcriptional regulators that will positively regulate the expression of genes involved in nodulation, collectively known as nodulins. Gene expression profiling of several legumes, such as *L. japonicus*, *M. truncatula*, and soybean (*Glycine max*), has revealed that genes involved in cell growth, cell division, and DNA synthesis are upregulated relatively early after bacterial perception (Kouchi et al., 2004; Lohar et al., 2006; Høglund et al., 2009; Libault et al., 2010; Maunoury et al., 2010).

The biological association between legumes and rhizobia is established under nitrogen-limited soil conditions and accounts for a significant amount of the nitrogen incorporated into soils (Gruber and Galloway, 2008). However, the process is suboptimal in agricultural systems, since highly efficient strains of rhizobia used as inoculants are usually out-competed by soil bacteria (Brockwell et al., 1995). On the other hand, plants that have coevolved with their symbiotic bacteria show preference for more efficient strains (Lie et al., 1987; Aguilar et al., 2004), suggesting the existence of a mechanism to avoid low quality partners. Common bean (*Phaseolus vulgaris*) is one of the legumes in which host-dependent competitiveness has been observed. This species is native to America, where two major centers of bean genetic diversification have been proposed, one in Mesoamerica and the other in the Andean region (Velasquez and Gepts, 1994). The predominant rhizobia found to be associated with common bean in both genetic diversification centers is *Rhizobium etli* (Amarger, 2001). Examination of polymorphism in the *R. etli nodC* gene showed that strains carrying the *nodC* allele type- α are predominant in Mesoamerican soils, whereas those carrying the *nodC* allele type- δ are predominant in the Andean region (Aguilar et al., 2004). Analysis of nodule occupancy and nodulation performance by these strains led to the finding that wild and cultivated bean accessions from the Mesoamerican genetic diversification center are preferentially and more efficiently nodulated by strains *nodC* type- α compared with strains *nodC* type- δ (Aguilar et al., 2004; Peltzer Meschini et al., 2008). This suggests that plants from the Mesoamerican diversification center can effectively recognize and select symbiotic partners that have coevolved in the same geographic region. This biological interaction, in which host-dependent selection has been observed, provides a challenging opportunity to explore the molecular mechanisms underlying bacterial strain preference by the plant, which ultimately will help in understanding the mechanisms that determine competence in the rhizosphere.

To identify genes that might be involved in the strain preference associated with symbiotic efficiency, we previously used a subtractive hybridization approach to isolate genes that are differentially induced in a Mesoamerican cultivar of common bean by a *R. etli* strain representative of this geographic region compared with a strain that is predominant in the Andean soils (Peltzer Meschini et al., 2008). One of these genes encodes a protein highly similar to the C subunit of the nuclear factor Y (NF-Y). NF-Ys are heterotrimeric transcription factors composed of NF-YA (also known as HAP2 or CBF-B), NF-YB (HAP3 or CBF-A), and NF-YC (HAP5 or CBF-C) subunits. These complexes display high affinity and sequence specificity for the CCAAT box, a *cis*-element present in ~25% of eukaryotic gene promoters (Mantovani, 1999), playing key roles in development and in response to adverse environmental conditions (Bhattacharya et al., 2003; Lee

et al., 2003; Ben-Naim et al., 2006; Nelson et al., 2007; Li et al., 2008). In mammals and yeast, each subunit of NF-Y is encoded by a single gene, whereas the *Arabidopsis thaliana* genome encodes 10 NF-YAs, 13 NF-YBs, and 13 NF-YCs (Gusmaroli et al., 2002). Although the presence of many genes encoding NF-Y subunits suggests a high degree of genetic redundancy in plants, mutations in single NF-Y genes have been linked to defects in development or enhanced stress sensitivity in *Arabidopsis*, suggesting that each member has a specialized function (Lotan et al., 1998; Kwong et al., 2003; Lee et al., 2003; Li et al., 2008). In addition, a member of the NF-YA family was identified in the model legume *M. truncatula* and designated *HAP2-1* (Combiere et al., 2006). *HAP2-1* plays a role in the persistence of nodule meristem, and its expression is spatially restricted to the meristematic zone of indeterminate nodules by a regulatory mechanism of mRNA decay involving microRNA169 at early stages of symbiosis and by a transacting small peptide derived from alternative splicing of the *HAP2-1* gene at later stages of the interaction (Combiere et al., 2006, 2008).

In this study, we used a reverse genetic approach to investigate the role of a common bean cDNA, which we named *NF-YC1*, in the selection of rhizobial strains that coevolved with the symbiont in the Mesoamerican genetic diversification center and fix nitrogen more efficiently. Our findings suggest that this transcription factor might link nodule organogenesis with the nodulation efficiency of rhizobial strains that coevolved in diversification centers.

RESULTS

Identification and Sequence Analysis of NF-YC Genes in Common Bean

A cDNA with high sequence similarity to the C subunit of NF-Y was initially identified in a survey of genes differentially induced in root hairs from a Mesoamerican cultivar of common bean by the highly efficient and competitive strain *R. etli* SC15, compared with the less efficient and poorly competitive strain 55N1 (Peltzer Meschini et al., 2008). This cDNA, initially named RSH24-27, is 1176 bp in length, including 81 bp of the 5'-untranslated region, an open reading frame of 783 bp encoding a predicted protein of 260 amino acids, and a 3'-untranslated region of 312 bp. A BLAST search (Altschul et al., 1997) with the RSH24-27 deduced amino acid sequence against the GenBank nonredundant database showed a high sequence similarity with the C subunit of NF-Y transcription factors from legume and nonlegume plants: 90% identity ($E = 6.0e^{-135}$) with the sequence of *M. truncatula* NF-YC and 63% identity ($E = 5.4e^{-70}$) with NF-YC9 protein from *Arabidopsis*. Best homolog/putative ortholog genes from soybean and *L. japonicus* were retrieved from genomic databases for these species. RSH24-27 displays 95% identity ($E = 2.4e^{-134}$) with the soybean putative protein product and 89% identity ($E = 1.0e^{-126}$) with the *L. japonicus* protein product. An alignment of amino acid sequences from RSH24-27 and other plant species is presented in Supplemental Figure 1 online. The highest sequence conservation was found in the histone fold domain, which contains four α -helices separated by short loops

(Gusmaroli et al., 2002). The C-terminal end has a domain rich in Gln and hydrophobic amino acids that has been proposed to play a role in transcriptional activation (Mantovani, 1999), whereas sequence at the N-terminal end is more divergent among the sequences analyzed here. Based on this analysis, and following the nomenclature recommended for members of CCAAT binding factors in plants (Gusmaroli et al., 2001, 2002; Siefers et al., 2009), RSH24-27 was renamed *NF-YC1*.

A TBLASTN search in the Dana-Farber Cancer Institute (DFCI) common bean gene index database (release 3.0) identified two tentative consensus sequences, designated *NF-YC2* and *NF-YC3*, which display 60% ($E = 2.1e^{-70}$) and 59% ($E = 3.6e^{-22}$) amino acid identity to common bean *NF-YC1*, respectively (see Supplemental Figure 2A online). Based on the current EST and genomic sequence information available for this legume, this analysis indicates that, as described for other plant species (Gusmaroli et al., 2001, 2002; Yang et al., 2005; Stephenson et al., 2007; Siefers et al., 2009), *NF-YC* is a gene family, with at least three members in common bean.

Expression Analysis of *NF-YC* Genes Revealed That *NF-YC1* Shows a Unique Strain-Specific Expression Pattern in Mesoamerican Common Bean Roots

In a previous work, we showed that *NF-YC1* is ubiquitously expressed in common bean, since transcripts were detected in several organs, including leaves, stems, roots, and nodules of different developmental stages (Peltzer Meschini et al., 2008). This study also showed that *NF-YC1* transcript levels were higher in nodules formed by the more competitive *R. etli* strain SC15 (*nodC- α*) than in those formed by strain 55N1 (*nodC- δ*) at 7 and 14 d after inoculation (DAI). Here, quantitative RT-PCR (qRT-PCR) experiments were conducted using gene-specific primers for each of the three *NF-YC* members identified in common bean. This analysis confirmed that *NF-YC1* mRNA levels increased ~10-fold 24 h after inoculation (HAI) with the more competitive *R. etli* strain SC15 and were not significantly modified by strain 55N1 as previously described (Peltzer Meschini et al., 2008), whereas the other two members of the *NF-YC* family showed no modification (*NF-YC3*) or only a slight increase (1.7-fold for *NF-YC2*) in transcript levels in response to both *R. etli* SC15 and 55N1 (Figure 1A). Based on this analysis, we conclude that *NF-YC1* shows a unique strain-specific upregulation among members of this family. Consistent with this, its putative ortholog in *M. truncatula* also shows a unique mRNA accumulation pattern during root nodulation compared with other members of the *NF-YC* gene family (see Supplemental Figure 3A online) based on analysis of data from the *M. truncatula* gene expression atlas (Benedito et al., 2008). In addition, expression analysis of the putative ortholog of *NF-YC1* in *L. japonicus* (chr1.CM0544.480.nd) using available transcriptome data (Högslund et al., 2009) revealed that transcripts of this gene also accumulate at higher levels in nodules at 14 and 21 DAI and in the whole root at 3 DAI with its symbiotic partner *M. loti* compared with uninoculated whole roots (see Supplemental Figure 3B online). In the susceptible zone of *L. japonicus* roots, increase in transcript levels of this gene was observed at 24 HAI with *M. loti*, and, interestingly, this

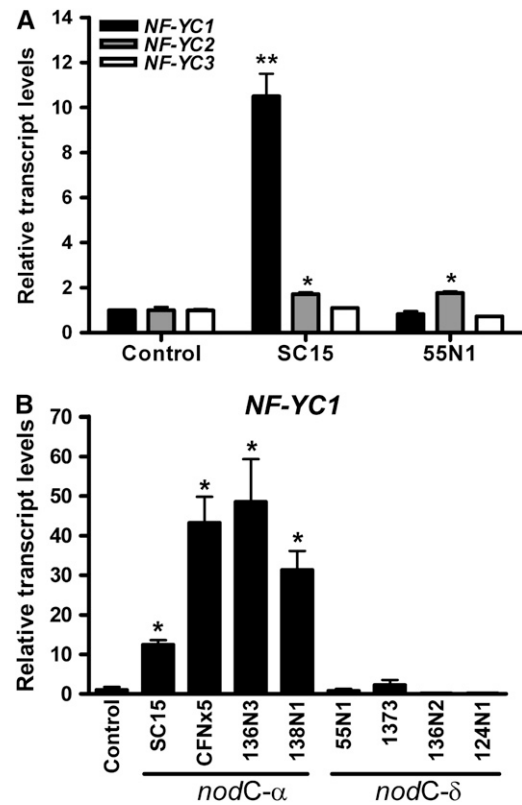


Figure 1. *NF-YC1* Expression Is Specifically Upregulated by *R. etli* Strains Carrying the Allele *nodC- α* .

(A) qRT-PCR analysis of *NF-YC1*, *NF-YC2*, and *NF-YC3* transcript levels in wild-type common bean roots 24 HAI with either strain SC15 (*nodC- α*) or 55N1 (*nodC- δ*) of *R. etli* or with the medium used to grow the bacteria (YEM) as control.

(B) qRT-PCR analyses of *NF-YC1* transcript levels in roots 24 HAI with either *nodC- α* (SC15, CFNx5, 136N3, and 138N1) or *nodC- δ* (55N1, 1373, 136N2, and 124N1) strains of *R. etli* or with YEM as control.

NF-YC1 mRNA levels were normalized to eukaryotic elongation factor 1 α (EF1 α) and presented relative to control roots. Error bars represent SD of at least three technical replicates. Results are representative of two independent biological experiments. Single and double asterisks indicate that values are significantly different from the control value in an unpaired two-tailed *t* test with $0.01 < P < 0.05$ and $0.001 < P < 0.01$, respectively.

increase was abolished in *nfr1*, *nfr5*, and *nin* single mutants (see Supplemental Figure 3C online).

To investigate whether transcript levels of *NF-YC1* are correlated with the recognition of strains that have coevolved in the Mesoamerican genetic diversification center, we selected a group of strains carrying either the allele type- α or - δ of the *nodC* gene: the allele type- α is predominant in Mesoamerican soils, whereas the *nodC* type- δ is found in strains from Andean soils (Aguilar et al., 2004). Twenty-four HAI, *NF-YC1* transcript levels were 10- to 50-fold (32-fold in average) higher in roots infected with *nodC- α* strains than in control roots, whereas no significant accumulation or only slight increases were observed after inoculation with *nodC- δ* bacterial strains (Figure 1B). This

difference in the response to *nodC*- α and *nodC*- δ strains was significantly different ($P < 0.001$, unpaired two-tailed t test). *NF-YC1* transcript accumulation was correlated with the higher nodulation performance exhibited by plants inoculated with *nodC*- α strains (Table 1), which developed an average of 20.07 ± 0.37 nodules per plant ($n = 34$), compared with *nodC*- δ strains, which only developed 10.40 ± 0.14 nodules per plant ($P < 0.0001$, unpaired two-tailed t test, $n > 24$) at 7 DAI. These results indicate that *NF-YC1* is differentially upregulated by lineages of *R. etli* that are more efficient in nodule formation and have coevolved in the Mesoamerican genetic diversification center.

NF-YC1 Localizes Predominantly in the Nucleus

The PSORT II program (Horton and Nakai, 1997) identified a nonclassical nuclear localization signal at position 103 of the NF-YC protein sequence (PXXRXKK) and predicted a nuclear localization with 76.7% reliability, based on Reinhardt's method for cytoplasmic/nuclear discrimination (Reinhardt and Hubbard, 1998). To assess the subcellular localization of this protein experimentally, a translational fusion between green fluorescent protein (GFP) and the N terminus of NF-YC1 was expressed in common bean roots by *Agrobacterium rhizogenes*-mediated transformation. Cells expressing GFP-NF-YC1 showed predominant GFP signal in the nucleus, whereas the free GFP was dispersed in the nucleus and the cytoplasm (see Supplemental Figure 4 online). This subcellular location is in agreement with that previously described for NF-YC9 and NF-YC2 proteins in *Arabidopsis* (Koroleva et al., 2005; Liu and Howell, 2010).

Nodule Organogenesis and Development Are Impaired in Roots with Reduced Levels of NF-YC1

To investigate the role of this nuclear factor in the establishment of a highly efficient interaction between common bean and its partner *R. etli*, we used an RNA interference (RNAi) approach to reduce *NF-YC1* mRNA levels in roots of common bean composite plants, which consist of wild-type shoots and transgenic hairy roots. Since the region of *NF-YC1* used for RNAi displays 79 and 66% identity at nucleotide level with *NF-YC2* and *NF-YC3*, respectively (see Supplemental Figure 2B online), we assessed the specificity of the silencing caused by the RNAi construct by measuring mRNA levels of *NF-YC1*, *NF-YC2*, and *NF-YC3* in root RNA samples from three independent *NF-YC1* RNAi composite plants. *NF-YC1* RNAi roots exhibited a >85% reduction of *NF-YC1* transcript levels, whereas *NF-YC2* and *NF-YC3* mRNAs levels showed no significant alterations compared with β -glucu-

ronidase (*GUS*) RNAi roots (Figure 2A). Based on this analysis, we concluded that the RNAi construct used in this study was specific for *NF-YC1*.

A time-course experiment of nodule formation showed that *NF-YC1* RNAi roots developed fewer nodules than did control *GUS* RNAi roots with either *R. etli* strain SC15 or 55N1 (Figure 2B; $P = 0.0003$ and 0.0391 , respectively for each strain at 10 DAI, unpaired two-tailed t test). Moreover, the number of nodules formed in *NF-YC1* RNAi roots inoculated with SC15 was not significantly different from those formed upon inoculation with 55N1 at 10 DAI (Figure 2B; $P = 0.5608$, unpaired two-tailed t test), showing that the difference in the efficiency of nodule formation observed between *R. etli* strains was lost in roots with reduced levels of *NF-YC1*.

Nodulation performance was also examined at later time points: a significant reduction of $\sim 80\%$ in the number of nodules was observed in *NF-YC1* RNAi roots compared with *GUS* RNAi roots at 21 DAI with *R. etli* SC15 (12 ± 2 nodules per root in *GUS* RNAi and 3.1 ± 0.6 in *NF-YC1* RNAi roots, respectively; $P < 0.0001$, unpaired two-tailed t test, $n > 45$). At this time point, *NF-YC1* RNAi plants showed obvious symptoms of nitrogen deficiency, such as leaf chlorosis and poor shoot development, whereas *GUS* RNAi plants developed abundant and green foliage (Figure 2C). The few nodules formed in *NF-YC1* RNAi roots were small and white or pale pink compared with those of control plants (Figures 2D and 2E). Viable bacteria was recovered from 98% ($n = 54$) of the nodules analyzed in *GUS* RNAi roots, but from only 48% ($n = 60$) of *NF-YC1* RNAi roots, suggesting that the nitrogen deficiency phenotype observed in *NF-YC1* RNAi plants might be caused by the reduction in both the number of nodules and their occupancy by nitrogen-fixing bacteria. This phenotype was observed upon inoculation with either *R. etli* genotype *nodC*- α or *nodC*- δ . Optical and transmission electron microscopy of nodule sections revealed that *GUS* RNAi nodules showed a central zone containing numerous infected cells surrounded by the peripheral tissue in which vascular bundles were developed (Figures 3A and 3C). Infected cells contained small vacuoles and numerous symbiosomes with one or more bacteroids filled with poly- β -hydroxybutyrate granules as previously described for common bean wild-type nodules (Tat e et al., 1994; Cermola et al., 2000). On the contrary, the central zone of *NF-YC1* RNAi nodules presented small, irregularly shaped, and highly vacuolated cells that were devoid of bacteroids (Figures 3B and 3D). Taken together, these results indicate that *NF-YC1* is required for both organogenesis and proper development of nodules in common bean and further support the idea that differential

Table 1. Number of Nodules Developed in Wild-Type Common Bean Plants after Inoculation with *R. etli* Strains Carrying Either the *nodC*- α or the *nodC*- δ Allele

<i>nodC</i> - α				<i>nodC</i> - δ			
SC15	CFNx5	136N3	138N1	55N1	1373	136N2	124N1
$20.8 \pm 2.7^{a,b}$	16.9 ± 2.5^a	16.8 ± 1.8^a	25.8 ± 2.4^b	8.4 ± 1.6^c	$12.3 \pm 0.9^{a,c}$	10.4 ± 2.2^c	nd

Nodules developed in Mesoamerican NAG12 common bean plants were recorded at 7 DAI with each strain of *R. etli*. Data represent the number of nodules developed per plant \pm SE ($n > 8$). Different letters indicate significantly different values in an unpaired two-tailed t test ($P < 0.05$). nd, not determined.

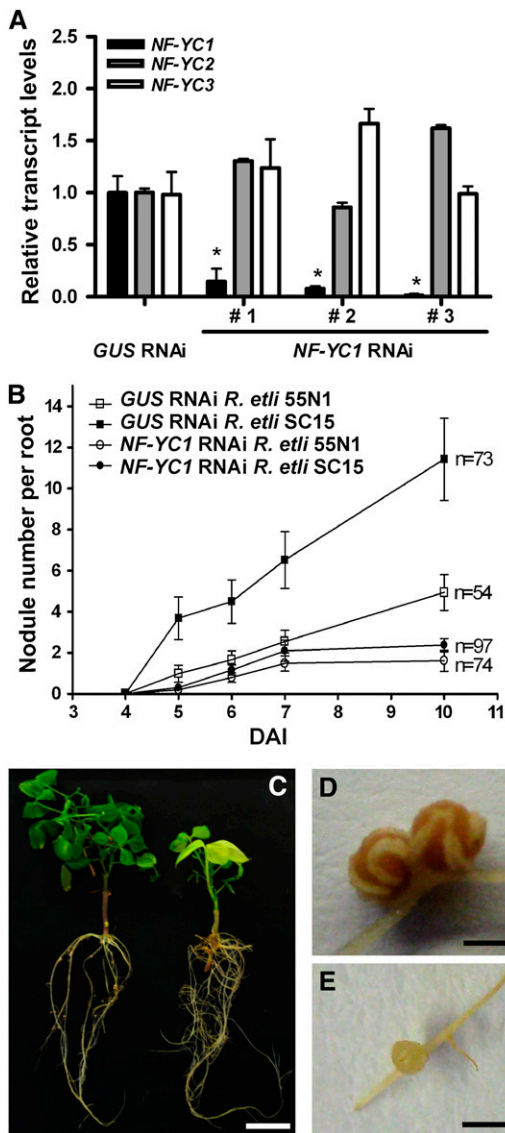


Figure 2. *NF-YC1* RNAi Roots Are Impaired in Nodule Organogenesis.

(A) qRT-PCR analysis of *NF-YC1*, *NF-YC2*, and *NF-YC3* mRNA levels in transgenic roots from *GUS* RNAi and three *NF-YC1* RNAi plants. Error bars represent SD of at least three technical replicates. Asterisk indicates that values are significantly lower than those of *GUS* RNAi roots in an unpaired two-tailed *t* test with $0.01 < P < 0.05$.

(B) Time course of the number of nodules per root. Error bars represent SE, and *n* is the number of hairy roots analyzed in each case. The experiment is representative of three independent biological replicates.

(C) Phenotype of a *NF-YC1* RNAi plant (right) compared with a *GUS* RNAi control (left) at 21 DAI with *R. etli* strain SC15.

(D) Nodules developed in *GUS* RNAi roots at 21 DAI with *R. etli* strain SC15.

(E) A nodule developed in *NF-YC1* RNAi roots at 21 DAI with *R. etli* strain SC15.

Bars = 1 cm in **(C)** and 5 mm in **(D)** and **(E)**.

regulation of *NF-YC1* is required for the establishment of highly efficient nodulation.

***NF-YC1* RNAi Roots Showed Excessive Root Hair Deformation, but IT Initiation and Progression Were Reduced**

The phenotype observed in *NF-YC1* RNAi nodules indicated that the infection process is defective in these roots. Thus, we examined infection events at 5 DAI with *R. etli*. First, we tested whether root hair morphological responses were affected in *NF-YC1* RNAi roots (Figures 4A to 4C). It was found that reduction of *NF-YC1* transcript levels resulted in excessive root hair deformation in response to rhizobia: 83% of root hairs showed swelling, branching, or curling compared with only 22% in *GUS* RNAi roots ($n > 100$). This increase in the number of root hairs that suffer deformation, illustrated in Figure 4B, was observed in several independent roots obtained from different *NF-YC1* RNAi composite plants. Next, infection events were visualized and quantified by the use of a GFP-expressing *R. etli* strain previously obtained in our laboratory (Blanco et al., 2009). Although bacteria microcolonies were observed on the surface of both *GUS* RNAi and *NF-YC1* RNAi root hairs, the number of infection foci and/or ITs was severely reduced in *NF-YC1* RNAi compared with *GUS* RNAi roots (Figure 4D; $P < 0.001$, unpaired two-tailed *t* test). In control roots, the majority of the ITs (69%) progressed, ramified, and released bacteria into cortical cells (Figures 4E and 4H). By

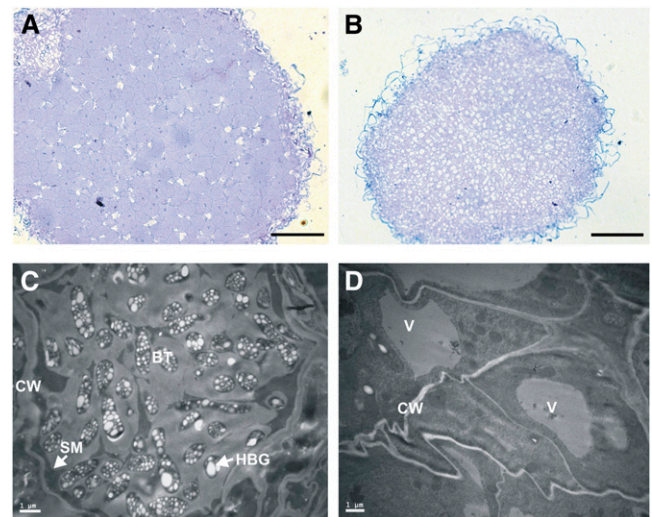


Figure 3. Ultrastructural Characterization of *NF-YC1* RNAi Nodules.

(A) and **(B)** Optical microscopy of semithin (1 to 2 μm) sections of nodules developed in *GUS* RNAi **(A)** or *NF-YC1* RNAi **(B)** roots at 21 DAI with *R. etli* strain SC15.

(C) and **(D)** Transmission electron microscopy of ultrathin (70 nm) sections of nodules developed in *GUS* RNAi **(C)** and *NF-YC1* RNAi **(D)** roots.

BT, bacteroids; CW, cell wall; HBG, poly- β -hydroxybutyrate granules; SM, symbiosome membrane; V, vacuole. Bars = 200 μm in **(A)** and **(B)** and 1 μm in **(C)** and **(D)**.

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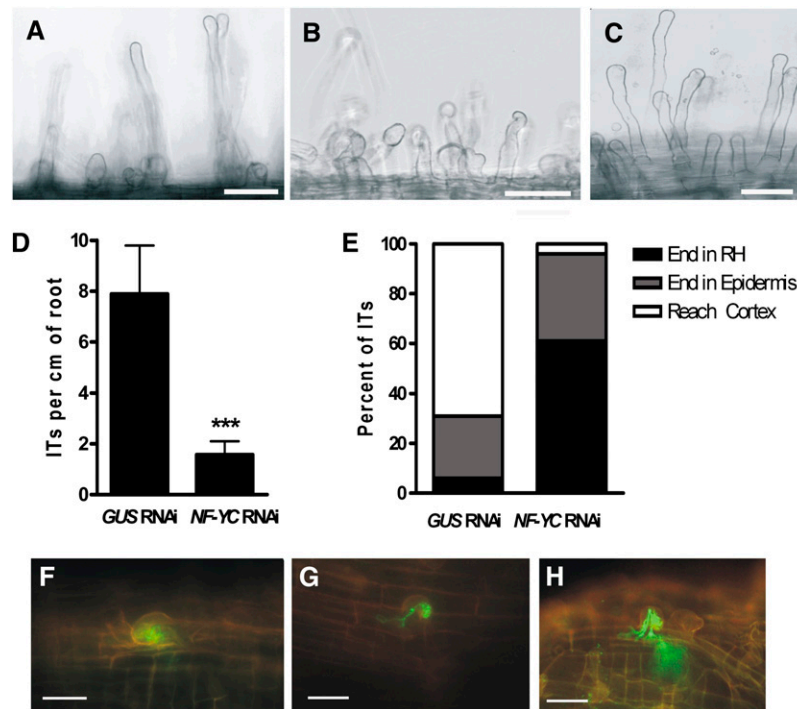


Figure 4. Root Hair Morphological Response and Infection Events in *NF-YC1* RNAi Roots.

(A) Root hair deformation in *GUS* RNAi roots inoculated with *R. etli*.

(B) Excessive root hair deformation in *NF-YC1* RNAi roots inoculated with *R. etli*.

(C) Nondeformed root hairs in noninoculated *NF-YC1* RNAi roots.

(D) Quantification of IT formation. ITs per centimeter of root were scored on >10 independent *GUS* RNAi or *NF-YC1* RNAi roots at 5 DAI with a GFP-expressing *R. etli* strain 55N1. Asterisks indicate that the value in *NF-YC1* RNAi roots was significantly different of that of *GUS* RNAi roots in an unpaired two-tailed *t* test with $P < 0.001$ ($n > 50$).

(E) Percentage of ITs that reach the root cortex (white), end in the epidermis (gray), or end in the root hair (black). Total number of infection events scored was 71 for *GUS* RNAi and 56 for *NF-YC1* RNAi.

(F) IT that aborted in the root hair in *NF-YC1* RNAi roots.

(G) IT that aborted in the epidermis in *NF-YC1* RNAi roots.

(H) IT releasing the bacteria into the cortical cells in *GUS* RNAi roots.

Bars = 50 μm in (A) to (C) and 25 μm in (F) to (H).

contrast, progression of most of the ITs observed in *NF-YC1* RNAi roots was blocked either at root hairs or epidermis, reaching the cortex in only 4% of the cases (Figures 4E to 4G). Based on these results, we conclude that *NF-YC1* is also required at early stages of the symbiosis for initiation and progression of ITs that guide bacteria to nodule primordia.

Expression of Cell Cycle Genes, but Not Early Nodulins, Is Affected in *NF-YC1* RNAi Roots

The putative role of *NF-YC1* as a transcriptional activator and the phenotype observed in RNAi composite plants suggested that it might act in regulating the expression of genes involved in nodulation, collectively known as nodulins (Oldroyd and Downie, 2008). Since transcripts of *NF-YC1* were previously found to accumulate as early as 3 HAI with *R. etli* strain SC15 (Peltzer Meschini et al., 2008), we tested whether expression of nodulin genes that are rapidly induced after Nod factor perception was

affected in *NF-YC1* RNAi roots. Three genes were selected: *RIP*, a peroxidase induced early during nodulation in epidermal cells (Cook et al., 1995); *ERN1* (ERF required for nodulation), an AP2-type transcription factor required for IT formation and nodule invasion (Middleton et al., 2007); and *ENOD40*, encoding an RNA involved in nodule primordia formation (Crespi et al., 1994). Transcripts of these genes accumulated in wild-type roots of common bean in response to both SC15 and 55N1 strains, but in the case of *ERN*, transcript levels were higher in roots inoculated with strain SC15 than in those inoculated with 55N1 (see Supplemental Figure 5 online). Likewise, transcripts of these early nodulation genes accumulated within 24 HAI with strain SC15 in transgenic hairy roots transformed with *GUS* RNAi (Figure 5, white bars). The expression pattern of these nodulins was not significantly affected by RNAi of *NF-YC1* at any of the time points analyzed (Figure 5, black bars), indicating that *NF-YC1* might act downstream or independently of these nodulation marker genes.

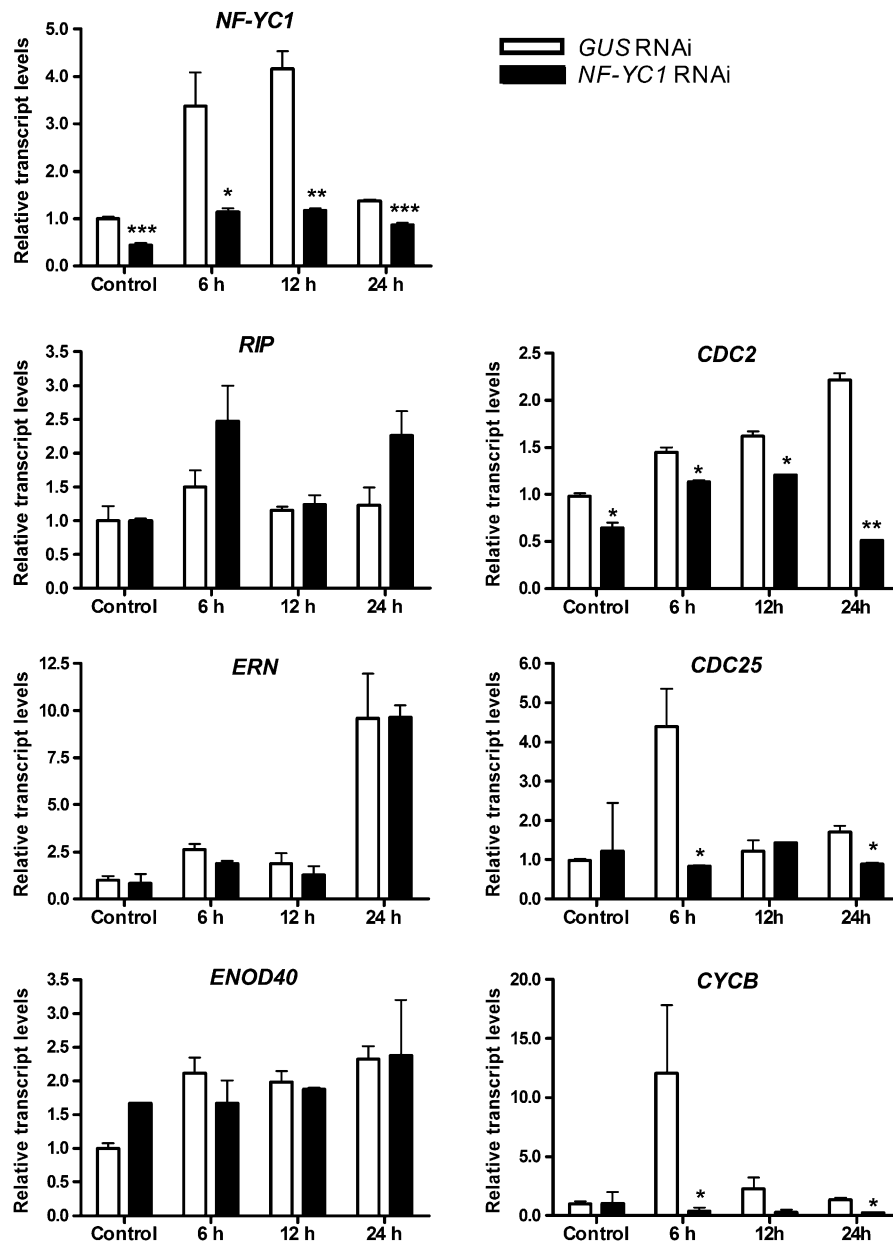


Figure 5. Expression Analysis of Early Nodulins and Cell Cycle Genes in *NF-YC1* RNAi Roots.

GUS RNAi (white bars) or *NF-YC1* RNAi (black bars) roots were inoculated with *R. etli* strain SC15 or with YEM (control). For plants inoculated with rhizobia, root tissue was harvested from at least three independent plants at the indicated times, and for YEM-inoculated plants, tissue from roots at 6, 12, and 24 HAI was harvested and pooled together. Transcripts levels were normalized to *EF1 α* and presented relative to the values of control *GUS* RNAi roots. Error bars represent SD of three technical replicates. Single, double, and triple asterisks indicate that expression values in *NF-YC1* RNAi are significantly different from those in *GUS* RNAi roots at the same time point, in an unpaired two-tailed *t* test with $0.01 < P < 0.05$, $0.001 < P < 0.01$, and $P < 0.001$, respectively.

In mammals, NF-Y transcription factors regulate the expression of genes involved in the control of cell cycle progression, such as G2/M transition genes *cdc2*, *cdc25*, and *cyclinB* (Caretti et al., 2003). In the context of legume nodulation, it has been shown that these cell cycle genes are activated at early time points after Nod factor application or rhizobial inoculation

(Savouré et al., 1994; Yang et al., 1994). Therefore, we assessed whether expression of cell cycle genes was affected by knock-down of *NF-YC1* at early stages of the common bean-*R. etli* interaction. In control roots, *CDC2* transcripts progressively increased up to 2-fold during the first 24 HAI with *R. etli* SC15, whereas *CDC25* and *CYCB* mRNAs accumulated at 6 HAI and

then decreased (Figure 5, white bars). By contrast, no significant induction of *CYCB* and *CDC25* mRNAs was detected in *NF-YC1* RNAi roots during the first 24 HAI, whereas *CDC2* transcripts showed only a slight induction at 6 and 12 HAI (Figure 5, black bars), suggesting that *NF-YC1* might directly or indirectly promote activation of some cell cycle genes at early stages of nodule formation. Based on this correlation between *NF-YC1* knock-down and the level of some G2/M genes, we propose that *NF-YC1* might regulate nodule organogenesis by promoting activation of the first cortical cell divisions that lead to the formation of the initial primordium in determinate-type nodules.

Overexpression of *NF-YC1* Improves Nodulation Performance of Less Efficient *R. etli* Strains

To investigate the role of *NF-YC1* on nodulation efficiency with different rhizobial strains, we generated transgenic roots expressing *NF-YC1* fused to the FLAG epitope under the control of the 35S promoter. Increases of 3- to 10-fold in *NF-YC1* transcript levels were detected in 35S:FLAG-*NF-YC1* transgenic roots from four independent composite plants compared with those transformed with the p35S:GFPGUS+ plasmid, which expresses GFP and GUS under the control of the 35S promoter (see Supplemental Figure 6A online). A protein gel blot assay using anti-FLAG antibodies confirmed expression of the FLAG-*NF-YC1* protein, although at different levels, in transgenic roots of individual composite plants (see Supplemental Figure 6B online). Overexpression of this protein did not produce any noticeable phenotypic effect in root growth as determined by the number of hairy roots (10.4 ± 0.9 in 35S:GUSGFP plants versus 10.2 ± 1.2 in 35S:FLAG-*NF-YC1* plants, $P = 0.8947$, unpaired two-tailed t test, $n > 8$) and their lengths (13.9 ± 1.4 cm in 35S:GUSGFP plants versus 14.3 ± 1.8 cm in 35S:FLAG-*NF-YC1* plants, $P = 0.7532$, unpaired two-tailed t test, $n > 15$). Inoculation with strain 55N1 (*nodC- δ*) led to the formation of a significantly higher number of nodules in 35S:FLAG-*NF-YC1* roots than in those transformed with p35S:GFPGUS+ ($P = 0.0023$, unpaired two-tailed t test; Figures 6A and 6B, black bars). To rule out an effect of the FLAG epitope on the nodulation performance, we transformed plants with the empty vector p35S:FLAG. Nodulation of these roots was comparable to that of 35S:GFPGUS composite plants (see Supplemental Figure 7 online). Since different levels of *NF-YC1* protein were observed in individual overexpressing composite plants (see Supplemental Figure 6B online), we examined whether *NF-YC1* protein levels were correlated with nodulation performance by strain 55N1. At 10 DAI with strain 55N1, 35S:FLAG-*NF-YC1* composite plants that accumulated low levels of *NF-YC1* protein (e.g., composite plant #1 in Supplemental Figure 6B online) developed 12.9 ± 3.4 nodules per root, whereas plants that accumulated higher levels of *NF-YC1* protein (e.g., plant #3 in Supplemental Figure 6B online) developed 18.8 ± 3.2 nodules per root, indicating that higher levels of *NF-YC1* protein correlated with a significantly higher nodule formation by strain 55N1. The higher nodulation performance of 35S:FLAG-*NF-YC1* composite plants inoculated with 55N1 was accompanied by an increase in the nitrogen fixation efficiency measured as dry weight of shoots (Figure 6C, black bars). On the other hand, upon inoculation with the efficient strain SC15

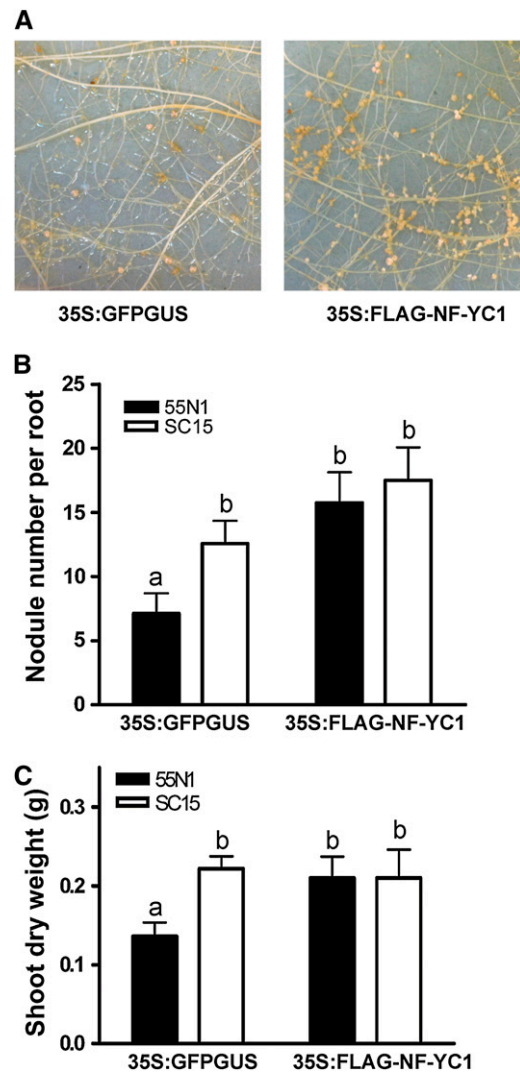


Figure 6. Nodulation Phenotype of *NF-YC1*-Overexpressing Roots.

(A) Representative pictures illustrating the higher nodule density found in 35S:FLAG-*NF-YC1* roots (right panel) compared with 35S:GFPGUS (left panel) at 21 DAI with strain 55N1.

(B) Number of nodules per root developed in 35S:GFPGUS or 35S:FLAG-*NF-YC1* roots at 10 DAI with *R. etli* strain 55N1 (black bars) or SC15 (white bars). Error bars represent SE of nodule per root counted on over 50 independent transgenic hairy roots.

(C) Dry weight of shoot tissue of 35S:GFPGUS or 35S:FLAG-*NF-YC1* composite plants at 21 DAI with *R. etli* strain SC15 or 55N1. Error bars represent SE of at least five independent plants.

Different letters indicate significantly different values in an unpaired two-tailed t test with $P < 0.05$. Results are representative of three independent biological experiments.

(*nodC- α*), the number of nodules developed in the *NF-YC1*-overexpressing roots (35S:FLAG-*NF-YC1*) was not significantly different from that of roots transformed with p35S:GFPGUS+ ($P = 0.1178$, unpaired two-tailed t test; Figure 6B, white bars). Interestingly, the nodulation performance and nitrogen fixation efficiency observed in 35S:FLAG-*NF-YC1* roots inoculated with

55N1 reached values that were comparable to those found upon inoculation with the more efficient strain SC15 ($P > 0.5$, unpaired two-tailed t test; Figures 6B and 6C). Similar results were obtained when transgenic roots were inoculated with a different pair of *R. etli* strains (see Supplemental Figure 8 online): the number of nodules formed in 35S:FLAG-NF-YC1 roots at 10 DAI with the *nodC*- δ strain 136N2 was ~ 2 -fold higher than those formed in roots transformed with the control construct inoculated with the same strain ($P = 0.0453$, unpaired two-tailed t test), whereas no difference was observed for plants inoculated with the *nodC*- α strain CFNx5. All together, these results demonstrate that an increase in NF-YC1 levels is sufficient to improve the symbiotic outcome of less efficient bacteria.

Overexpression of NF-YC1 Is Sufficient to Overcome the Predominant Occupation of Common Bean Nodules by *R. etli* Strain *nodC*- α

Coinoculation experiments in Mesoamerican cultivars had shown that strains carrying the *nodC*- α allele out-competed strains with the *nodC*- δ allele, occupying most of the nodules formed in the roots (Aguilar et al., 2004). Based on this previous observation, we investigated whether an increase of NF-YC1 protein levels affects this preferential selection of rhizobial strains. Control and NF-YC1-overexpressing roots were coinoculated with a mixture of *R. etli* strains SC15 and 55N1. Twenty-one days after coinoculation, nodules were excised from 35S:GFPGUS and 35S:FLAG-NF-YC1 roots, and the genotype of the bacteria isolated from individual nodules was determined by polymorphism analysis of the *nodC* gene (Figure 7A). As shown in Figure 7B, only 6% of the nodules developed in transgenic roots harboring the p35S:GFPGUS+ construct were occupied by strain 55N1. This is in agreement with what was previously observed in wild-type roots (Aguilar et al., 2004). Interestingly, overexpression of NF-YC1 raised this occupancy to 31%, showing that increased levels of NF-YC1 have a major impact in the selection of rhizobium strains (Figure 7B). This result indicates that changes in the expression level of a single gene are sufficient not only to improve nodulation performance with less efficient strains, but also to enhance the rate of nodule occupancy by less competitive strains, and further demonstrates that competence can be modulated by the host.

Expression of G2/M Transition Cell Cycle Genes Is Augmented in Plants Overexpressing NF-YC1

Since RNAi of NF-YC1 affected the induction of G2/M transition cell cycle genes in response to rhizobia, we questioned whether expression of these genes was modified by the overexpression of NF-YC1. In 35S:GFPGUS control roots, transcript levels of *CDC2* and *CDC25* increased ~ 1.8 - and 4-fold, respectively, at 24 HAI with strain SC15 (Figure 8, white bars), in accordance with that described in Figure 5 for *GUS* RNAi roots. Notably, mRNA levels of both genes were lower in roots inoculated with strain 55N1 than in uninoculated roots at this time point. On the other hand, roots overexpressing NF-YC1 exhibited higher levels of both *CDC2* and *CDC25* mRNAs compared with 35S:GFPGUS roots in all tested conditions (Figure 8, black bars). Increase in

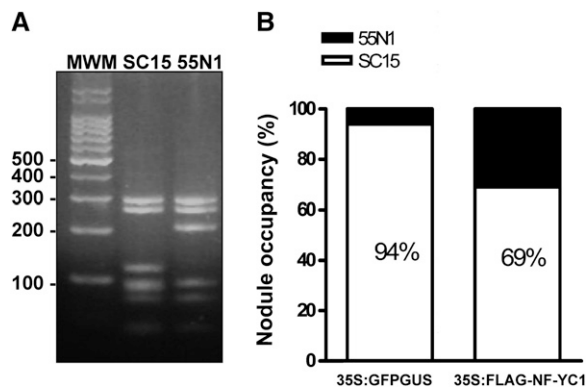


Figure 7. Nodule Occupancy by *R. etli* Strains SC15 and 55N1 in NF-YC1-Overexpressing Roots.

(A) Polymorphic profiles of the *nodC* gene in *R. etli* strains SC15 (*nodC*- α) and 55N1 (*nodC*- δ). DNA was extracted from each *R. etli* strain. A fragment of the *nodC* gene was PCR amplified and digested with *Hinf*I. Restriction fragments were resolved by electrophoresis in an ethidium bromide-stained 2% agarose gel. MWM is a 100-bp ladder molecular weight marker.

(B) Percentage of nodule occupancy by strains SC15 (white) and 55N1 (black) in coinoculation experiments. Nodule occupancy was determined by examination of *nodC* polymorphic profiles of bacteria isolated from individual nodules ($n = 120$) excised from control 35S:GFPGUS and 35S:FLAG-NF-YC1 roots at 21 d after coinoculation with strains SC15 and 55N1.

transcript levels in response to strain SC15 was also verified in overexpressing roots, most likely due to the induction of the endogenous NF-YC1 gene in response to this strain. These results show that constitutive expression of NF-YC1 is correlated with the higher expression of these G2/M transition cell cycle genes. However, constitutive expression of these cell cycle genes is not sufficient for nodule formation, since spontaneous nodule or nodule primordia were not observed in uninoculated NF-YC1-overexpressing roots. This indicates that additional host components activated by *R. etli* are needed to initiate nodule formation.

DISCUSSION

The Role of NF-YC1 in Nodule Organogenesis and Bacterial Infection

In the past decade, several genes encoding transcriptional regulators required for the development of symbiotic nodules have been genetically identified through the screening of legume mutants that are defective in nodulation. Nodule inception (NIN) is a transcriptional regulator required for bacterial infection and development of both determinate and indeterminate types of nodules (Schauser et al., 1999; Marsh et al., 2007). Two GRAS domain transcriptional regulators, Nodulation Signaling Pathway 1 (NSP1) and NSP2, were also identified (Kaló et al., 2005; Smit et al., 2005). Both genes are required for nodulation, since *nsp1* and *nsp2* single mutants failed to form nodules. Afterward, ERN1

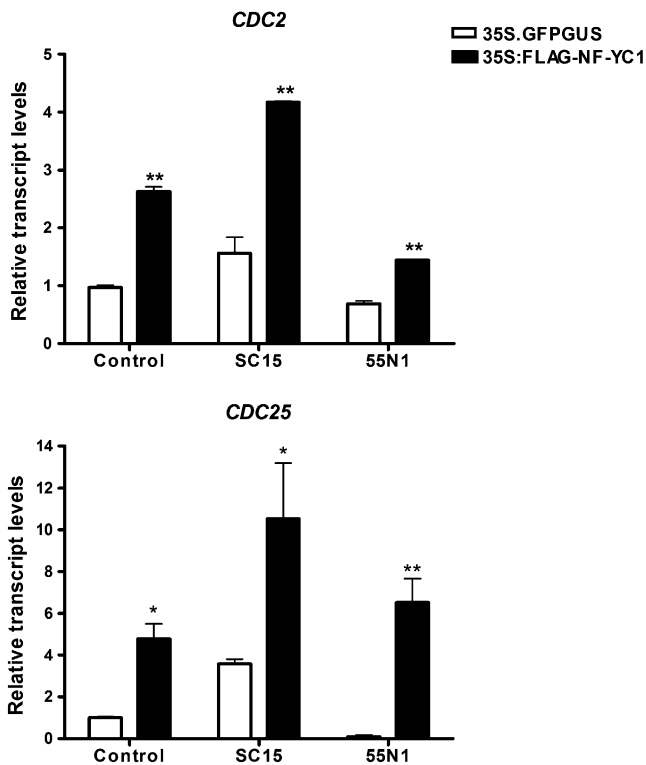


Figure 8. Expression Analysis of Cell Cycle Genes in 35S:FLAG-NF-YC1 Roots.

qRT-PCR analysis of *CDC2* and *CDC25* in 35S:GFPUGS (white bars) or 35S:FLAG-NF-YC1 (black bars) roots at 24 HAI with YEM (control) or with either *R. etli* strain SC15 or 55N1. Transcripts levels were normalized to EF1 α and presented relative to the values of control 35S:GFPUGS roots. Error bars represent SD of three technical replicates. Single and double asterisks indicate that expression values in 35S:FLAG-NF-YC1 roots are significantly different from those in 35S:GFPUGS roots in an unpaired two-tailed *t* test with $0.01 < P < 0.05$ and $0.001 < P < 0.01$, respectively.

was identified as a transcription factor required for nodulation that mediates Nod factor-dependent transcriptional activation of *ENOD11* (Andriankaja et al., 2007; Middleton et al., 2007). More recently, it has been reported that NSP1 and NSP2 proteins associate in vitro and in vivo with the promoter of the early nodulin gene *ENOD11* (Hirsch et al., 2009), leading to the suggestion that NSP1, NSP2, and ERN1 proteins could act together to promote transcriptional activation of *ENOD11*. The list of transcriptional regulators required for nodulation also includes the previously mentioned *HAP2-1*, which encodes an A subunit of an NF-Y heterotrimeric transcription factor (Combiér et al., 2006). Although no complete functional NF-Y A/B/C complex has been reported yet in plants, NF-Y subunits are emerging as transcriptional regulators with essential roles in diverse plant processes. In our study, a C subunit of NF-Y was characterized through RNAi and overexpression studies. *NF-YC1* was not only required for nodule organogenesis and rhizobia infection, but also influences partner selection in common bean-*R. etli* symbiosis. NF-YCs constitute a gene family in plants (Yang et al., 2005; Stephenson et al., 2007; Siefers et al., 2009), and common

bean is not an exception, with three members identified based on the current EST data set. Both *NF-YC1* and *NF-YC2* were upregulated by rhizobium infection, but only *NF-YC1* was found to be specifically upregulated by highly efficient *R. etli* strains at 24 HAI (Figure 1). In addition, our analyses indicate that not only common bean *NF-YC1* exhibits a transcriptional response to rhizobia, but also its best homologs/putative orthologs from *M. truncatula* and *L. japonicus* (see Supplemental Figures 3A and 3B online), suggesting that this particular member of the NF-YC family might have a specialized function in legume symbiosis. According to expression data from *L. japonicus*, Nod factor receptors and *NIN* genes are required for upregulation of this NF-Y subunit in response to rhizobia (see Supplemental Figure 3C online), suggesting that this nuclear factor might act downstream of *NIN* to promote the formation of nitrogen-fixing nodules.

Coordinated infection and nodule organogenesis are required for the development of functional nitrogen-fixing nodules (Oldroyd and Downie, 2008; Oldroyd et al., 2009). Here, we present evidence that silencing of *NF-YC1* affects both nodule organogenesis and IT formation/progression (Figures 2B and 4D to 4H). This dual function of transcription factors in symbiosis has been reported also in two recent studies in which *L. japonicus* mutants that nodulate spontaneously were used to uncouple organogenesis from infection (Hayashi et al., 2010; Madsen et al., 2010). These studies revealed that *NIN*, *NSP1*, and *NSP2* play key roles in both infection and cortical cell divisions, since plants mutated in any of these genes in a spontaneous nodulation background failed to form nodules and ITs. In the case of *NF-YC1* RNAi roots, we cannot exclude the possibility that nodule primordium organogenesis is primarily affected and that infection is blocked as a consequence of the lack of initial cell divisions within the cortex. The requirement of initial cortical cell divisions for progression of ITs was previously suggested for the development of indeterminate nodules of *M. truncatula* (Gonzalez-Rizzo et al., 2006). In addition, the sequence of cytoskeleton rearrangements after rhizobia inoculation revealed that initial cortical cell division precedes IT formation (Timmers et al., 1999) and is accompanied by the expression of cell cycle genes (Savouré et al., 1994; Yang et al., 1994). This induction of cell cycle genes is transient in determinate nodules, supporting the notion that only initial cell divisions are required to form the nodule primordium and then it grows by increasing the cell size rather than the cell number (Kouchi et al., 2004). Common bean roots that express the *NF-YC1* RNAi construct failed to induce the expression of *CYCB* and *CDC25* significantly and showed reduced induction of *CDC2* at early stages of the interaction (Figure 5). In addition, overexpression of *NF-YC1* was correlated with constitutive augmented levels of *CDC2* and *CDC25* mRNAs (Figure 8). Based on these correlations, we hypothesize that *NF-YC1* might function in promoting the initial cortical cell divisions that lead to the primordium formation in determinate nodules and that infection could be blocked as a consequence of the lack of cortical cell divisions. This hypothesis is in agreement with the previously proposed function of another subunit of NF-Y in *M. truncatula* indeterminate nodules, *HAP2-1*, which is required for meristem persistence during nodule development (Combiér et al., 2006, 2008). Although ITs were formed in *HAP2-1*-RNAi roots, bacteria were not released from ITs in mature nodules. Whether the

defects in IT progression or bacterial release observed in *NF-YC1* knockdown roots are the consequence of defects in cell division remains to be established and are worth investigating.

NF-YC1 RNAi roots showed a significant reduction in the total number of infection events as well as in the percentage of ITs that reached the root cortex. However, root hairs retained the capacity to form the so-called Shepherd's crook structures, which entrap the bacterial microcolony within the root hair (Figure 4B). Moreover, root hairs of *NF-YC1* RNAi roots exhibited excessive deformation in response to rhizobia, a phenotype that has been observed also in several infection-defective legume mutants (Schauser et al., 1999; Endre et al., 2002; Stracke et al., 2002; Esseling et al., 2004; Marsh et al., 2007). This abnormal deformation is usually interpreted as evidence of a mechanism that negatively regulates epidermal cell competence in wild-type plants, in which *NF-YC1* might be involved. Previous studies in common bean identified a member of the Rab family of small GTPases, *RabA2a*, which is specifically upregulated by *R. etli* strain SC15 and required for preinfection stages during symbiosis (Peltzer Meschini et al., 2008; Blanco et al., 2009). Roots with reduced levels of *RabA2a* are impaired in root hair deformation and failed to induce early nodulin genes in response to *R. etli*. Interestingly, induction of *NF-YC1* was also abolished in *RabA2a* silenced roots, indicating that upregulation of *NF-YC1* is mediated by a signaling pathway that requires *RabA2a* (Blanco et al., 2009). The phenotype observed in *NF-YC1* RNAi roots, in which infection is affected but root hairs retain the ability to deform in response to rhizobia also is consistent with the idea that *NF-YC1* might act downstream of *RabA2a* to promote IT formation and growth.

The Role of *NF-YC1* in Symbiotic Partner Selection

Development of a functional fixing nodule is a highly complex and coordinated process that depends on a continuous molecular exchange between the plant and bacteria. Only rhizobia producing the correctly decorated Nod factor can induce epidermal and cortical responses on a particular host. In addition to the Nod factor, surface polysaccharides (both exopolysaccharides and lipopolysaccharides) also play an important role in IT expansion (Downie, 2010). Bacterial release into cortical cells also can be blocked if the right signals are not present. These layers of sequential recognition are necessary to avoid not only penetration by pathogenic bacteria but also to discriminate good nitrogen-fixing strains from others that will use plant resources without reciprocating (i.e., providing nitrogen in a reduced form to the host) (Perret et al., 2000). In natural communities, plants that can discriminate between strains of bacteria, selecting and rewarding partners that provide a better benefit for the host, will be favored by selection. Discrimination among different partners can be established early during chemical communication in the rhizosphere, through honest signals that indicate partner quality (Simms and Taylor, 2002).

Our analysis of plants overexpressing *NF-YC1* indicates that this transcriptional regulator is involved in the more efficient nodulation by microsymbionts that have coevolved in the same geographical region (Figure 6; see Supplemental Figure 8 online). Transcript levels of *NF-YC1* increased at early time points (e.g., 24 and 48 h) after inoculation with strain SC15 and also remained

high in nodules of 7 DAI with this strain, but not with 55N1 (Figure 1; Peltzer Meschini et al., 2008). This suggested that only strains from the same geographical region produced a sustained induction of *NF-YC1* transcript levels, which is correlated with the higher nodulation performance observed with these strains (Table 1).

Based on the findings of this study and the current knowledge on legume symbiosis, we propose a model that provides a link between an early molecular dialogue and the capacity of the plant to select high quality bacteria. In this model, the specific recognition between Mesoamerican common beans and their cognate *R. etli* strains, through yet unknown molecules, induces an increase of *NF-YC1* over the basal level, which rapidly activates, directly or indirectly, the transcription of cell cycle genes to initiate nodule primordium development. Initial cortical cell divisions precede and are required for IT progression, allowing bacteria to penetrate the root and reach the developing nodules. By contrast, *R. etli* strains from a different bean genetic diversification center failed to produce a significant and sustained upregulation of *NF-YC1* and downstream gene activation; therefore, both the infection process and nodule organogenesis proceed normally but are delayed compared with cognate strains, resulting in a competitive disadvantage. Strains that can initiate the infection process and nodulation faster will out-compete slower ones, since initial nodules suppress further nodule organogenesis, probably via a systemic autoregulation of nodulation. The identification of the molecules that act upstream of *NF-YC1* and are directly involved in the specific recognition between Mesoamerican common bean and *R. etli* strains from the same geographic region is an important aim to better define and support the proposed model.

Optimizing functional compatibility between legumes and rhizobia is necessary to improve crop productivity, since nitrogen fixation efficiency in a single legume species can vary by >10-fold depending of the quality of the symbiotic partners (Den Herder and Parniske, 2009). This can reflect the fact that crops are introduced to ecosystems where they do not share a common evolution with indigenous soil populations, usually well adapted to their environment. As a consequence, native rhizobia can out-compete and persist longer than highly efficient strains introduced as inoculants. Efficiency of bacteria present in the soil can vary widely, and cheating (using plant resources without providing fixed nitrogen in return) can occur. Persistence of mutualism depends on mechanisms that constrain cheating, preventing a shift toward parasitism. One of these possible mechanisms, partner choice, requires different partners to choose from (a market scenario) and the ability to discriminate among these different strains, with the condition that this selection provides more benefit than the cost (Heath and Tiffin, 2009; Marco et al., 2009). Another proposed mechanism, referred as host sanctioning, is related to the capacity of the plant to allocate resources (oxygen and carbohydrates) differentially according to the nitrogen fixation efficiency in the nodules formed by different strains (Kiers et al., 2003; Heath and Tiffin, 2009; Marco et al., 2009).

We believe that the model presented features an interesting link between coevolution of the symbiotic partners and a molecular mechanism that explains the plant-driven preference for more efficient bacteria. Further research in this area would help in

better understanding the molecular basis of this functional compatibility in symbiotic interactions. This is the key to manipulating the genetic regulation of the host to select more efficient strains that are also good competitors in the soil, to the detriment of less efficient bacteria.

METHODS

Biological Material

Seeds of *Phaseolus vulgaris* cultivar NAG12 (Mesoamerican) were provided by Susana García Medina (Instituto Nacional de Tecnología Agropecuaria, Salta, Cerrillos, Argentina). *Rhizobium etli* strains SC15, CFNx5, 136N3, 138N1, 55N1, 1373, 136N2, and 124N1 were previously isolated and characterized (Aguilar et al., 2004). The GFP-expressing strain used for visualization of ITs was previously described (Blanco et al., 2009). *Agrobacterium rhizogenes* strain K599 (Bond and Gresshoff, 1993) and the same strain carrying the p35S:GFPGUS+ construct were a kind gift from Federico Sánchez (Universidad Nacional Autónoma de México, Cuernavaca, Mexico).

Sequence Analysis

Arabidopsis thaliana sequence was retrieved from the GenBank nonredundant database in a BLASTP (Altschul et al., 1997) search using the *P. vulgaris* NF-YC1 amino acid sequence as the query. *Medicago truncatula*, *Glycine max*, and *Lotus japonicus* sequences were retrieved from the genomic database for each species, <http://www.medicago.org/genome/>, <http://www.phytozome.net/soybean> (Schmutz et al., 2010), and <http://www.kazusa.or.jp/lotus/> (Sato et al., 2008), respectively, using the *P. vulgaris* NF-YC1 amino acid sequence and the TBLASTN program (Altschul et al., 1997). Nucleotide sequences of common bean NF-YC2 and NF-YC3 were retrieved from the DFCI bean gene index database (<http://compbio.dfci.harvard.edu/tgi/plant.html>) by a TBLASTN search (Altschul et al., 1997) using NF-YC1 amino acid sequence as the query. Alignments were generated with ClustalW (Thompson et al., 1994) and shaded with BOXSHADE 3.21 software (K. Hofmann and M.D. Baron, pretty printing and shading of multiple alignment files, 1996; http://www.ch.embnet.org/software/BOX_form.html).

ESTs with high similarity to *M. truncatula* RIP, ERN, ENOD40, cdc2, and Cyclin B (*cyc2*) and to *Arabidopsis* cdc25 were identified in a BLASTN search (Altschul et al., 1997) of the DFCI bean gene index database and used to design the gene-specific primers for qRT-PCR experiments listed in Supplemental Table 1 online.

Vector Construction for Reverse Genetic and Subcellular Localization

The NF-YC1 RNAi construct was generated by PCR amplification using primers NF-YC RNAi F and NF-YC RNAi R (see Supplemental Table 1 online). The PCR product was introduced into the pENTR/D-TOPO vector (Invitrogen) and recombined into the destination vector pK7GWIWG2D (II), which carries EgfpER as a screenable marker for early visualization and selection of the transgenic roots (Karimi et al., 2007). For overexpression of NF-YC1, the open reading frame was amplified by PCR using NF-YC OE F and NF-YC OE R (see Supplemental Table 1 online), cloned into the pENTR/D-TOPO vector, creating pENTR-NF-YC1, and recombined into the destination vector p35S:HF-GATA (Mustroph et al., 2010). For subcellular localization, a translational fusion of NF-YC1 to the C-terminal end of GFP was generated by recombination of the pENTR-NF-YC1 with pMDC43 (Curtis and Grossniklaus, 2003). All constructs were verified by sequencing and restriction digestion. pK7GWIWG2D (II) was obtained from the Department of Plant System

Biology, University of Ghent, Belgium, and pMDC43 was obtained from the ABRC. All binary vectors were introduced into *A. rhizogenes* strain K599 by electroporation.

Common Bean Growth, Root Transformation, and Inoculation with Rhizobia

Wild-type common bean seeds were surface sterilized and germinated for 2 d as described by Peltzer Meschini et al. (2008). Sprouts were then transferred to acrylic boxes (17 × 22 × 4.5 cm) containing slanted agar-Fahraeus media (Fahraeus, 1957). Plants were grown in a MLR-350HT growth chamber (Sanyo Electric) at 26°C in a day/night cycle of 14 h/10 h and 80% of humidity. Transformation of common bean roots was performed as previously described (Blanco et al., 2009). Composite plants, consisting of wild-type shoots and transgenic roots, were transferred to acrylic boxes containing slanted agar-Fahraeus media. Five days after transplantation to acrylic boxes, wild-type or transgenic roots were inoculated with *R. etli* strains as described (Peltzer Meschini et al., 2008). In coinoculation experiments, composite plants transformed with the p35S:GFPGUS+ or p35S:FLAG-NF-YC1 constructs were transferred to pots containing vermiculite, and 5 d after transplantation, roots were inoculated with 10 mL of a mixture of *R. etli* strains SC15 and 55N1 (ratio 1:1) as previously described (Aguilar et al., 2004).

Nodule Quantification and Dry Weight Determination

For wild-type plants, the number of nodules per plant was recorded at 7 DAI with each *R. etli* strain using at least eight plants per strain. In the case of composite plants, the number of nodules per root was recorded every 2 to 3 d, starting at 4 DAI, on over eight individual composite plants containing 7 to 10 independent transgenic roots. Twenty-one DAI with *R. etli*, the aerial part of individual composite plants was collected, dried in an oven at 80°C for ~72 h, and weighed to determine dry weight. Statistical significance was determined by unpaired two-tailed *t* test comparing mean ± SE of nodules per root for each construct and time point.

Bacterial DNA Recovery from Nodules and *nodC* Polymorphism Analysis

Four weeks after coinoculation, 120 nodules from five independent plants for each construct were excised, crunched, and individually plated on yeast extract mannitol (YEM) agar media supplemented with congo-red. After incubation for 3 d at 28°C, bacterial DNA was extracted, and the *nodC* polymorphic profile was examined in DNA samples of *R. etli* isolated from individual nodules by amplification of the *R. etli nodC* genes and restriction digestion with *Hinf* I endonuclease as previously reported (Aguilar et al., 2004).

RNA Extraction and qRT-PCR

Root tissue from individual wild-type or composite plants containing 8 to 10 independent transgenic roots was harvested. Total RNA extraction, first-strand cDNA synthesis, and qPCR reactions were performed as previously described (Peltzer Meschini et al., 2008). For each primer pair, the presence of a unique product of the expected size was checked on ethidium bromide-stained agarose gels after PCR reactions. Amplification of common bean elongation factor 1 α (EF1 α) was used to normalize the amount of template cDNA as previously described (Peltzer Meschini et al., 2008). Statistical significance between samples was determined by unpaired two-tailed *t* test comparing mean ± SD of at least three technical replicates for each data point. Primers qNF-YC₇₈₄₋₈₀₅ F and qNF-YC₉₅₁₋₉₃₁ R were used to evaluate abundance of NF-YC1 transcripts in wild-type, NF-YC1 RNAi, and GUS RNAi roots, whereas primers qNF-YC₅₆₂₋₅₈₁ F and qNF-YC₇₅₇₋₇₃₄ R were used to quantify NF-YC1 mRNAs in 35S:

FLAG-NF-YC1 and 35S:GFPUS roots. All primers used for qPCR are listed in Supplemental Table 1 online.

Protein Gel Blots

Proteins from transgenic root tissue of individual composite plants containing 8 to 10 independent transgenic roots were extracted, separated into 15% SDS-PAGE, and subjected to immunoblot analysis using anti-FLAG horseradish peroxidase-conjugated monoclonal antibody (1:500; Sigma-Aldrich) as previously described (Zanetti et al., 2005).

Microscopy and Imaging

Bright-field and epifluorescence imaging of root hair deformation and IT formation by the GFP-labeled *R. etli* strain were performed with Nikon Eclipse Ti inverted microscope (Nikon Instruments) using white light and UV light with appropriated GFP filters. Images were captured using a Nikon digital sight high-resolution DS-Ri1 camera (Nikon Instruments) and NIS-Elements Imaging software F3.0 (Nikon Instruments).

For optical and electron microscopy, individual nodules were excised from *GUS* RNAi and *NF-YC1* RNAi hairy roots at 21 DAI after infection with *R. etli* strain SC15 and fixed in 50 mM potassium phosphate buffer, pH 7.4, containing 2% paraformaldehyde during 2 h at 4°C. During fixation, samples were subjected to short pulses of gentle vacuum until they sank. Nodules were post fixed in 50 mM potassium phosphate buffer, pH 7.4, containing 1% osmium tetroxide for 1 h at 4°C, rinsed three times in the same buffer, dehydrated by passing through a series of graded ethanol, and embedded in epoxy resin. Ultrathin sections (70 nm) were obtained with a microtome, stained with uranyl acetate and lead citrate, and observed in a JEM 1200 EX II (JEOL USA) transmission electron microscope. Semithin (1 to 2 μm) sections of the same samples were stained with 0.04% toluidine blue and observed under white light in a Nikon Eclipse Ti inverted microscope (Nikon Instruments).

Confocal microscopy was performed on several roots transformed with the pMDC43-NF-YC1 construct or with the empty vector expressing free GFP using an Olympus FV300 (Olympus Optical) following the previously described settings (Blanco et al., 2009). Images were captured with FluView 3.3 (Olympus Optical) and processed with ImageJ 1.42q (W.S. Rasband, ImageJ, National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>).

Accession Numbers

Sequence data from this article can be found at the GenBank/EMBL or DFCI gene index data libraries under the following accession numbers: *NF-YC1* (GQ913690), *NF-YC2* (TC9603 composed by ESTs CV532047, CV531056, CV544140, FE698328, and FE707163), *NF-YC3* (TC10494 composed of ESTs FE681706 and FE681701), *ENOD40* (CV536158), *ERN* (CV535404), *RIP* (CV537859), *CDC2* (EX305547), *CDC25* (CV536989), and *CYCB* (CV542569). Sequences used for the alignment are as follows: *M. truncatula* (Medtr7g139450.1 or DFCI Medicago Gene Index database TC107348), *Arabidopsis NF-YC9* (At1g08970), *G. max* (Glyma19g42460.1), and *L. japonicus* (chr1.CM0544.480.nd).

Author Contributions

M.E.Z., F.A.B., and O.M.A. designed the research. M.E.Z., F.A.B., M.B., and M.P.B. performed the research. M.E.Z. and F.A.B. analyzed the data and wrote the manuscript.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of Common Bean NF-YC1 Amino Acid Sequence with Those from Other Plant Species.

Supplemental Figure 2. Alignment of Common Bean NF-YC Gene Family Members.

Supplemental Figure 3. Expression Analysis of *NF-YC1* Homologs in *M. truncatula* and *L. japonicus*.

Supplemental Figure 4. Subcellular Localization of NF-YC1.

Supplemental Figure 5. Expression Analyses of Early Nodulins in Common Bean Roots Inoculated with Strains SC15 and 55N1 of *R. etli*.

Supplemental Figure 6. Analysis of *NF-YC1* Transcript and Protein Levels in 35S:FLAG-NF-YC1 and 35S:GFPUS Roots.

Supplemental Figure 7. Nodulation Performance of 35S:FLAG and 35S:FLAG-NF-YC1 Roots.

Supplemental Figure 8. Time Course of Nodule Formation in 35S:FLAG-NF-YC1 and 35S:GFPUS Roots after Inoculation with *R. etli* Strain CFNx5 or 136N2.

Supplemental Table 1. List of Primers Used in This Study for Cloning and PCR.

Supplemental References.

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A C Subunit of the Plant Nuclear Factor NF-Y Required for Rhizobial Infection and Nodule Development Affects Partner Selection in the Common Bean–*Rhizobium etli* Symbiosis

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