A Nuclear Factor Y Interacting Protein of the GRAS Family Is Required for Nodule Organogenesis, Infection Thread Progression, and Lateral Root Growth[1][C][W][OPEN]

Marina Battaglia[2,3], Carolina Rípodas[2], Joaquín Clúa, Maël Baudin, O. Mario Aguilar, Andreas Niebel, María Eugenia Zanetti, and Flavio Antonio Blanco*

Instituto de Biotecnología y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Centro Científico y Tecnológico-La Plata, Consejo Nacional de Investigaciones Científicas y Técnicas, 1900 La Plata, Argentina (M.Bat., C.R., J.C, O.M.A., M.E.Z., F.A.B.); Institut National de la Recherche Agronomique, Laboratoire des Interactions Plantes-Microorganismes, Unité Mixte de Recherche 441, F–31326 Castanet-Tolosan, France (M.Bau., A.N.); and Centre National de la Recherche Scientifique, Laboratoire des Interactions Plantes-Microorganismes, Unité Mixte de Recherche 2594, F–31326 Castanet-Tolosan, France (M.Bau., A.N.)

A C subunit of the heterotrimeric nuclear factor Y (NF-YC1) was shown to play a key role in nodule organogenesis and bacterial infection during the nitrogen fixing symbiosis established between common bean (*Phaseolus vulgaris*) and *Rhizobium etli*. To identify other proteins involved in this process, we used the yeast (*Saccharomyces cerevisiae*) two-hybrid system to screen for NF-YC1-interacting proteins. One of the positive clones encodes a member of the Phytochrome A Signal Transduction1 subfamily of GRAS (for Gibberellic Acid-Insensitive (GAI), Repressor of GAI, and Scarecrow) transcription factors. The protein, named Scarecrow-like13 Involved in Nodulation (SIN1), localizes both to the nucleus and the cytoplasm, but in transgenic *Nicotiana benthamiana* cells, bimolecular fluorescence complementation suggested that the interaction with NF-YC1 takes place predominantly in the nucleus. SIN1 is expressed in aerial and root tissues, with higher levels in roots and nodules. Posttranscriptional gene silencing of *SIN1* using RNA interference (RNAi) showed that the product of this gene is involved in lateral root elongation. However, root cell organization, density of lateral roots, and the length of root hairs were not affected by *SIN1* RNAi. In addition, the expression of the RNAi of *SIN1* led to a marked reduction in the number and size of nodules formed upon inoculation with *R. etli* and affected the progression of infection threads toward the nodule primordia. Expression of *NF-YA1* and the G2/M transition cell cycle genes *CYCLIN B* and *Cell Division Cycle2* was reduced in *SIN1* RNAi roots. These data suggest that SIN1 plays a role in lateral root elongation and the establishment of root symbiosis in common bean.

One of the main entry points of molecular nitrogen into ecosystems is the mutualistic symbiosis between legume plants and rhizobia, which results in the formation of a new root organ, the nodule. This interaction involves two separated but coordinated processes, the infection that leads bacteria to the interior of the root and the organogenesis of the nodule, where bacteria reduce atmospheric nitrogen to metabolic intermediates that the plant can incorporate (Oldroyd and Downie, 2008). Entry of rhizobia occurs mainly through an invagination of the root hair plasma membrane that expands toward the interior of the root, forming a cylindrical channel where bacteria allocate and proliferate. This penetration structure formed by the plant is called infection thread (IT). At the same time that infection begins, a yet unknown signal triggers the reactivation of cell divisions in the cortex, leading to the development of the nodule primordia. When the IT reaches the dividing cortex, bacteria are released into the cytoplasm in a process similar to endocytosis, producing a new organelle-like structure, the symbiosome, where the enzymatic nitrogen fixation takes place.

Establishment of symbiosis is highly specific, involving several recognition steps between both partners. In response to iso/flavonoids exuded by legume roots, rhizobia synthesize the Nod Factor, a lipochitooligosaccharide molecule that acts as a signal in root cells. Perception of

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[2] These authors contributed equally to the article.

[3] Present address: Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, 62250 Cuernavaca, Morelos, Mexico.

* Address correspondence to fablanco@biol.unlp.edu.ar.

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Nod Factor requires two extracellular lysin motif receptor-like kinases and activates a signaling pathway that involves a series of downstream acting proteins, including a Leu-rich repeat receptor-like kinase, cation channels, a calcium/calmodulin dependent protein kinase (DMI3), nucleoporins, and Interacting Protein of DMI3, a protein of unknown function (Oldroyd et al., 2011).

In the past 10 years, a number of transcription factors that act downstream of this signaling pathway and are required for nodulation has been identified. Two of them, named Nodulation Signaling Pathway1 (NSP1) and NSP2, belong to the GRAS family, whose name derives from the three members initially identified: Gibberellic Acid-Insensitive (GAI), Repressor of GAI, and Scarecrow (SCR; Pysh et al., 1999). GRAS transcription factors are part of a large gene family of plant-specific proteins with at least 33 and 60 members in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), respectively (Pysh et al., 1999; Tian et al., 2004; Lee et al., 2008). Members of this family play critical and diverse functions in signaling and transcription associated with plant developmental processes, such as root radial patterning and growth, development of adventitious roots, hypocotyl elongation, axillary shoot formation, and maintenance of shoot meristem. They also act as integrators of regulatory and environmental signals, such as abiotic and biotic stresses and the phytohormones gibberellic acid, brassinosteroids, jasmonic acid, and auxins (Bolle, 2004; Sun et al., 2012). In the legume symbiosis, NSP1 and NSP2 are essential for nodulation, mediating different nuclear factor-induced responses, such as root hair deformation, IT formation, cortical cell divisions, and expression of nodulin genes (Catoira et al., 2000; Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005). Both proteins are also involved in mycorrhization (Liu et al., 2011), together with Required for Arbuscular Mycorrhization1 (RAM1), which plays a specific role in the formation of arbuscular mycorrhiza through its interaction with NSP2 but is dispensable for nodule formation (Gobbato et al., 2012).

Nuclear factors Y (NF-Ys) are heterotrimeric transcription factors composed by the NF-YA, NF-YB, and NF-YC subunits. This complex recognizes and binds with high affinity and sequence specificity to the CCAAT box, one of the most frequent eukaryotic promoter elements (Dolfini et al., 2012). Whereas each subunit of NF-Y is encoded by one or two members in yeasts (*Saccharomyces cerevisiae*) and mammals, these gene families have largely expanded in plants. Approximately 10 members were identified for each subunit in the genomes of both mono- and dicotyledonous plants (Petroni et al., 2012; Laloum et al., 2013). Individual NF-Y subunits have been implicated in a number of plant developmental processes, including embryogenesis, seed production, photoperiod-dependent flowering, pollen tube development, and root elongation, and in the transcriptional response to drought and endoplasmic reticulum stresses. NF-Y complexes or individual subunits have been shown to associate with other transcriptional regulators. For example, the Arabidopsis NF-YA4/NF-YB3/NF-YC2 trimeric complex associates with the basic leucine zipper protein bZIP28 to promote transcriptional up-regulation of endoplasmic reticulum stress-responsive genes (Liu and Howell, 2010). Likewise, bZIP67 interacts with the NF-YC2/LEAFY COTYLEDON 1 (NF-YB9) heterodimer to activate abscisic acid-responsive elements of seed-specific genes (Yamamoto et al., 2009). In rice, NF-YB1 interacts with the MADS box protein MADS18 (Masiero et al., 2002). The heterotrimeric nature of NF-Y complexes, the association with other classes of transcription factors, and the expansion of the gene family of each subunit in plants result in a flexible combinatorial system that provides the versatility required to integrate endogenous and exogenous signals, allowing plants to growth and adapt to changing environmental conditions.

In legumes, members of the *NF-YA* and *NF-YC* gene families have been implicated in the development of indeterminate and determinate nodules, respectively. The A subunit of the NF-Y trimeric complex of *Medicago truncatula* (NF-YA1, formerly known as *Heme-Activated Protein2-1* [*MtHAP2-1*]) plays a central role during early symbiotic signaling and rhizobial infection, but it is also required for nodule meristem function and maintenance (Combier et al., 2006, 2008). In *Lotus japonicus*, knockdown of *LjNF-YA1* inhibited root nodule organogenesis but not the infection process (Soyano et al., 2013). Reverse genetic studies carried out in common bean (*Phaseolus vulgaris*) revealed that the NF-YC1 subunit is required for nodule organogenesis and rhizobial infection, as well as the activation of cell cycle genes at early stages of the symbiotic interaction. On the other hand, NF-YC1 plays a key role in the selection of rhizobial strains that are more efficient in the interaction between common bean and *Rhizobium etli* (Zanetti et al., 2010).

In this work, we aimed to identify new components of the signaling pathway in which NF-YC1 participates. We screened a yeast two-hybrid complementary DNA (cDNA) library, searching for interacting partners of NF-YC1. This study focuses on one of these partners, which encodes a transcription factor of the GRAS family that is required for nodule organogenesis, progression of ITs, and growth of lateral roots at postemergence stages.

RESULTS

Identification of a GRAS Transcription Factor from Common Bean

In a search for proteins that potentially interact with NF-YC1, we screened a common bean cDNA library using the yeast two-hybrid system. Forty-five positive clones corresponding to eight nonredundant cDNAs were obtained. Two of the cDNA clones showed high-sequence similarity to a GRAS protein according to a BLASTX search against the nonredundant GenBank database. A BLASTN search against the common bean genome v1.0 (http://www.phytozome.org/commonbean.php) showed that the gene with the highest sequence

identity was located on chromosome 3 (gene identification Phvul.003G085000). Analysis of the gene model revealed the presence of a single intron of 504 bp in the 5′ untranslated region, which is 716 bp in length in the predicted mature transcript, a coding sequence of 1,650 bp, and a 3′ untranslated region of 826 bp (Supplemental Fig. S1A). The predicted protein contains 549 amino acids, with a molecular mass of 61.2 kD and an isoelectric point of 10.8. The only conserved domain is the GRAS in the C terminus, where characteristic motifs and amino acids are highly conserved (Supplemental Fig. S1B).

The Arabidopsis GRAS family has been divided into several branches based on sequence homology (Bolle, 2004; Sun et al., 2012). The phylogenetic analysis of Arabidopsis and common bean sequences presented in Figure 1A shows that the protein encoded by Phvul.003G085000 belongs to the Phytochrome A Signal Transduction1 (PAT1) branch of GRAS proteins, displaying highest sequence similarity with Scarecrow-like 13 (SCL13) from Arabidopsis. SCL13 was shown to play a role in light responses mediated by phytochrome A and B but is also expressed at high levels in Arabidopsis roots (Torres-Galea et al., 2006). The PAT1 family is represented by six members in Arabidopsis and 11 members in common bean (Fig. 1A). A phylogenetic analysis of the SCL13 branch, including the best homologs of AtSCL13 of *M. truncatula* and *L. japonicus*, is shown in Figure 1B. NSP1 and NSP2, two GRAS proteins identified in *M. truncatula* as required for nodulation (Kaló et al., 2005; Smit et al., 2005), as well as their best homologs/putative orthologs in common bean (Phvul.001G129400 and Phvul.009G122770), belong to the SHORT-ROOT (SHR) and HAIRY MERISTEM (HAM) families, respectively (Fig. 1A), revealing that

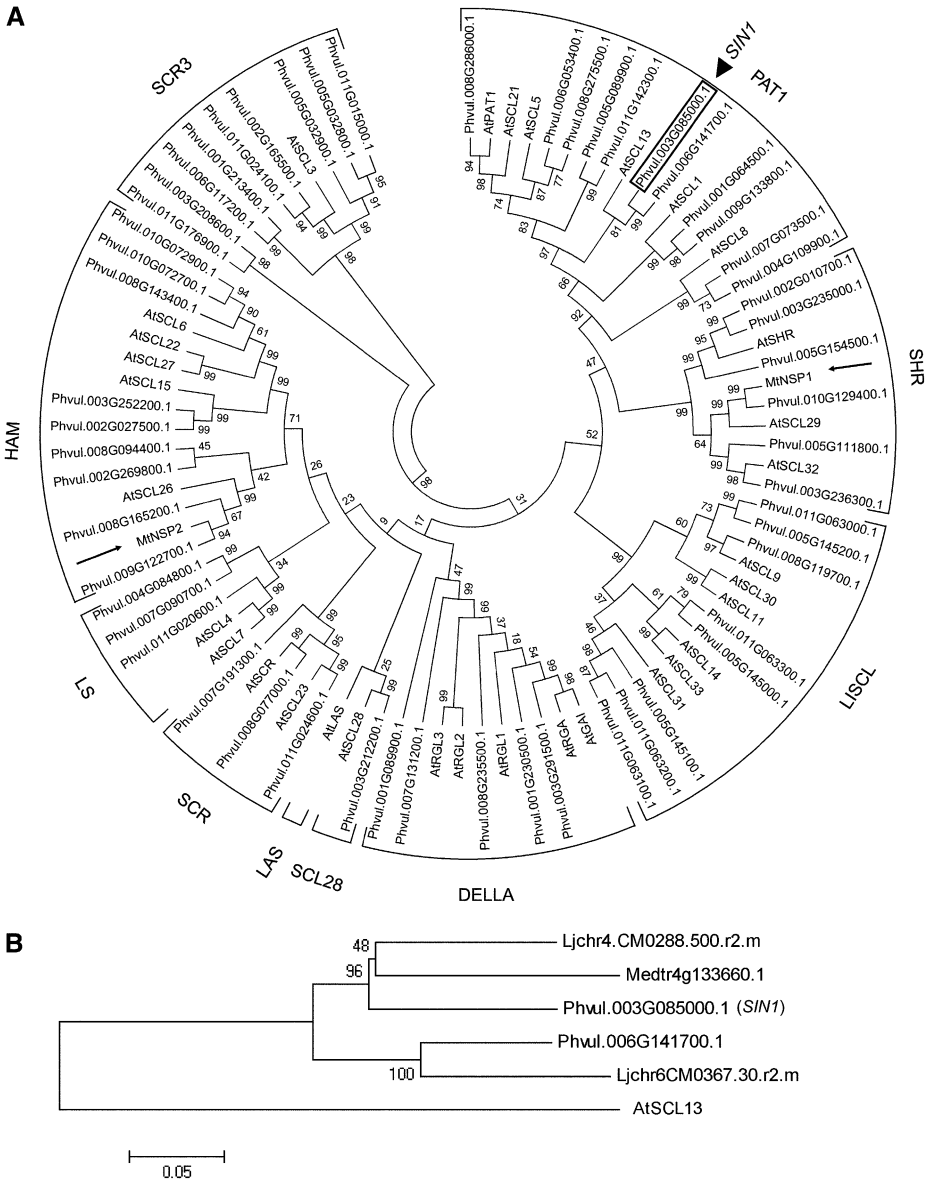

Figure 1. *SIN1* encodes a GRAS transcription factor of the PAT1 subfamily. A, Phylogenetic analysis of the GRAS family of common bean and Arabidopsis based on the classification in subfamilies performed by Bolle (2004) and Pysh et al. (1999). The position of SIN1 (Phvul.003G085000) is indicated by a rectangle. NSP1 and NSP2 from *M. truncatula* are indicated with arrows. The phylogram was constructed using the neighbor-joining method based on the multiple sequence alignment analysis. The phylogenetic tree was generated using MEGA5 from a ClustalW analysis. Numbers represent bootstrap values obtained from 1,000 trials. B, SCL13 and its homologs from common bean, *M. truncatula*, and *L. japonicus*.

they are not close homologs of the protein encoded by Phvul.003G085000. Based on this analysis, we named the gene Phvul.003G085000 as *SCL13 Involved in Nodulation1* (*SIN1*).

SIN1 Interacts with NF-YC1

The partial cDNA of *SIN1* isolated in the yeast two-hybrid screening corresponds to a portion of the GRAS domain that contains the conserved Leu heptad II and one-half of the PFYRE motif (Supplemental Fig. S1B). Therefore, the interaction between NF-YC1 and SIN1 was tested in yeast by retransformation with the full-length open reading frames (ORFs) of each protein. Growth under high stringent selection conditions and measurement of β-galactosidase activity confirmed the interaction between the complete proteins in yeast (Fig. 2). Deletion of either the C or N termini of NF-YC1 (Fig. 2A) partially interfered with the interaction, reducing the β-galactosidase activity to approximately 32% in both cases (Fig. 2, B and C). On the other hand, expression of the central region of NF-YC1, which harbors the conserved histone fold motifs required for DNA binding and the interaction with the A and B subunits of the NF-Y complex, may not be sufficient to interact with SIN1. This result indicates that regions other than the core domain of NF-YC1 are required for the interaction with SIN1. The physical interaction was confirmed in planta by bimolecular fluorescence complementation (BiFC) after agroinfiltration of *Nicotiana benthamiana* leaves (Fig. 2D). SIN1 and NF-YC1 were fused to both the split C and N termini of the yellow fluorescent protein (YFP) and expressed in complementary pairs in agroinfiltrated leaves. Two days after transformation, a fluorescent signal corresponding to the wavelength of YFP emission was detected predominantly in the nucleus of *N. benthamiana* leaf epidermal cells by confocal laser microscopy (Fig. 2D). No signal was observed when the split C terminus of YFP (CYFP) and NF-YC1 fused to the split N terminus of YFP (NFYP; Fig. 2D, left) or NYFP and SIN1-CYFP were coexpressed (Supplemental Fig. S2A). A strong fluorescence signal in the nucleus was visualized when NSP1 and NSP2 from *M. truncatula* were used as a positive control of a BiFC interaction (Hirsch et al., 2009). This fluorescence signal was not observed when the interaction of either NF-YC1 or SIN1 was tested against NSP1 or NSP2 (Supplemental Fig. S2, B–E). Taken together, our results suggest that SIN1 and NF-YC1 interact both in yeast and in planta and that the interaction takes place mainly in the nuclei of plant cells.

SIN1 Is Localized Predominantly in the Nucleus

A nuclear localization signal known as pat7, which consists in a pattern starting with a P followed within three residues by a basic segment containing three K/R residues out of four, was identified in the amino acid 452 of the SIN1 sequence (PRDDKKR) according to a pSORTII analysis (Horton and Nakai, 1997) and predicted a nuclear localization with 70.6% reliability, based on Reinhardt's method for cytoplasmic/nuclear discrimination (Reinhardt and Hubbard, 1998). To investigate the subcellular localization of SIN1, a translational fusion between the GFP and SIN1 (GFP-SIN1) was expressed in *N. benthamiana* leaves after agroinfiltration. Observation of the fluorescent signal indicates that SIN1 is distributed between the nucleus and the cytoplasm (Fig. 2E). The integrity and stability of the GFP-SIN1 fusion protein was verified by western blot (Fig. 2F).

SIN1 Is Expressed in Roots and Mature Nodules

Expression of *SIN1* in different organs from common bean was analyzed by quantitative reverse transcription followed by PCR (RT-qPCR). *SIN1* transcripts were detected in both photosynthetic and nonphotosynthetic organs; however, *SIN1* transcripts accumulated at higher levels in roots than in leaves or stems of 7-d-old plants and in nodules of 14 d post inoculation (dpi) as compared with younger nodules (i.e. 7 dpi; Fig. 3A). Because we have previously shown that *NF-YC1* mRNAs increase in roots at early time points after *R. etli* inoculation (Meschini et al., 2008; Zanetti et al., 2010), we examined the accumulation of *SIN1* transcripts at 24 h post inoculation (hpi) and 4 dpi. No differences were observed between uninoculated and inoculated roots at any of these time points (Fig. 3B). On the other hand, the expression pattern of the closest homolog of *SIN1*, Phvul.006G141700, revealed a completely distinct expression pattern: transcripts of this gene accumulated at higher levels in leaves compared with other organs, such as stem, root, and nodules (Supplemental Fig. S3).

Expression data of the putative ortholog/best homolog from *M. truncatula* (Medtr4g133660) was retrieved from RT-qPCR data (Moreau et al., 2011). Transcripts of this gene exhibited a similar expression pattern to that of *SIN1* in common bean, with highest expression in mature nodules of 14 and 16 dpi (Supplemental Fig. S4). The expression pattern observed in both legume plants is compatible with a role of SIN1 at late stages of the symbiotic interaction between legumes and rhizobia. Based on gene expression data measured by microarray (Høgslund et al., 2009), the best homologs from *L. japonicus* (Fig. 1B) are accumulated in inoculated tissue at late stages of nodule formation (Supplemental Fig. S5). Mutation of *NODULE INCEPTION* or other components of the Nod Factor signaling pathway did not significantly affect the expression of the best homolog of *SIN1*, *chr4.CM0288.500.r2.m* (Supplemental Fig. S5).

Posttranscriptional Silencing of *SIN1* Using RNAi

To functionally characterize SIN1, we took advantage of the common bean root transformation system using *Agrobacterium rhizogenes*. Expression of an RNA interference (RNAi) construct containing a region of the C

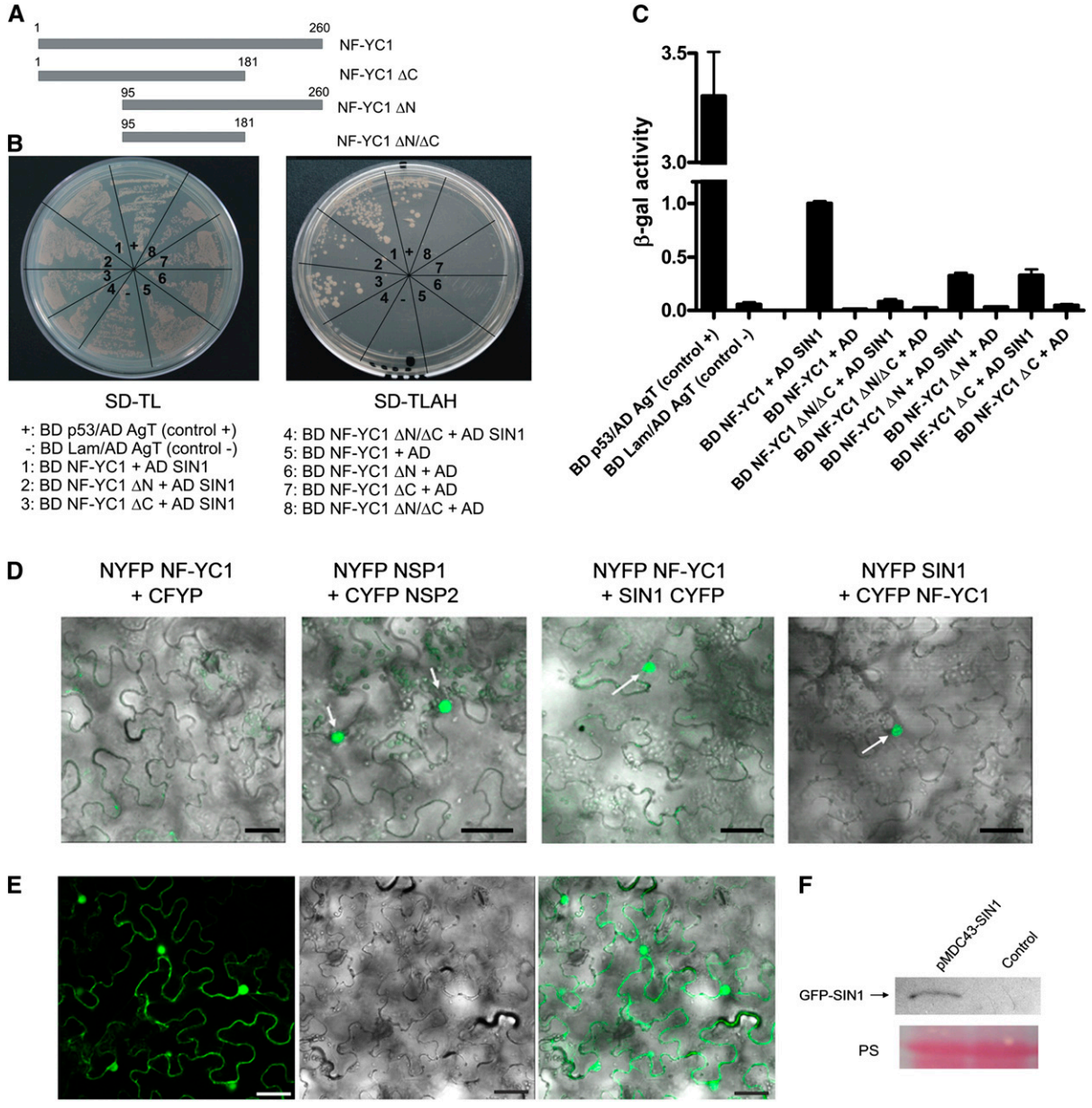

Figure 2. Interaction of NF-YC1 and SIN1. A, Schematic representation of NF-YC1 and deleted versions designed to test the interaction. B, Interaction in yeast using the two-hybrid system. The pGBKT7 plasmid containing the binding domain of the yeast transcription factor Gal4 (BD) was fused to different versions of NF-YC1 and introduced into the Y187 strain by transformation, whereas pGADT7, containing the activation domain of Gal4 (AD), was fused to SIN1 and introduced into the AH109 strain. These strains were mated in the combinations indicated (1 to 8) and selected in synthetic defined media (SD) lacking Leu, Trp (SD-TL) or Leu, Trp, adenine, and His (SD-TLAH). Positive and negative controls are p53 interacting with AgT and LamC, respectively. C, β-Galactosidase activity measured on the diploid yeasts. D, BiFC assay showing the interaction of NF-YC1 and SIN1 in *N. benthamiana* epidermal cells. The interaction between *M. truncatula* NSP1 and NSP2 is shown as a positive control. Arrows indicate the fluorescent signal in the nuclei. Bar = 25 μm. E, Subcellular localization of SIN1: the ORF of SIN1 was cloned in pMDC43 for the construction of the GFP-SIN1 fusion and introduced in *N. benthamiana* leaf cells by agroinfiltration. The same image, obtained with a confocal microscope, is shown under UV (left), white light (middle), and merged (right). Bar = 25 μm. F, Proteins were extracted from leaves agroinfiltrated with GFP-SIN1 or an untransformed strain of *Agrobacterium tumefaciens* (control), subjected to SDS-PAGE, and analyzed by immunoblot with anti-GFP antibodies, revealing the presence of a single band with the expected size of 88 kD (left). PS, Ponceau stain.

terminus of *SIN1* produced a reduction of *SIN1* mRNA levels ranging from 80% to 90%, as compared with control roots expressing *GUS* RNAi (Supplemental Fig. S6). To assess the specificity of the posttranscriptional silencing generated by the RNAi strategy, we designed gene-specific primers for other genes of the PAT1 subfamily.

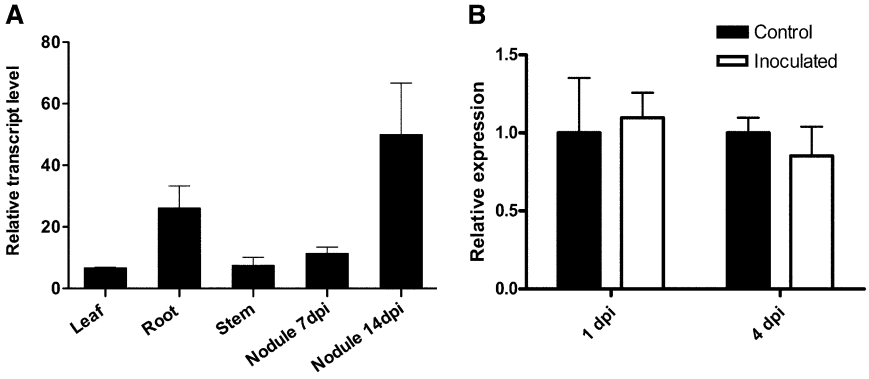

Figure 3. Relative expression of *SIN1* in different organs. A, RT-qPCR analysis of *SIN1* accumulation in different organs of common bean. Total RNA was extracted from different organs (leaf, root, and stem) of 7-d-old plants grown under optimal conditions and nodules formed by *R. etli* SC15 strain at 7 and 14 dpi. B, Levels of *SIN1* mRNA in roots after inoculation with *R. etli* strain SC15 or with yeast-extract mannitol media (control) at 1 or 4 dpi. Expression of the *SIN1* gene was measured by RT-qPCR and then normalized with *PvEF1α* expression values. Data are the media of three technical replicates and are representative of two independent experiments. In B, values are presented relative to the control at 1 dpi. Error bars represent the SD. Expression in roots and 14-dpi nodules were significantly higher than in other tissues in an unpaired two-tailed Student's *t* test with $P < 0.05$. No significant differences were observed between control and inoculated values at 1 or 4 dpi.

The closest homolog of *SIN1*, Phvul.006G141700, showed a reduction of 46% compared with control roots, indicating that the RNAi construct designed to knockdown *SIN1* partially affected transcript levels of this gene (Supplemental Fig. S6). The expression of two other members of the PAT1 branch, the best homologs of Arabidopsis PAT1 and SCL8 in common bean (Phvul.008G286000 and Phvul.007.G073500, respectively), was not modified by expression of the *SIN1* RNAi construct. Because it is well described that *NSP1* and *NSP2* GRAS transcription factors play essential roles during nodule formation in *M. truncatula*, we also quantified the mRNA levels of the putative orthologs/best homologs of these genes in common bean, Phvul.010G129400 and Phvu1.009G122700, respectively. No significant differences in transcript levels of these genes were observed between *GUS* and *SIN1* RNAi roots, indicating that the small RNA/s generated by expression of the *SIN1* RNAi construct is/are not targeting *NSP1* or *NSP2* (Supplemental Fig. S6). Our results indicate that the RNAi construct we generated strongly silences *SIN1* and to a lesser extent Phvul. 006G141700, a gene that exhibits 74% of identity with SIN1 at the amino acid level, but with a very distinct expression pattern (Supplemental Fig. S3).

SIN1 Is Involved in Lateral Root Elongation

Several members of the GRAS family have been involved in different root developmental processes, including root radial pattering, root growth, and root cell elongation. Therefore, we compared the root architecture of *SIN1* RNAi composite plants with controls that express the *GUS* RNAi construct. Macroscopic and microscopic observations did not reveal any obvious difference in the cellular organization of the root apical zone between *GUS* and *SIN1* RNAi plants (Fig. 4A). The root length was measured in the primary root that emerges from the callus (main root), as well as the first and secondary lateral roots (LR1 and LR2, respectively; Fig. 4B). The length of the main root was not affected by expression of the *SIN1* RNAi construct (Fig. 4C). However, the length of lateral roots was significantly reduced in *SIN1* as compared with *GUS* RNAi roots (Fig. 4C); lateral roots emerging from the main hairy roots (LR1) or from the second branch point (LR2) were 46% and 80% shorter than those of control roots, respectively. On the other hand, the density of lateral roots and length of root hairs were not affected by *SIN1* silencing (Fig. 4, D and E). This result suggests this member of the PAT1 family could be specifically involved in the postemergence elongation stage of lateral roots.

SIN1 Is Required for Proper Nodule Development and Progression of Infection

SIN1 was identified as a protein that interacts with NF-YC1, a transcriptional regulator required for nodule organogenesis and development. Thus, we investigated whether posttranscriptional silencing of *SIN1* affected nodule formation. Expression of *SIN1* RNAi in common bean hairy roots produced a marked reduction (approximately 75% to 80%) in the number of nodules formed upon inoculation with *R. etli* (Fig. 5, A and B). This reduction was observed as early as 7 dpi and persisted until 18 dpi, the last time point analyzed here (Fig. 5C). In addition, the few nodules formed in the *SIN1* RNAi roots were significantly smaller than those developed in *GUS* RNAi roots (Fig. 5, A, B, and D). However, nodule growth was delayed but not arrested in *SIN1* RNAi roots because the diameter of nodules increased continuously over the

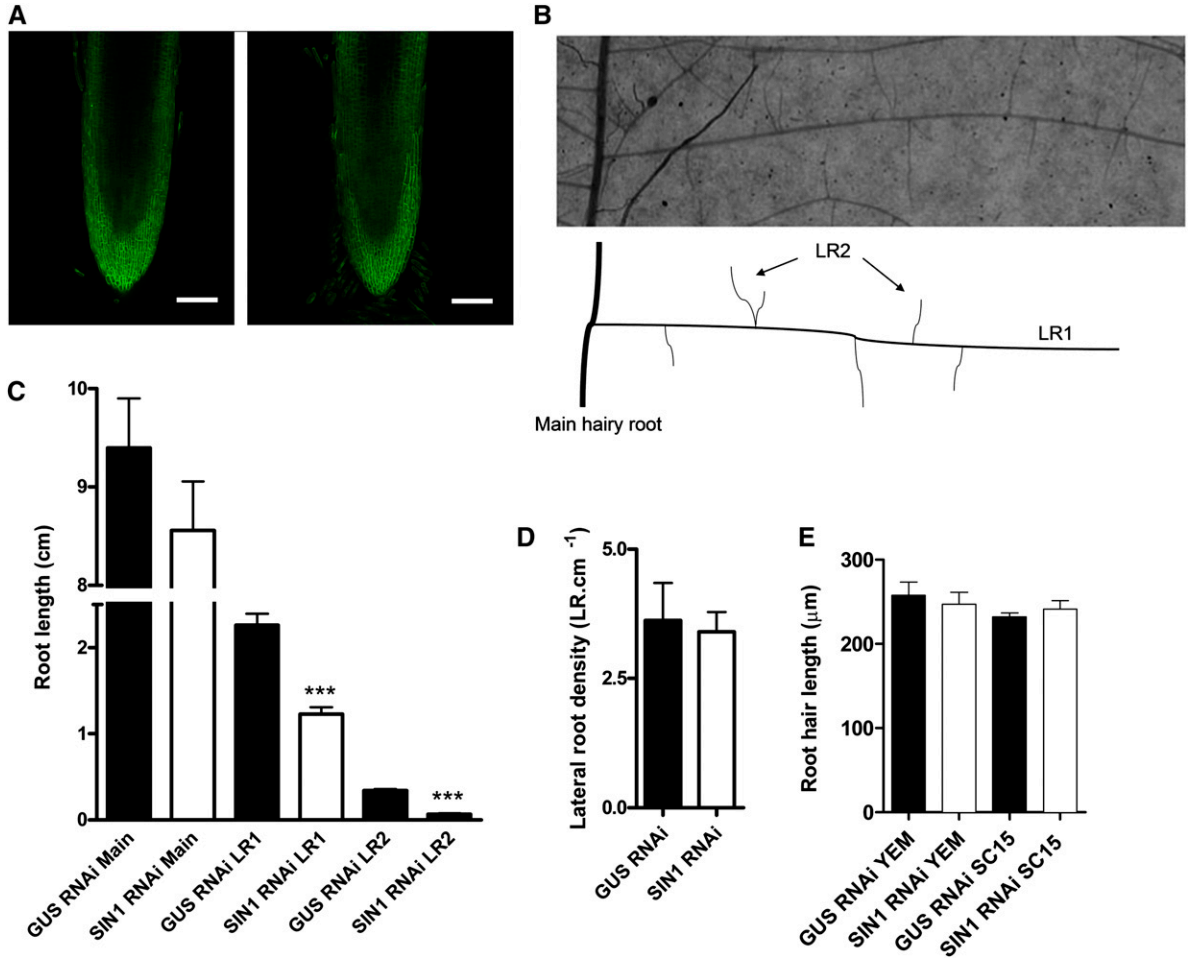

Figure 4. The length of lateral roots is affected in *SIN1* RNAi composite plants. A, General cell organization of the root tip of *GUS* (left) or *SIN1* (right) RNAi plants. Cells were observed in a confocal fluorescent microscope with optimal settings for GFP. Images were obtained by *z* integration of 5-μm sections. Bar = 100 μm. B, The first and second branches of roots emerging from the main hairy root were referred as LR1 and LR2, respectively. Length of the main root, LR1, and LR2 (C) and density of LR1 (D) were measured in *GUS*- and *SIN1* RNAi-transformed roots (black and white bars, respectively). E, Root hair length was also determined in roots inoculated with *R. etli* SC15 or control (yeast-extracted mannitol). Asterisks in C indicate significant differences in an unpaired two-tailed Student's *t* test with $P < 0.001$ ($n > 100$). No significant differences were observed in D and E between *GUS* and *SIN1* RNAi values ($n > 100$). [See online article for color version of this figure.]

time (from 6 to 19 dpi; Fig. 5D). At 7 dpi, most of the nodules formed in *SIN1* RNAi roots were infected by bacteria, but the occupancy of these nodules by a strain of *R. etli* that expresses the fluorescent protein DsRed was reduced as compared with control nodules (Fig. 5, E–H). Bright-field micrographs of mature nodule sections stained with toluidine blue revealed that the central tissue of *SIN1* RNAi nodules contained both infected and noninfected cells, but the colonized area was reduced as compared with GUS RNAi nodules (Fig. 5, I and J).

The phenotype produced by *SIN1* RNAi expression suggests that this gene might be involved in nodule organogenesis and growth but also led us to hypothesize that SIN1 might have a role in the rhizobial infection of common bean roots. Therefore, we quantified the number and the progression of infection events by inoculating roots with the *R. etli* strain that expresses the DsRed protein. The number of ITs per centimeter of root was slightly reduced in *SIN1* RNAi roots at 4 and 8 dpi; however, these differences were not statistically significant (Fig. 6A). On the other hand, whereas most of the ITs formed in control plants were found to reach the dividing cells in the cortex, they ended more frequently in the root hair or in the epidermal cells in *SIN1* RNAi roots at 4 or 8 dpi (Fig. 6B). Taken together, our results suggest that SIN1 is not required for initiation of ITs but plays a key role in their progression.

Expression of *SIN1* RNAi Affects Expression of Cell Cycle Genes

To study whether SIN1 is required for activation of early molecular responses of legumes to rhizobia, we

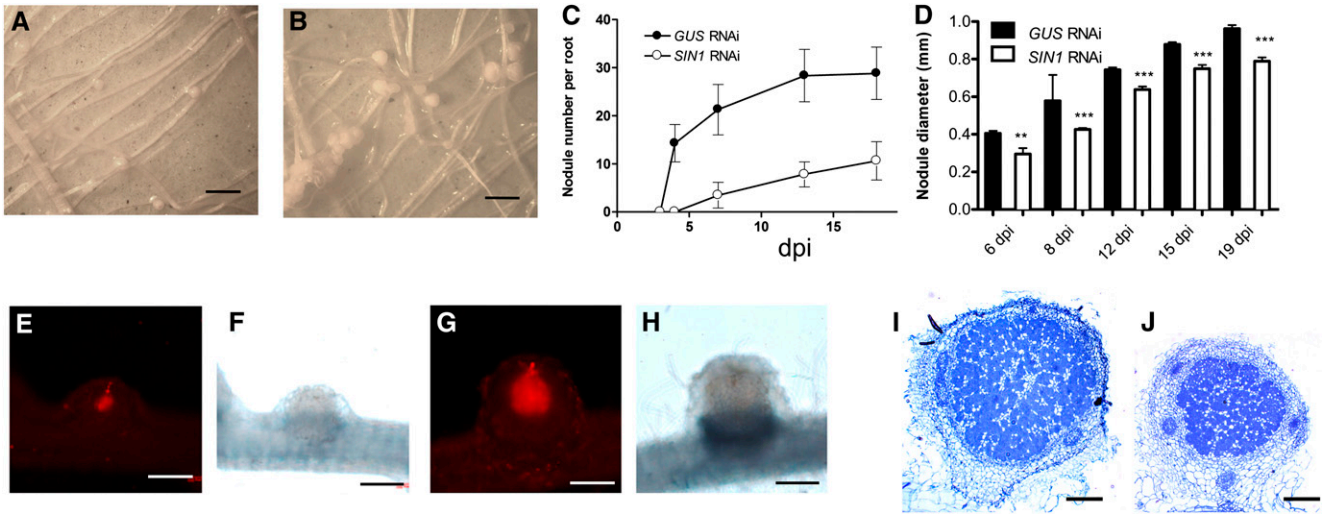

Figure 5. *SIN1* RNAi plants developed less and smaller nodules than controls. Nodulation phenotype of *SIN1* (A) and *GUS* RNAi (B) roots at 7 dpi with *R. etli* SC15. The number (C) and size (D) of nodules were recorded in both types of plants at different times after inoculation. Asterisks in D indicate significant differences in an unpaired two-tailed Student's *t* test with $P < 0.001$ ($n > 120$). Occupancy of nodules was examined 5 dpi with a strain of *R. etli* that expresses the DsRed protein by fluorescent microscopy in *SIN1* (E and F) and *GUS* RNAi roots (G and H) under UV (E and G) or visible light (F and H). Longitudinal sections of nodules formed in *GUS* (I) or *SIN1* (J) RNAi roots were observed at 21 dpi with *R. etli* strain SC15. Bars = 1 cm (A and B), 500 μm (E–H), and 300 μm (I and J).

monitored the expression of genes that are induced at early stages of the interaction. For this analysis, we selected *Rhizobium-induced peroxidase* (*RIP*), *ethylene responsive factor required for nodulation* (*ERN*), and *early nodulin 40* (*ENOD40*), a group of genes that have been previously shown to increase their steady-state mRNA levels in common bean upon rhizobia inoculation (Blanco et al., 2009; Zanetti et al., 2010). As expected, the three selected genes were induced by *R. etli* infection at 24 hpi (Fig. 7; Supplemental Fig. S7); however, expression of *SIN1* RNAi did not significantly affect the expression of these genes in control roots or in those inoculated with rhizobia, as compared with *GUS* RNAi plants. Considering the interaction between SIN1 and NF-YC1, we wondered if *SIN1* silencing had an effect on transcript levels of two subunits of the

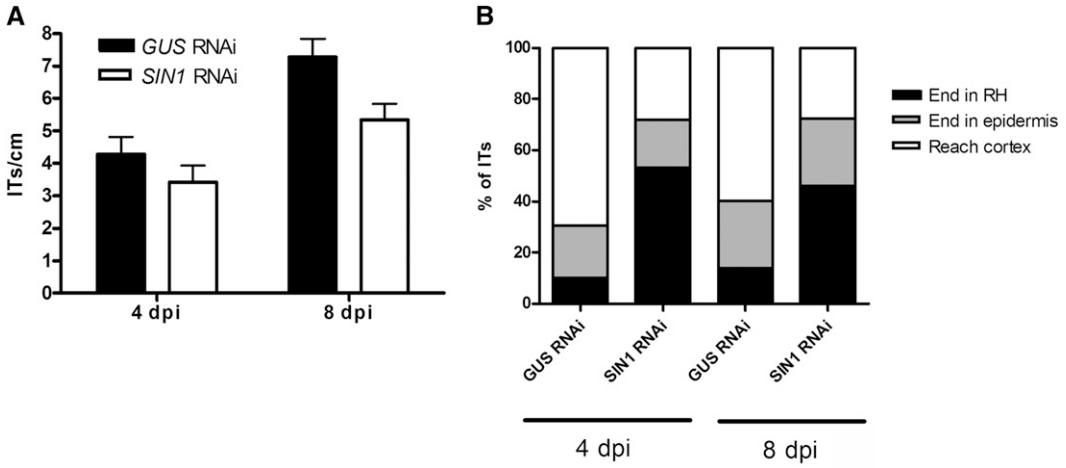

Figure 6. Effect of *SIN1* RNAi on infection events. The density of ITs (number of IT per root centimeter) formed in *GUS* and *SIN1* RNAi composite plants was quantified at 4 and 8 dpi with a *R. etli* strain that expresses the DsRed protein. There were no significant differences between *GUS* and *SIN1* RNAi at 4 or 8 dpi in an unpaired two-tailed Student's *t* test with $P < 0.05$ (A). ITs formed were classified as events that reach the cortex (white bars), end in the epidermis (gray bars), or end in the root hair (RH; black bars) and expressed as percentage of the total. The number of ITs that end in RH or reach the cortex were significantly higher in *SIN1* than in *GUS* RNAi plants in an unpaired two-tailed Student's *t* test with $P < 0.05$, whereas ITs that end in epidermis did not show significant variations (B). Approximately 60 infection events were recorded for *GUS* and *SIN1* RNAi at 4 or 8 dpi.

heterotrimeric complex, NF-YC1 and NF-YA1; the last is the best homolog/putative ortholog in common bean of *M. truncatula NF-YA1/MtHAP2-1*. As observed in Figure 7 and Supplemental Figure S7, transcripts of both genes increased in response to rhizobia, but no significant differences were observed between *GUS* and *SIN1* RNAi for *NF-YC1*. On the other hand, accumulation of *NF-YA1* mRNAs in response to rhizobia inoculation was impaired in *SIN1* RNAi as compared with *GUS* RNAi roots, suggesting that SIN1 might directly or indirectly be involved in the transcriptional or posttranscriptional response of *NF-YA1* to rhizobial infection.

In a previous report, we showed that NF-YC1 controls the expression of genes involved in the G2/M transitions of the cell cycle, which are activated during nodule organogenesis (Zanetti et al., 2010). Thus, we examined whether silencing of *SIN1* affected the expression of two of these genes: *Cell Division Cycle 2* (*CDC2*) and *CYCLIN B* (*CYCB*). As observed in Figure 7B and Supplemental Figure S7, the silencing of *SIN1* also produced a reduction of *CDC2* and *CYCB* in inoculated

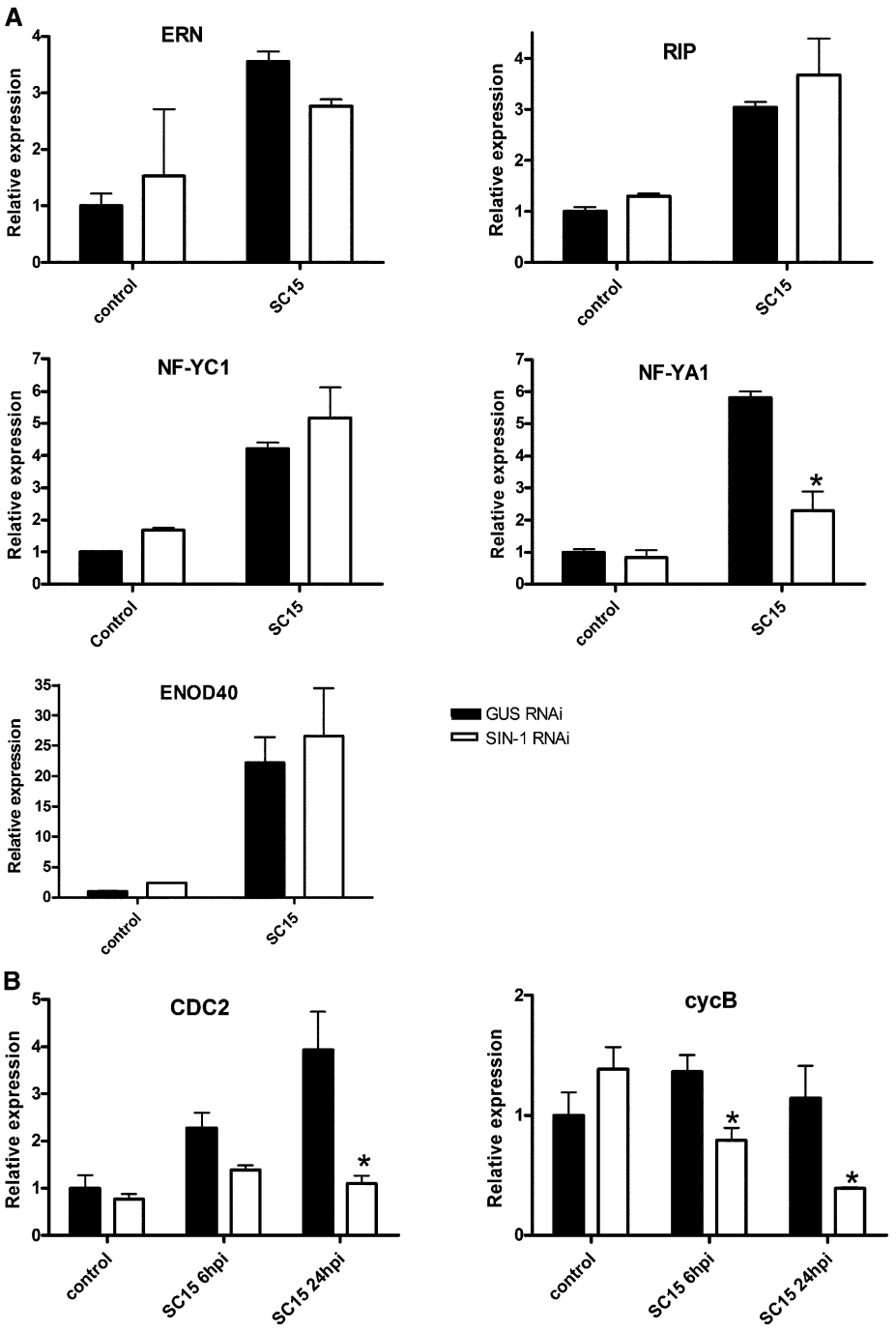

Figure 7. Effect of *SIN1* RNAi on the expression of early nodulins (A) and cell cycle genes (B). *GUS* (black bars) or *SIN1* RNAi (white bars) roots were inoculated with the *R. etli* strain SC15 and tissue was collected 6 or 24 hpi. Controls were treated with yeast-extract mannitol for 24 h. Expression of indicated genes was measured by RT-qPCR, normalized with *PvEF1α* expression values, and presented relative to the values of *GUS* RNAi controls. Data are the media of three technical replicates, and two other independent experiments are shown in Supplemental Figure S7. Error bars represent the SD. Asterisks indicate that expression values in *SIN1* RNAi are significantly different from those in *GUS* RNAi roots at the same time point, in an unpaired two-tailed Student's *t* test with $P < 0.01$.

roots as compared with controls, indicating that SIN1 and NF-YC1 might act together to trigger the molecular responses that lead to reactivation of cortical divisions during nodule formation.

DISCUSSION

In this work, we identified a member of the plant-specific family of GRAS proteins through its physical interaction with NF-YC1. Both proteins are necessary for nodule organogenesis during the symbiotic interaction between common bean and rhizobia. However, SIN1 seems to have an additional role in lateral root growth, a phenotype that was not observed in NF-YC1 silenced roots. The posttranscriptional silencing mediated by RNAi produced a strong reduction of *SIN1* transcript levels but also partially reduced mRNAs levels of Phvul.006G141700 (Supplemental Fig. S6). Even though we cannot exclude an effect of the partial silencing of this *SIN1* homolog on the RNAi-mediated phenotype, it is more likely that changes in lateral root growth and the nodulation process are due to *SIN1* silencing. This assumption is based on the different levels of silencing, as well as the distinct expression pattern of both genes: whereas *SIN1* is accumulated at higher levels in the affected organs, namely roots and nodules, Phvul.006G141700 is mainly expressed in leaves (Supplemental Fig. S3).

As mentioned previously, GRAS proteins are involved in different developmental processes. Some members of the family are required for root patterning (SHR and SCR families), axillary meristem development (Lateral Supressor), maintenance of shoot apical meristem (HAM), gibberellic and jasmonic acid response (DELLA), and light signaling (PAT1). In addition, three members of the GRAS family were previously identified as required for symbiotic interactions in *M. truncatula*: NSP1, NSP2, and RAM1. The heterodimers formed by NSP1 and NSP2 associate with promoters of Nod Factor-inducible genes, leading to molecular responses specific to legume-rhizobia interaction, whereas dimerization of NSP2 with RAM1 activates a molecular program that leads to arbuscular mycorrhization (Hirsch et al., 2009; Gobbato et al., 2012). On the other hand, SHR and SCR, other members of the GRAS gene family, can also form a heterodimer that defines a single cell layer of endodermis in plants (Cui et al., 2007). The primary sequence of SIN1 contains all the domains defined in GRAS proteins: Leu heptad repeats I and II, VHIID, PFYRE, and SAW. Phylogenetic analysis of *SIN1* showed that it belongs to a branch that is present in legume and nonlegume plants, PAT1, whose members have been involved in phytochrome-dependent red and far-red light signaling (Bolle et al., 2000; Torres-Galea et al., 2006). SIN1, as NSP1 and NSP2, is involved in nodulation but also plays a role in lateral root growth, a developmental process that has not yet been associated with other members of the GRAS transcriptional regulators. Interestingly, two members of the *L. japonicus* NF-Y complex have been associated with lateral root formation by activating cell divisions in the primordia (Soyano et al., 2013). Ectopic expression of LjNF-YA1 and LjNF-YB1 resulted in the formation of lateral roots with abnormal tips, suggesting a connection between NF-Y transcription factors and the organogenesis of lateral roots.

Symbiosis between legumes and rhizobia relies in the coordinated development of two processes, nodule organogenesis and bacterial infection (Oldroyd and Downie, 2008). We have previously reported that knockdown of *NF-YC1* leads to a reduction of the number and size of nodules (Zanetti et al., 2010). Here, we showed that expression of *SIN1* RNAi produced a similar effect: *NF-YC1* and *SIN1* RNAi roots formed only 20% of the nodules developed in *GUS* RNAi control plants. However, the infection phenotype was more severe in *NF-YC1* RNAi plants, showing a higher proportion of abortive events, a strong reduction of the density of ITs, and an excessive root hair deformation, whereas these defects were not observed in *SIN1* RNAi roots. This comparison suggests that NF-YC1 can play a role in early events that lead to IT formation independently of SIN1. This idea is consistent with the expression pattern of both genes, because *NF-YC1* mRNAs levels increased as early as 3 hpi and remained high at 24 hpi, whereas *SIN1* transcript levels are not changed during early stages of the interaction. Comparison with the phenotype observed in NSP1 and NSP2 mutants also suggests that SIN1 would act at later steps of the interaction. The expression of ERN (a target of the NSP1-NSP2 heterodimer) and other early nodulins was not affected by silencing of *SIN1*. The association of a GRAS protein of the PAT1 subfamily with root symbiosis suggests that transcriptional networks governing root nodule formation involve a sequential activation of different and specific members of this large family of transcription factors. On the other hand, SIN1 and NF-YC1 control, directly or indirectly, the expression of cell cycle genes, which are necessary to trigger the division of cortical cells (Savouré et al., 1994; Yang et al., 1994). The fact that expression of *NF-YA1* is affected by knockdown of *SIN1*, together with the requirement of NF-YA1 for cortical cell divisions (Soyano et al., 2013), points to a strong connection between SIN1 and NF-Y transcription factors. Expression of *NF-YA1/MtHAP2-1* during nodule formation is controlled, in part, at posttranscriptional level by the action of the microRNA169 (Combier et al., 2006). An in silico analysis of microRNA putative targets in common bean identified NF-YA1 as a target of miR169 (Peláez et al., 2012). In this context, SIN1 might be required either for transcriptional activation of *NF-YA1* or stabilization of its mRNAs upon rhizobial inoculation.

Our results showed that SIN1 is located mainly in the nucleus but also in the cytoplasm of *N. benthamiana* leaves. Other GRAS proteins were found to locate in the nucleus or in the cytoplasm or to be distributed between different subcellular compartments (Bolle, 2004). For example, the two GRAS proteins involved in legume-rhizobia symbiosis showed different localizations inside the cell; NSP1 is located in the nucleus, whereas NSP2 is visualized in the nuclear membrane and the endoplasmic

reticulum, and it is translocated to the nucleus of epidermal root cells after Nod Factor perception (Kaló et al., 2005; Smit et al., 2005). BiFC experiments indicated that SIN1 and NF-YC1 are able to form a complex mainly in the nuclei of *N. benthamiana* cells, as expected for two transcriptional regulators forming a complex that modulates gene expression. Based on the yeast two-hybrid assays, both the C and N termini of NF-YC1 seem to be required for an efficient interaction with SIN1. Considering that NF-YC1 localization is predominantly nuclear (Zanetti et al., 2010), our results suggest that the molecular complex between both proteins is formed directly and stabilized in the nucleus. However, it is not known whether SIN1 is rapidly translocated to the nucleus upon interaction with NF-YC1 or Nod Factor perception.

SIN1 is expressed at higher levels in roots than aerial tissue, with maximum levels at late steps of nodule formation. However, mRNA levels did not change during early time points of the interaction with rhizobia. This expression pattern is comparable to *NSP1*, which is constitutively expressed in roots and remained unchanged upon rhizobia inoculation, but it is different from *NSP2*, which is induced 2-fold in *M. truncatula* roots at 24 hpi with rhizobia or after Nod Factor treatment according to Affymetrix and reverse transcription-PCR data (Kaló et al., 2005). On the other hand, *NF-YC1* is induced at very early time points after rhizobium inoculation, but its expression is sustained during later stages of the interaction. In addition to transcript accumulation, a recent report showed that genes involved in the nodulation signaling pathway are regulated at translational level upon rhizobia inoculation (Reynoso et al., 2012). For this reason, an increase of protein levels of SIN1 at early time points cannot be excluded. The phenotypic analysis presented here suggests that the biological action of the complex formed by SIN1 and NF-YC1 would take place at the time of cortical cell divisions and IT growth but also at later time points, when nodules are developing and actively growing.

Several genes involved in the nodulation signaling pathway have been identified by forward genetics, transcriptomic analysis followed by reverse genetics, or identification of partners by two-hybrid screenings. This report constitutes an example of how new components of the root nodule symbiotic pathway can be identified based on the physical interaction with a protein already known to be involved in this process. The screen for proteins that are part of the same transcriptional complex as NF-YC1 led us to identify SIN1 as a new player of root biology of common bean, a species that is emerging as a new model for grain legumes that are important for human alimentation.

MATERIALS AND METHODS

Biological Material and Plant Transformation

Plant growth and transformation were performed essentially as previously described (Blanco et al., 2009; Zanetti et al., 2010). *Rhizobium etli* strain SC15 was previously reported (Aguilar et al., 2004). The *R. etli* strain expressing the DsRed protein was generated by electroporation with the plasmid pBHRDsRED T3 (Smit et al., 2005).

Yeast Two-Hybrid Screening

A cDNA library in yeast (*Saccharomyces cerevisiae*; AH109 strain, MATa) was constructed in pGADT7-Rec using the Matchmaker Library Construction and Screening Kit (Clontech). cDNA from roots of common bean (*Phaseolus vulgaris*) inoculated with *R. etli* for 24 h was prepared using oligo(dT) and random primers in separated reactions and then pooled for yeast transformation. The library titer was estimated in $6.4.10^6$ clones. Bait vectors were obtained by cloning the complete ORF and truncated versions of NF-YC1 in pGBKT7 and introduced in Y187 (MATα). Primers for each construct are detailed in Supplemental Table S1. The complete ORF of SIN1 was obtained by amplification with SIN1 ORF yeast two-hybrid primers (Supplemental Table S1) and recombined in pGADT7-Rec as indicated (Clontech). Activation of reporter genes and growth of yeast containing bait or empty plasmid were tested following manufacturer's instruction. Screening of the library was performed by mating and plating in synthetic defined media lacking Leu, Trp, His, and adenine. Approximately 10^7 colonies were tested for interactions. Plasmids were purified from yeast using the Yeastmaker Yeast Plasmid Isolation Kit (Clontech). To test interactions between SIN1 and different versions of NF-YC1, and to obtain positive and negative controls, haploid yeasts were mated in a small scale as described by the kit manufacturer. The liquid β-galactosidase assay using *ortho*-Nitrophenyl-β-galactoside as substrate was performed according to the Yeast Protocols Handbook (Clontech).

Plasmid Construction

To create a construct for RNAi-mediated silencing of *SIN1*, a fragment of 397 bp corresponding to the C terminus of the coding region of this gene was amplified by PCR using the primers SIN1 RNAi (Supplemental Table S1) and cDNA from common bean roots as template. The PCR product was cloned into the pENTR/D-TOPO entry vector following manufacture's instructions (Invitrogen) and recombined into the Gateway compatible destination vector pK7GWIWG2D(II) (Karimi et al., 2002) to produce the RNAi construct. The *GUS* RNAi plasmid was previously generated (Blanco et al., 2009). For subcellular localization, the complete ORF of SIN1 was amplified with primers SIN1 stop (Supplemental Table S1), cloned into pENTR/D-TOPO, and recombined into the Gateway compatible vector pMDC43 for the construction of the 2X35S:GFP6-SIN1 fusion (Curtis and Grossniklaus, 2003). For BiFC assays, the ORF of *NF-YC1* and *SIN1* were amplified with M13 primers from the corresponding pENTR/D-TOPO vectors and then recombined into the pGPTVII.Bar.YN-GW, pGPTVII.Bar.YC-GW, and pGPTVII.Hyg.GW-YC for fusion to N and C termini of the N fragment of split YFP and the C terminus of the C fragment of split YFP, respectively (Hirsch et al., 2009).

Phenotypic Analysis

Composite plants were generated as described (Blanco et al., 2009). Root length was measured from the tip to the site of hairy root emergence on the stem 10 d after transplantation to boxes. Lateral root length was measured as the distance between the primary root and the tip of the lateral root as described (Rípodas et al., 2013). Lateral and emerging roots were counted and normalized by lineal centimeter of root. Five roots per plant were selected from 10 independent plants for each construct (*GUS* or *SIN1* RNAi). Statistical significance was evaluated by unpaired two-tailed Student's *t* tests. Length of root hairs was measured as previously reported (Blanco et al., 2009). Wild-type or transgenic roots were inoculated with *R. etli* strains as described (Meschini et al., 2008). Phenotypic analysis of nodules, quantification, and classification of infection structures were performed as previously described (Zanetti et al., 2010). The total number of infection events recorded was 58 for *GUS* RNAi and 64 for *SIN1* RNAi at 4 dpi and 60 for *GUS* and *SIN1* RNAi at 8 dpi.

Quantitative Reverse Transcription-PCR Assays

RNA extraction, cDNA synthesis, and RT-qPCR assays in common bean were performed as described (Meschini et al., 2008). For each primer pair, the presence of a unique product of the expected size was verified on ethidium bromide-stained agarose gels after PCR reactions. Absence of contaminant genomic DNA was confirmed in reactions with DNAse-treated RNA as template. Amplification of common bean elongation factor 1α (EF1α) was used to

normalize the amount of template cDNA. At least three biological replicates were performed per condition. Conditions and primers used to quantify *EF1α*, *ENOD40*, *ERN*, *NF-YC1*, *CDC2*, *CYCB*, and *RIP* from common bean were previously described by Zanetti et al. (2010). Primers for amplification of *NF-YA1*, *SIN1*, and homolog genes are listed in Supplemental Table S2. RT-qPCR experiments in *Medicago truncatula* were performed as in Moreau et al. (2011).

Sequence Analysis

DNA sequences of common bean were obtained from the Dana-Farber Cancer Institute gene index (http://compbio.dfci.harvard.edu/tgi/plant.html) and the 1.0 version of the genome produced by the U.S. Department of Energy Joint Genome Institute, available at Phytozome (http://www.phytozome.org/), by a TBLASTN search. Sequences from Arabidopsis (*Arabidopsis thaliana*) and *M. truncatula* were retrieved from public databases (http://www.arabidopsis.org and http://www.medicagohapmap.org). Phylogenetic analysis and sequence alignment were performed as described (Rípodas et al., 2013). *Lotus japonicus* sequences were retrieved from the Kasuza DNA Research Institute Web site (http://www.kazusa.or.jp/lotus/index.html) and expression data from the *L. japonicus* Gene Expression Atlas at the Noble Foundation (http://ljgea.noble.org/v2/).

Agroinfiltration of *Nicotiana benthamiana* Leaves

The *Agrobacterium tumefaciens* strain GV3101 was transformed by electroporation. Overnight cultures of the strains carrying each construct or the p19 silencing suppressor plasmid (Voinnet et al., 2003) were centrifuged and pellets resuspended in 5 mL of Agromix (10 mM $MgCl_2$, 10 mM MES/KOH, pH 5.6, and 150 mM acetosyringone) and incubated at room temperature for 2 h. Suspensions were brought to an optical density at 600 nm of 1 with Agromix. Different construct combinations were prepared by mixing equal volumes of each culture, diluted to obtain a final optical density at 600 nm of 0.25. The *Agrobacterium tumefaciens* mixture was infiltrated into *N. benthamiana* leaves as described previously (Voinnet et al., 2003). The fluorescence was observed 50 to 70 h after agroinfiltration.

Microscopy and Imaging

Optical microscopy of nodule section was performed as previously described (Zanetti et al., 2010). Imaging of IT formation and nodule occupancy by the DsRed-labeled *R. etli* strain was performed with an Olympus IX51 inverted microscope using white and UV light with appropriated filters. Images were captured using a Q-Color3 high-resolution camera (Olympus Corporation). An inverted SP5 microscope (Leica Microsystems) was used for confocal microscopy.

SDS-PAGE and Immunoblots

Proteins were separated on 12% (w/v) SDS-PAGE and detected by immunoblotting as described (Zanetti et al., 2005) using an anti-GFP antibody (1:1,000; BD Bioscience).

Supplemental Data

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

Supplemental Figure S1. Analysis of SIN1 sequence.

Supplemental Figure S2. Negative interaction of NF-YC1 and SIN1 with NSP1 and NSP2.

Supplemental Figure S3. Relative expression of Phvul.006G141700 in different organs.

Supplemental Figure S4. Expression of the best homolog of SIN1 in *M. truncatula*.

Supplemental Figure S5. Expression of the best homologs of SIN1 in *L. japonicus*.

Supplemental Figure S6. Posttranscriptional gene silencing of *SIN1*.

Supplemental Figure S7. Biological replicates of the effect of *SIN1* RNAi on the expression of early nodulins and cell cycle genes.

Supplemental Table S1. Primer sequences used for cloning.

Supplemental Table S2. Primer sequences used for quantitative PCR.

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