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A Small GTPase of the Rab Family Is Required for Root Hair Formation and Preinfection Stages of the Common Bean–*Rhizobium* Symbiotic Association[W]

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Legume plants are able to establish a symbiotic relationship with soil bacteria from the genus *Rhizobium*, leading to the formation of nitrogen-fixing root nodules. Successful nodulation requires both the formation of infection threads (ITs) in the root epidermis and the activation of cell division in the cortex to form the nodule primordium. This study describes the characterization of RabA2, a common bean (*Phaseolus vulgaris*) cDNA previously isolated as differentially expressed in root hairs infected with *Rhizobium etli*, which encodes a protein highly similar to small GTPases of the RabA2 subfamily. This gene is expressed in roots, particularly in root hairs, where the protein was found to be associated with vesicles that move along the cell. The role of this gene during nodulation has been studied in common bean transgenic roots using a reverse genetic approach. Examination of root morphology in RabA2 RNA interference (RNAi) plants revealed that the number and length of the root hairs were severely reduced in these plants. Upon inoculation with *R. etli*, nodulation was completely impaired and no induction of early nodulation genes (ENODs), such as ERN1, ENOD40, and Hap5, was detected in silenced hairy roots. Moreover, RabA2 RNAi plants failed to induce root hair deformation and to initiate ITs, indicating that morphological changes that precede bacterial infection are compromised in these plants. We propose that RabA2 acts in polar growth of root hairs and is required for reorientation of the root hair growth axis during bacterial infection.

INTRODUCTION

Roots serve a multitude of plant functions. They anchor and supply plants with water and nutrients and exchange several compounds with shoots, such as hormones and metabolites from the photosynthesis. At the root-soil interface, numerous interactions between plants and their environment take place, which extend to interactions with both pathogenic or beneficial microbes and other organisms as nematodes. Within this context, plants from the Leguminosae family are able to establish a symbiotic relationship with the soil bacterial population collectively named as rhizobia, in which nitrogen-fixing root nodules are formed in response to rhizobial infection. The interaction is initiated by the exchange of signals between the plant and the bacteria. Nod factor, a lipochito-oligosaccharide molecule, is produced by the bacteria in response to flavonoids exuded by the plant. Perception of Nod factor by the plant triggers different responses responsible for changes in young epidermal cells and, at the same time, is necessary for the molecular events that will lead to nodule organogenesis (Oldroyd and Downie, 2008). These responses can occur independently, as was shown by the phenotype of different mutants, in which bacterial infection, and related epidermal responses, is observed in the absence of nodule formation and vice versa (Gleason et al., 2006; Tirichine et al., 2006a, 2006b, 2007; Murray et al., 2007). After the initial chemical communication, the physical association begins with the attachment of the bacteria to the surface of the root through plant lectins and polysaccharides present on the surface of the bacteria (Smit et al., 1992; Diaz et al., 1995). A colony is formed on the surface of the root hair, and the normal polar growth of this cell is arrested in a process that is dependent on Nod factor perception (Esseling et al., 2003). Approximately 1 h later, the swollen tip of the root hair restarts growing as an outgrowth that produces root hair curling, resulting in structures called shepherd's crooks that surround the microcolony. Entrapped bacteria enter the plant through a channel formed by invagination of the plant cell wall and plasma membrane, the infection thread (IT), that extends from the infection focus toward the dividing cortical cells, where the bacteria are released to differentiate into nitrogen-fixing bacteroids. Deposition of cell wall material for the IT formation depends on intracellular vesicle trafficking, a process that has been shown to be regulated by small GTPases and by phosphatidylinositide signaling molecules (Jurgens, 2004; Wang, 2004).

Root hair curling after Nod factor perception is an interesting case of polar growth, in which the unique developmental program of the root hair is redefined in response to environmental conditions by redirectioning of cell polarity. Understanding the processes involved in root hair tip growth may be useful to shed light on the process of early stages of symbiotic interaction, such as root hair curling and IT formation.

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Studies of polarized growth conducted in pollen tubes and root hairs have significantly contributed to elucidate the mechanisms of tip growth in plant cells. These cells are expanded by the addition of new plasma membrane and cell wall in an outgrowth of pollen grains or trichoblast for pollen and root hairs, respectively. This growth needs material that is provided by an active secretory pathway, a process coupled to an active recycling of precursors for membrane and cell wall resulting from continuous synthesis and degradation. Calcium gradients in the tip and changes in the cytoskeleton are involved in movement, stabilization, and fusion of vesicles with target membranes at the tips of growing cells. Vesicle movement is regulated by signaling pathways, including those involving small GTPases, protein kinases, calcium influx, reactive oxygen species (ROS), and phospholipids (Hepler et al., 2001; Cole and Fowler, 2006; Šamaj et al., 2006).

Rab GTPases of the Ras superfamily have emerged as key regulators of targeting specificity in eukarionts, influencing each of the four major steps in membrane traffic: vesicle budding, delivery, tethering, and fusion of the vesicle membrane with the target compartment (Grosshans et al., 2006). GTP binding proteins cycle between an inactive GDP-bound and an active GTP-bound state. Numerous homologs to mammalian Rabs have been identified in different plant species, including 57 members in the *Arabidopsis thaliana* genome (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003). The *Arabidopsis* Rabs are predicted to encode eight functional families, which may be further divided into 18 different structural subclasses (Rutherford and Moore, 2002). The number of genes in the Rab11 family arises from proliferation and diversification in the plant lineage, with 26 members in *Arabidopsis* that are homologs to the three genes present in humans and two in *Saccharomyces cerevisiae*. Although analysis of *Agrobacterium tumefaciens* T-DNA insertional mutants of *Arabidopsis* has not provided functional clues, overexpression and antisense silencing of different Rabs in other species resulted in developmental and morphological phenotypes. Tomato (*Solanum lycopersicum*) Rab11 silencing produced defects in secretion of cell wall degrading enzymes, cell wall loosening, determinate growth, and reduced apical dominance (Lu et al., 2001). Another member of the Rab11 family from tobacco (*Nicotiana tabacum*) has been implicated in pollen tube growth (de Graaf et al., 2005). Overexpression of either dominant-negative or constitutively active forms of this protein led to reduced tube growth rate and reduced male fertility. Tobacco Rab11 has been shown to localize in the apical zone of the pollen tube in a region rich in transport vesicles, overlapping the region stained by FM4-64, a lipophilic dye internalized by endocytosis. This apical localization of Rab11 and polarized actin organization are disrupted in dominant-negative and constitutively active mutants. Alteration of Rab11 activity also inhibited targeting of exocytic and recycled vesicles to the inverted cone region of the pollen tube and compromised the delivery of secretory and cell wall proteins to the extracellular matrix, suggesting that a proper regulation of Rab11 activity is essential for tip-focused membrane trafficking and growth at the pollen tube apex (de Graaf et al., 2005). Additional evidence of a connection between Rab proteins and polar growth comes from the characterization of a member of the *Arabidopsis* RABA4 subclass, RABA4b, which localizes to the trans-Golgi network compartment in the tip of growing root hairs (Preuss et al., 2004). Even though no phenotype was observed in RABA4b mutants, this protein has been implicated in tip growth in root hairs via its interaction with phosphatidylinositol-4-kinases, which are essential for root hair morphogenesis (Preuss et al., 2006).

While Nod factor perception by the root hair cell leads to root hair curling and IT formation, the molecular mechanisms underlying these processes are largely unknown. Rab proteins have been associated with symbiosis through expression studies in *Lotus japonicus* (Borg et al., 1997), *Medicago truncatula* (Schiene et al., 2004), and soybean (*Glycine max*; Cheon et al., 1993), but a role of small GTPases in symbiosis has not been clearly established. Taking into consideration the involvement of vesicle trafficking in curling and ITs, it is worthwhile to investigate whether members within the family of Rabs are important during the very early stages of the symbiotic interaction.

In a previous work based on a suppressive subtractive approach, we described identification of 41 differentially expressed cDNAs from common bean (*Phaseolus vulgaris*): transcripts of these genes were accumulated in response to infection with *Rhizobium etli* strains from the same geographical center of host diversification (Peltzer Meschini et al., 2008). It was found that one of these cDNAs, RHS24-03, shows similarity to small GTPases of the Rab subfamily based on a BLASTX search (Altschul et al., 1997). Quantitative PCR showed this gene is induced in response to the cognate strain of *R. etli* in a Mesoamerican bean cultivar, but not in a variety from the Andean domestication center. In this work, we show that this Rab GTPase is required for nodulation and affects the normal development of root hairs. Phenotypic analysis of RNA interference (RNAi)–mediated silencing of this gene using composite plants, consisting of a wild-type shoot and a transgenic hairy root, revealed that neither root hair curling nor IT formation occurs in these roots in response to *R. etli* inoculation. Based on these findings, we propose that this small GTPase acts at the preinfection stages of root hair–*Rhizobium* interaction.

RESULTS

RHS24-03 Belongs to the Rab Family of Small GTPases

Analysis of the RHS24-03 sequence indicates that this cDNA is 1189 bp in length, including 233 bp corresponding to the 5′ untranslated region (UTR), 305 bp of the 3′ UTR, and an open reading frame of 651 bp encoding a protein of 216 amino acids (see Supplemental Figure 1A online). A phylogenetic tree based on the amino acid sequences of the 57 members of the Rab family of *Arabidopsis* (Vernoud et al., 2003) and the sequence of RHS24-03 was constructed using the neighbor-joining method from a ClustalW alignment. RHS24-03 clustered in a branch together with the four members of the *Arabidopsis* RABA2 clade (Figure 1). The A group is part of one of the eight clades of Rabs detected in *Arabidopsis*, which is divided into subclasses 1 to 6 (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). Members of the A group are homologs to Rab11a, Rab11b, and Rab25 from mammals and Ypt31/Ypt32 from *S. cerevisiae*.

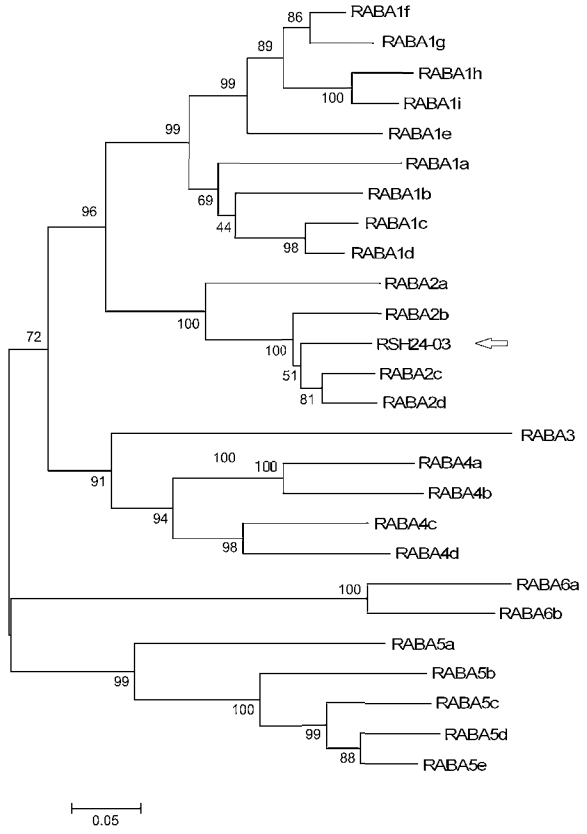

Figure 1. RHS24-03 Encodes a Protein Closely Related to Members of the RabA2 Subfamily.

A phylogenetic tree of *Arabidopsis* Rab proteins and RHS24-03 (arrow) was generated using MEGA4 from a ClustalW alignment (available as Supplemental Data Set 1 online). Numbers represent bootstrap values obtained from 1000 trials. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances are displayed in units of the number of amino acid substitutions per site.

However, a high amount of diversification has occurred in plants, with >20 members in the A group, making it difficult to predict which member corresponds to true orthologs of mammalians and yeast Rabs and how functions have diverged in the different kingdoms. The deduced amino acid sequence of RHS24-03 was aligned with Rab11 sequences from other legumes and the two members of *Arabidopsis* with the highest sequence similarity (see Supplemental Figure 1B online). Protein sequences are highly conserved among species, with 97% sequence similarity between the common bean and soybean members. Sequence motifs proposed to be involved in GTP binding and conserved among Rab proteins, as defined by Rutherford and Moore (2002) are also invariable in the RHS24-03 sequence. As it was described for the Rabs, the C-terminal end is the most divergent region of RHS24-03. Interestingly, it has been proposed that this region plays a role in targeting of these proteins to distinct intracellular membranes (Rutherford and Moore, 2002). Localization of Rab proteins at the cytosolic face of membranes depends on the posttranslational modification (addition of a geranyl-geranyl group) of a Cys motif located at the very C-terminal end. This motif, represented by the two Cys, is observed in all Rab sequences we have examined. Based on the results of this sequence analysis, we conclude that RHS24-03 belongs to the A2 group of Rabs; accordingly, hereafter this cDNA clone is renamed as RabA2.

RabA2 Is Expressed in Root Hairs and Nodules

We have previously shown that the Rab gene identified in the suppressive subtractive hybridization is expressed in roots, stem, and, to a lesser extent, leaves (Peltzer Meschini et al., 2008). This pattern of expression was found to be similar to those observed in homologous genes from other species (see Supplemental Table 1 online). Accumulation of RabA2 transcripts in root hairs of common bean was 50% higher than in roots (Figure 2). Furthermore, expression in the root hair elongation zone (zone II) and root hair zone (zone III) was 12 and 19 times higher, respectively, than in the tip of the root (zone I), a region depleted of root hairs. This spatial pattern is consistent with microarray data from *Arabidopsis* for *RabA2c* and *RabA2d*, in which transcripts are preferentially accumulated in the root hair enriched region of the root (Birnbaum et al., 2003).

A similar analysis was performed on roots inoculated with two strains of *R. etli*, SC15 and 55N1. These strains are representative lineages of the geographic regions corresponding to the two common bean domestication centers, Mesoamerican and Andean. According to our previous findings, common bean is preferentially and more efficiently nodulated by *R. etli* strains that coevolved in the same geographic region (Aguilar et al., 2004; Peltzer Meschini et al., 2008). Upon rhizobial inoculation, RabA2 mRNA levels increased in the entire root and to a higher extent in

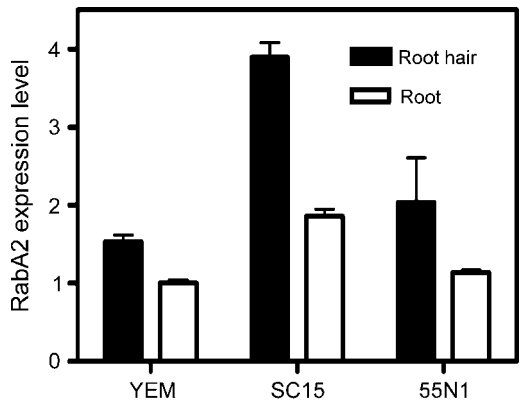

Figure 2. mRNA of RabA2 Accumulates in Rhizobia-Inoculated Roots and Root Hairs.

Total RNA was extracted from root hairs or the whole root from plants inoculated with yeast extract mannitol medium (YEM) or *R. etli* strain SC15 or 55N1 at 24 HAI. Levels of RabA2 were measured by qRT-PCR and normalized to elongation factor 1α (EF1α) values. Expression was then normalized to values obtained from roots of plants inoculated with YEM. Means ± SD of three technical replicates are presented. Results are representative of three independent experiments.

root hairs (Figure 2). The increases were approximately twofold relative to control tissue in the entire root and threefold in root hairs when the SC15 strain was used, whereas these values were more moderate in response to the 55N1 strain. This result is consistent with the previously reported expression pattern in response to these strains (Peltzer Meschini et al., 2008). Levels of RabA2 transcripts remained high in young (7 d) and mature (14 d) nodules formed upon inoculation with strain SC15 but not in those formed after infection with 55N1 (Peltzer Meschini et al., 2008). Microarray data (Kuwata et al., 2004) for the putative ortholog in *L. japonicus* (Gene ID MWM039d05_r) showed a moderate increase of expression in nodules between 30 and 100% during the 12 d following inoculation (see Supplemental Table 2 online). On the other hand, homologs from *M. truncatula* and *Medicago sativa* showed lower levels in nodules than in uninoculated roots (see Supplemental Table 2 online) according to the Medicago Gene Atlas (Benedito et al., 2008).

Subcellular Localization of RABA2 Protein in Root Hairs

Rab proteins are typically localized to the membrane of vesicles where, in conjunction with SNARE proteins, they participate in the targeting specificity of the endomembrane system. In polarized cells, such as pollen tubes and root hairs, vesicle trafficking is necessary to sustain tip cell growth. In order to determine RABA2 distribution in root hairs, transgenic bean roots expressing a green fluorescent protein (GFP)-RABA2 fusion under the control of the cauliflower mosaic virus (CaMV) 35S promoter were generated. Several independent transgenic roots were analyzed by confocal microscopy. GFP fluorescence was observed in the periphery of roots hairs, mainly associated with punctuate structures (Figure 3B). By contrast, GFP lacking the Rab fusion was evenly distributed along the root hair (Figure 3A). Compartments labeled by the GFP-RabA2 fusion moved along the root hair as showed in the time course presented in Figure 3B and Supplemental Movie 1 online.

Upon inoculation with *R. etli*, the growth of some root hairs is reoriented to form the typical curling that entraps the associated bacteria. Distribution of the GFP signal in inoculated but unresponsive root hairs was found similar to that of uninoculated roots, whereas root hairs that reoriented in response to the bacteria showed a higher concentration of fluorescence in the newly established axis of growth (Figure 3C). This subcellular localization is consistent with a role of RABA2 in the post-trans-Golgi movement of vesicles associated with polar growth.

Silencing of RabA2 in Composite Plants of Common Bean

Although several GTPases have been reported as induced during late stages of nodule formation (Cheon et al., 1993; Borg et al., 1997; Schiene et al., 2004), a role for members of the Rab family in the early symbiotic interaction has not been clearly established or further supported by genetic evidence. In an attempt to asses the biological function of RABA2 during the symbiotic association between common bean and *R. etli*, a reverse genetic approach was applied using the two following strategies: RNAi-based silencing and overexpression of the open reading frame (ORF) sequence. Since a highly efficient method for stable transformation of common bean has not been reported yet, we used a method mediated by *Agrobacterium rhizogenes* to generate transgenic hairy roots from common bean (Estrada-Navarrete et al., 2006). To validate this approach, the efficiency of the silencing mediated by RNAi was evaluated over the period of time required to observe phenotypic effects related to nodule

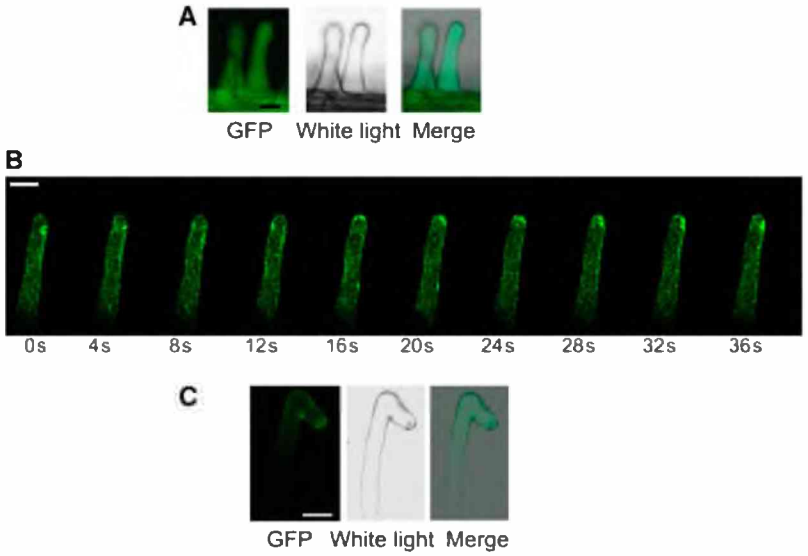

Figure 3. RABA2 Localizes to Vesicles inside of Root Hairs.

Roots from common bean plants expressing soluble GFP **(A)** or the GFP-RABA2 fusion (**[B]** and **[C]**) were observed by confocal microscopy. Time-lapse images of a root hair expressing GFP-RABA2 were collected every 4 s. Individual frames presented in **(B)** are part of Supplemental Movie 1 online. A root hair tip from a plant 24 HAI with *R. etli* is shown in **(C)**. Bars = 20 μm.

formation. Relative mRNA levels of RabA2 were monitored by quantitative RT-PCR (qRT-PCR) in a time course after tumor formation in RabA2 RNAi transgenic roots and in hairy roots transformed with a β-glucuronidase (GUS) RNAi construct as a control (Figure 4). At the beginning of hairy root differentiation, when visible-globular tumors have developed on the stem, accumulated RabA2 mRNA was greatly reduced (~65%) in the RabA2 RNAi plants compared with control plants. Maximum reduction of mRNA levels, ~95% of silencing, was detected between 6 and 9 d, but silencing remained above 70% up to 27 d after tumor formation. This silencing was restricted to transgenic roots, since aerial parts showed no differences in the mRNA levels of RabA2 when RNA samples obtained from leaves of the RabA2 RNAi and GUS RNAi composite plants were compared with each other (see Supplemental Figure 2 online). We conclude that silencing in transgenic roots of common bean is achieved by a specific RNAi and therefore useful to assess the root phenotype during symbiosis in further experiments.

In order to characterize the expression of RabA2 at early stages of nodule formation in composite plants, inoculation with *R. etli* was performed on roots of control plants and plants with reduced levels of RabA2. Twenty-four hours after inoculation (HAI), infection with rhizobia produced an increase of ~3.5-fold in RabA2 transcript levels in GUS RNAi roots compared with mock-inoculated plants, decreasing progressively at 48 and 72 HAI (Figure 4, dashed lines). Although RabA2 RNAi roots also showed increased levels of RabA2 transcript at 24 HAI compared with mock-inoculated roots, the accumulated RabA2 levels represented <30% of those present in inoculated GUS RNAi roots. By 48 HAI, RabA2 mRNA levels in inoculated RabA2 RNAi plants returned to those found in roots of mock-inoculated controls. These results showed that *R. etli* inoculation triggers

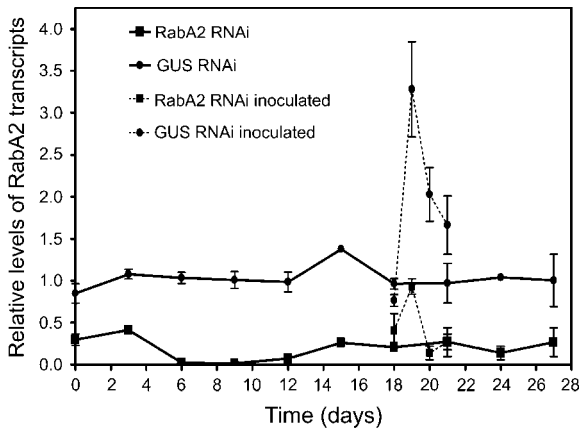

Figure 4. Temporal Pattern of Silencing of RabA2 after *A. rhizogenes* Transformation.

RNA was extracted from globular callus (time 0) and hairy roots at different times after emergence from the callus. Levels of RabA2 were measured by qRT-PCR in GUS and RabA2 RNAi transgenic tissue and normalized to EF1α values. Inoculation with *R. etli* was performed on a group of plants at day 18 and samples collected daily until 3 d after infection (dashed lines). Means ± SD of three technical replicates are presented.

RabA2 mRNA accumulation in composite plants, resembling what was observed in wild-type roots (Peltzer Meschini et al., 2008), and moreover, that RNAi-mediated silencing of this gene continues throughout the period of time in which events of the plant–rhizobia interaction leading to nodule formation take place.

RabA2-Silenced Hairy Roots Have Root Hairs Altered in Number and Length

To evaluate the phenotypic effects caused by the silencing of RabA2 in transgenic hairy roots, plants were examined for morphological alterations. No obvious differences in the general morphology of the plants were detected: traits such as shoot length, leaf number and size, or root length in RabA2 RNAi and control plants were found to be similar each other (see Supplemental Figure 3A online). However, microscopy examination of RabA2 silenced roots revealed that the number and length of root hairs were significantly reduced (Figures 5A and 5B). As shown in Figure 5C, the number of root hairs was reduced by ~50% in RabA2 RNAi plants (significantly different in a Student's *t* test, $P < 0.05$) compared with GUS RNAi plants. In addition, the length of the root hairs was severely reduced to ~20% of the control plants values (Figure 5D; Student's *t* test, $P < 0.05$). These effects, reduction in number and shortening of the root hairs, were observed consistently in several roots of independent transformation experiments conducted on different days. Since these assays were performed with plants that were transferred to nitrogen-free medium for 3 d, we asked whether changes in the morphology and number of root hairs could be related to nitrogen availability. Therefore, these results were compared with those obtained with transgenic plants that were grown on a medium supplied with a readily assimilable source of nitrogen (KNO_3). No differences either in number or length of the root hairs were observed in RabA2-silenced plants regardless the source of nitrogen (see Supplemental Figure 4 online), indicating that the altered root hair phenotype is independent of nitrogen availability for the plant. Moreover, nitrogen availability did not affect the mass of the plant as estimated by dry weight (see Supplemental Figure 3B online). The reduction of root hair length and number in RabA2 RNAi plants did not affect the dry weight of the plant when compared with control GUS RNAi plants (see Supplemental Figure 3B online) in the conditions used in our experiments. Next, we questioned whether overexpression of RabA2 had phenotypic effects on the development and growth of hairs in transformed roots. As shown in Figures 5C and 5D (see Supplemental Figures 3B and 4 online), 35S:RabA2-transformed hairy roots did not show any significant change (Student's *t* test, $P > 0.05$ in all comparisons) in the number, length of the root hairs, or dry weight compared with control plants transformed with the p35S: GFPGUS+ plasmid.

Nodulation Is Impaired in RabA2 RNAi-Silenced Plants

As describe above, RabA2 was identified in a suppressive subtractive hybridization approach as induced in the more efficient interactions between common bean and lineages of *R. etli*. To asses the role of RabA2 in nodulation, *A. rhizogenes*–transformed hairy roots were inoculated with *R. etli* lineages that exhibit

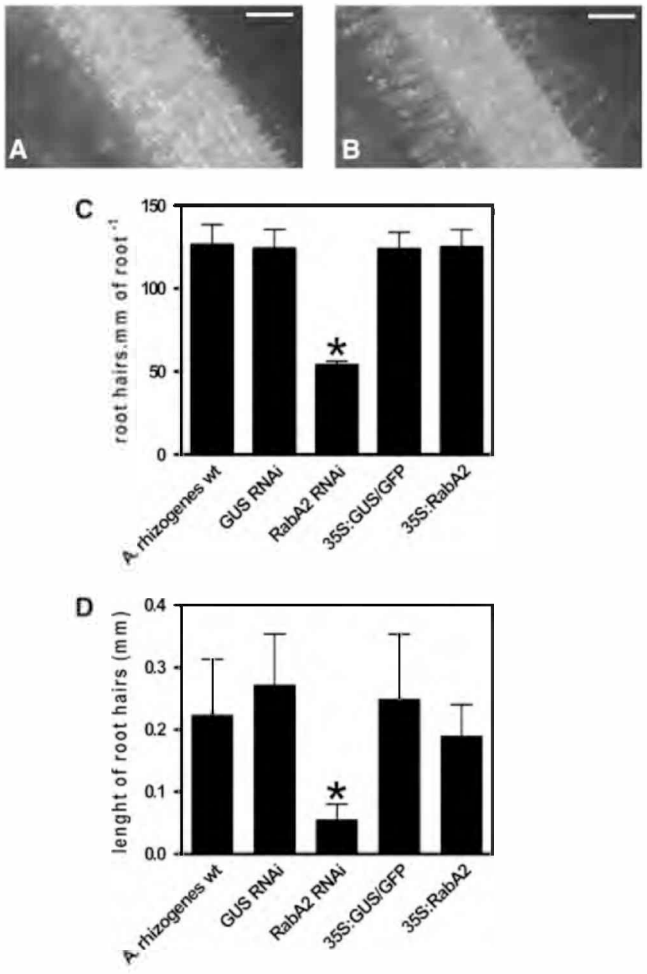

Figure 5. Effect of RabA2 Silencing and Overexpression on Root Hairs.

RabA2 RNAi **(A)** or GUS RNAi **(B)** root hairs were observed under the microscope. Root hairs were counted **(C)** and measured **(D)** from at least five different composite plants. Mean values were compared with GUS RNAi according to a Student's *t* test (*, $P < 0.05$; $n > 500$). Bars = 100 μm.

different degrees of nodulation efficiency. The number of nodules per root formed in RabA2- and GUS-RNAi plants was scored periodically. No nodule formation was observed in RabA2-silenced roots during the 9 d following infection with *R. etli* strain SC15 (Table 1) or 55N1 (data not shown), whereas control plants (GUS RNAi) displayed the normal nodulation kinetics observed previously on wild-type plants (Peltzer Meschini et al., 2008). The absence of nodule formation in RabA2 RNAi plants was repeatedly observed in hairy roots of composite plants obtained from independent transformation experiments. To confirm that this effect was caused by silencing of RabA2, mRNA levels were measured in the plants used for nodulation analysis. Indeed, RabA2 RNAi roots showed levels of silencing above 95% compared with control roots (GUS RNAi or transformed with *A. rhizogenes* K599) in several independent transformed plants (Figure 6A).

In order to asses whether the defective symbiotic phenotype was due to silencing of specific members of the RabA2 family, the mRNA level of RabA1, a member of the most closely related family of Rabs, was evaluated in these plants using gene-specific primers. The bottom panel of Figure 6A shows that no reduction of mRNA levels corresponding to RabA1 occurred in RabA2 RNAi transformed roots. These results demonstrate that silencing of RabA2 does not affect level of transcripts of another member of the Rab family, indicating that silencing was restricted to the RabA2 subfamily and that at least one member within this subfamily is required for nodule formation. The fact that identical results, absence of nodule formation, were obtained using either strain SC15 or 55N1, shows that the requirement for RabA2 is independent of the *R. etli* strain used in the nodulation experiments.

The infection process is initiated by the attachment of bacteria to the surface of the root hair, followed by a change in the direction of growth to produce the root hair curling. In order to examine whether root hairs of RabA2 silenced roots showed these characteristic morphological responses to rhizobial inoculation, microscopy observation of inoculated roots was performed. Control and RabA2 RNAi hairy roots were inoculated with a *R. etli* strain expressing GFP to facilitate observation of the bacteria within root tissues by fluorescence microscopy (Figure 7). Microcolonies were formed on the surface of both GUS RNAi and RabA2 RNAi root hairs (Figure 7A). By contrast, reorientation of the root hair axis of growth was observed in 22% of the GUS RNAi root hairs in inoculated plants, but only in 1.4% of inoculated RabA2 RNAi silenced roots and 1.6% of control uninoculated roots (Figure 7B). IT formation was observed in 9% of root hairs of control plants at 48 HAI, whereas RabA2 RNAi-silenced plants failed to form these structures along the root epidermis (Figure 7C). These observations were consistent among different roots from different plants, where no ITs were ever observed in RabA2 RNAi plants. Taken together, these results and those presented in Table 1 show that the nodulation phenotype observed in silenced plants for RabA2 is most probably due to the lack of preinfection responses, since they failed to induce root hair curling and IT formation in response to *R. etli*.

To investigate whether overexpresion of RabA2 had a phenotypic effect on nodulation efficiency, several independent composite plants with increased mRNA levels of RabA2 were obtained by *A. rhizogenes* transformation (Figure 6B). In contrast with what was observed in silenced plants, these transgenic roots did not show significant differences compared with control roots carrying the p35S:GFPGUS+ construct in terms of the number of nodules formed when inoculated with either *R. etli* strain SC15 or 55N1 (Table 1). These results indicate that overexpression of the wild-type form of RabA2 does not produce any appreciable effect on the nodulation efficiency observed in the interaction between common bean and these two lineages of *R. etli*.

Expression of Nodulins in Transgenic Hairy Roots

Since RabA2 was found to be required for both root hair deformation and nodule organogenesis, we asked whether molecular responses associated with the perception of rhizobial nodulation factors were also affected. Three different nodulins known to be induced in the epidermis or in cortical cells upon rhizobial

Table 1. Number of Nodules per Root in Hairy Roots after Inoculation with *R. etli*

DAI	GUS RNAi SC15	RabA2 RNAi SC15	35S:GFP-GUS 55N1	35S:GFP-GUS SC15	35S:RabA2 55N1	35S:RabA2 SC15
3	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
4	0.49 ± 0.61	0.00 ± 0.0	0.43 ± 0.1	0.80 ± 1.0	0.38 ± 0.1	0.96 ± 0.2
5	9.85 ± 0.7	0.00 ± 0.0	7.80 ± 0.6	13.00 ± 5.0	9.70 ± 1.4	13.54 ± 1.3
6	15.13 ± 0.84	0.00 ± 0.0	17.77 ± 1.1	22.80 ± 9.4	18.64 ± 2.1	24.68 ± 2.5
7	26.19 ± 1.07	0.00 ± 0.0	30.30 ± 1.2	35.37 ± 8.9	29.48 ± 2.3	40.96 ± 3.5
8	28.73 ± 0.61	0.00 ± 0.0	31.30 ± 1.2	35.00 ± 8.0	36.02 ± 2.5	42.16 ± 3.4
9	31.01 ± 1.65	0.00 ± 0.0	32.30 ± 1.3	36.37 ± 9.0	39.44 ± 2.5	43.88 ± 3.4

Fluorescent hairy roots were inoculated with *R. etli* and nodule number recorded at the indicated times after inoculation (DAI). Each value represents the number of nodules per transformed root ± SE ($n > 65$).

infection were selected: ERN1 (ERF required for nodulation) is a transcription factor of the ERF family that is part of the Nod signal transduction pathway downstream of the *dmi3* gene and upregulated in root hairs of *M. truncatula* upon Nod factor treatment (Andriankaja et al., 2007; Middleton et al., 2007); Hap5 was identified in the same suppressive subtractive hybridization screening as RabA2 (Peltzer Meschini et al., 2008), and it encodes the C subunit of the CCAAT transcription factor, a regulator of nodule development (Combier et al., 2006); and Enod40 encodes an RNA containing two small conserved ORFs that is induced by Nod factor in the pericycle and in dividing cells of the cortex (Crespi et al., 1994). Nucleotide sequences of common bean ESTs with high similarity to *M. truncatula* ERN and Enod40 were obtained by a BLAST search on the Dana-Farber Cancer Institute (DFCI) Bean Gene Index database and used to design gene-specific primers for qRT-PCR experiments. Quantification of mRNA levels of these nodulins was performed on RabA2 RNAi and control (GUS RNAi) transformed hairy roots. Reduced levels of RabA2, but not of RabA1, were confirmed in YEM-inoculated roots (Figure 8A). As shown in Figure 8B, all three tested genes showed increased levels of transcript upon *R. etli* inoculation in GUS RNAi plants. By contrast, mRNA levels of all three tested nodulins were not significantly modified over the first 24 HAI in RabA2 RNAi roots (Student's *t* test, $P > 0.05$ in all comparisons). Six days after inoculation, the nodulin transcript levels remained high in control GUS RNAi roots. At that time after rhizobia inoculation, Enod40 and Hap5 mRNA levels in RabA2 RNAi roots reached values that were ~30% of those detected in GUS RNAi roots, whereas transcript levels were almost undetectable for ERN (see Supplemental Figure 5 online). The lack of or reduced induction of these nodulation molecular markers upon rhizobial infection was associated with an efficient reduction of RabA2 transcript levels in all tested RabA2 RNAi roots (Figure 6). These results indicate that RabA2 is required for the rhizobial-activated signal transduction pathways that lead to the induction of gene expression in the root.

DISCUSSION

Signal transduction after Nod factor perception in roots of legume plants is highly complex, involving specific molecular and cellular responses in different cell layers of the root that ultimately lead to nodule organogenesis. The epidermis regulates bacterial infection, whereas the root cortex mediates the formation of the nodule primordia. Results reported here show that RabA2 is important for the early events that take place after Nod factor perception. Nodulation is completely abolished in RabA2-silenced hairy roots. Absence of root hair deformation and IT formation in silenced plants indicates that RabA2 is necessary in the epidermis for the morphological changes that lead to the infection process. Consistent with this, reduction or lack of nodulin induction upon rhizobial infection suggests that RabA2 is also necessary for molecular responses in both epidermal and cortical cells. These data support a function of RABA2 proteins early after Nod factor perception and upstream of the signal connecting changes in the epidermis with responses in the cortical cells.

Phenotypic changes observed during nodulation were found to be independent of the strain of *R. etli* used, since results were similar for strains SC15 and 55N1. These strains were chosen as representatives of the main polymorphic forms of the *nodC* gene associated with domestication centers of common bean (Aguilar et al., 2004). Despite the fact that RabA2 was obtained in a screening as differentially expressed in root hairs treated with either of these strains, overexpression of RabA2 did not change the strain preference previously reported (Peltzer Meschini et al., 2008) or have any noticeable effect on nodulation. However, it is noteworthy to considerer that small GTPases are regulated through the coordinated activities of the accessory proteins GTPase activating proteins and guanine-exchange factors, which allow Rab proteins to switch between active and inactive forms. In addition, the activity of Rabs is determined by post-translational modifications and membrane localization; therefore, overexpression of the protein might not result in an increase in the active form of the protein. As overexpressing RabA2 has no effect on the phenotype of hairy roots, it would be of interest to assess the effect of overexpressing a constitutively active form of the protein on the strain preference in bean nodulation.

RabA2 Is Important for Genesis and Growth of the Root Hair

Root hairs are cylindrical projections of epidermal cells that extend the surface for uptake of water and nutrients. They grow by extension of the tip with no elongation along sidewalls, a process that resembles extension of the pollen tube. Both cell types require membrane cycling, vesicle trafficking, arrangement of the actin filaments, and a tip gradient of Ca^{2+} to maintain tip

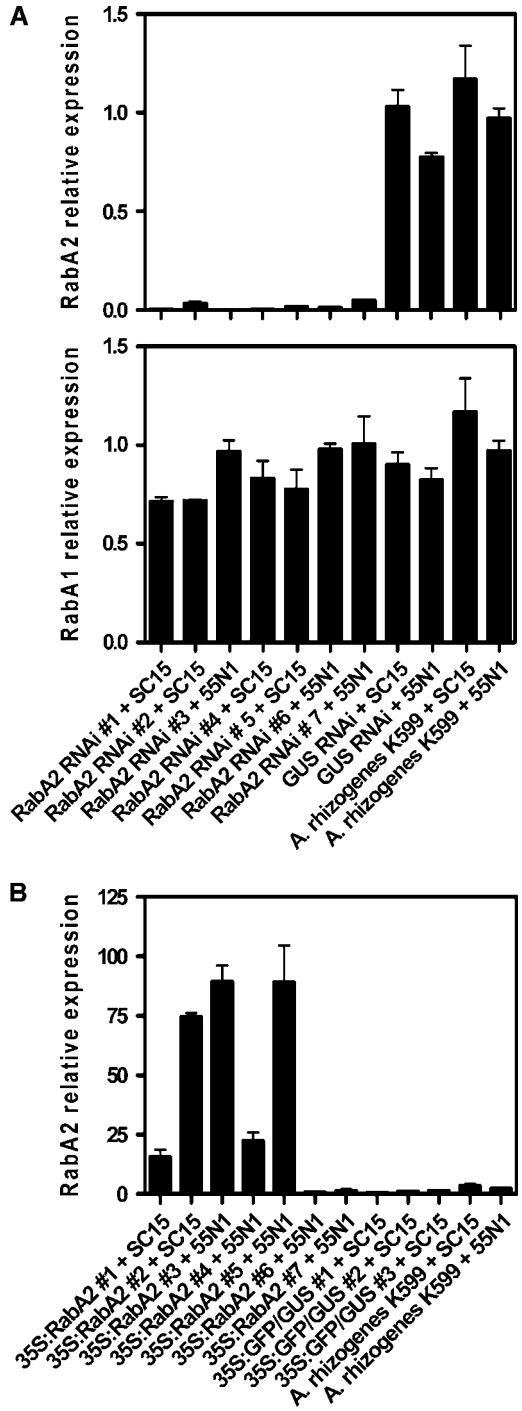

Figure 6. RNAi Posttranscriptional Silencing and Overexpression of RabA2 in Transformed Hairy Roots.

Hairy roots of composite plants grown on solid Fahraeus mineral medium were inoculated with *R. etli* strain SC15 or 55N1. At 6 d after inoculation, several roots harvested from the same plant were used for RNA extraction. Relative transcript levels of RabA2 (top panel) and RabA1 (bottom panel) in RabA2 RNAi plants were compared with those from plants transformed with the GUS RNAi construct or inoculated with the untransformed *A. rhizogenes* strain K599 **(A)**. Relative mRNA expression levels of RabA2 in hairy roots overexpressing RabA2 under the control of the CaMV35S promoter (35S:RabA2) were compared with plants transformed with control vector (35S:GFP/GUS) or with the untransformed *A. rhizogenes* **(B)**. Roots from plants #6 and #7 carrying the 35S:RabA2 construct showed levels of RabA2 similar to 35S:GFP/GUS plants. Transcripts levels were measured by qRT-PCR and normalized to EF1α values. Means ± SD of three technical replicates are presented.

growth directionality (Wymer et al., 1997; Smith and Oppenheimer, 2005; Šamaj et al., 2006). Several reports have shown that small G-proteins and their effectors play important roles in polar growth (e.g., Molendijk et al., 2001; Jones et al., 2002; Preuss et al., 2004; de Graaf et al., 2005; Carol et al., 2005; Gu et al., 2005, 2006; Song et al., 2006; Nielsen et al., 2008). Rab11 proteins from mammals are localized in the recycling endosome of polarized epithelial cells. It has been suggested that members of the Rab11 family may regulate distinct transport routes between the recycling endosome and the Golgi or plasma membrane, facilitating interaction between membranes and the actin cytoskeleton (Casanova et al., 1999; Rodman and Wandinger-Ness, 2000; Yokota et al., 2009). In this study, we have shown that RabA2 from common bean is associated with vesicles along the root hair and that reduction of mRNA levels of the corresponding gene produces a noticeable root phenotype. Indeed, RabA2 silencing leads to reduction in both number and length of root hairs, but it does not affect directionality of growth since a change of direction or branching of the tip was not observed (Figure 5). Alterations of directional growth have been linked to the microtubule structure formed in the tip of the growing root hair. Bibikova et al. (1999) have shown that application of a microtubule antagonist to root hairs of *Arabidopsis* led to a loss of growth directionality and the formation of multiple, independent growth points in a single root hair. Within this context, a possible scenario may be that RABA2 regulates the vesicle trafficking and membrane recycling required for the initiation and maintenance of tip growth in root hairs but does not affect the formation of the microtubule structures that determine the directionality of root hair expansion. This putative role is in agreement with findings in *Arabidopsis* that have shown that RABA4b localizes at the growing tip of root hairs in a compartment presumably involved in the secretion of cell wall components to the plasma membrane, a step necessary for expanding the tip of the cell (Preuss et al., 2004). Accordingly, it was shown recently that At RABA2 and At RABA3 localize at a novel post-Golgi membrane in *Arabidopsis* root tip cells (Chow et al., 2008).

Tip-focused root hair growth is regulated by the activity of several cellular components. For instance, ROS were shown to accumulate at the growing tip of root hairs (Foreman et al., 2003; Carol et al., 2005), suggesting that spatial regulation of ROS production is important for the polar growth of root hairs. It has been described that *Arabidopsis RHD2/rbohC* (for *ROOT HAIR DEFECTIVE2/respiratory burst homolog C*), which encodes a NADPH oxidase involved in the production of ROS, is required for root hair elongation, since *rhd2* homozygous mutants have root hairs 20% shorter than the wild type (Foreman et al., 2003; Renew et al., 2005). These mutants are also defective in setting

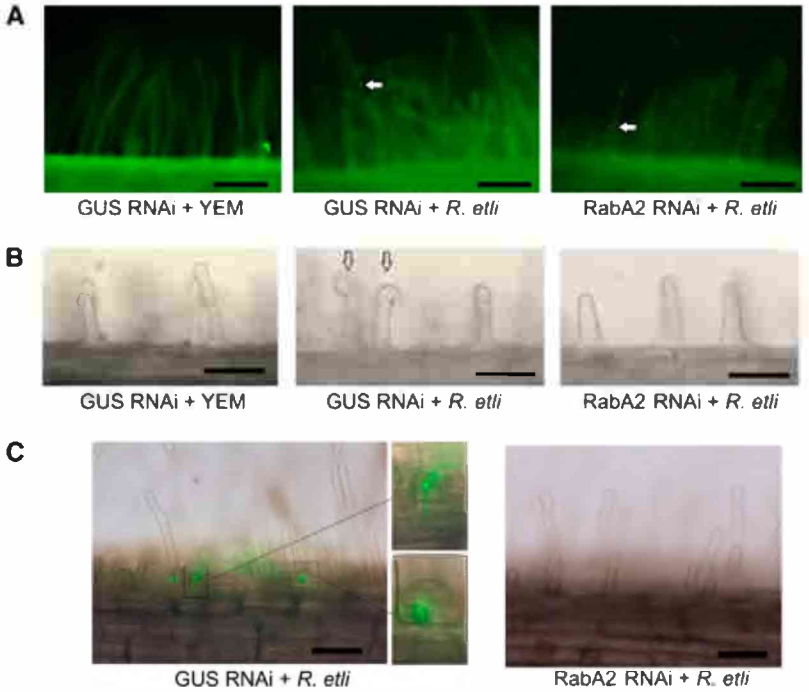

Figure 7. Effect of RabA2 RNAi Silencing on the Early Symbiotic Responses to Rhizobial Infection.

(A) Attachment of *R. etli* expressing GFP to the surface of root hairs: GUS RNAi transformed roots inoculated with YEM medium (left) or inoculated with GFP-labeled *R. etli* strain 55N1 at 24 HAI (middle) and RabA2 RNAi transformed roots inoculated with the same strain at 24 HAI (right). Attachment can be visualized as green dots on the surface of root hairs, as indicated by the arrows. Bars =100 μm.

(B) Root hair curling observed 24 HAI with *R. etli* in GUS RNAi (middle) compared with plants treated with YEM (left) or RabA2 RNAi roots (right). Arrows point to curled root hairs. Bars = 50 μm.

(C) ITs formed at 48 HAI on GUS RNAi plants (left), showing two ITs in detail. Root hair deformation was absent in RabA2 RNAi roots (right). Bars = 50 μm.

Digital photographs shown in **(A)** were obtained under UV light with the appropriate filter for GFP; images in **(B)** were obtained with visible light. Epifluorescence and visible light were merged in **(C)**.

up the tip-high Ca^{2+} gradient in root hairs, suggesting that RHD2/rbohC-mediated ROS production is required to stimulate Ca^{2+} influx during root hair elongation and cell expansion (Foreman et al., 2003). Phosphoinositides also have been postulated to contribute to root hair development. Evidence of this contribution comes from the analysis of *Arabidopsis* T-DNA mutants disrupted in phosphatidylinositol-4-phospahte 5-kinase and phosphatidylinositol 4-OH kinase that displayed aberrant root hair morphology (Preuss et al., 2006; Stenzel et al., 2008). Results presented here show that root hairs are also stunted in RabA2-silenced plants (Figures 5A and 5B), leading to the speculation that RabA subfamily of small GTPases, phosphoinositides, and ROS-dependent formation of tip-focused Ca^{2+} gradients may be components of the same signal transduction pathway that determines cell polarity in growing root hairs. This was partially supported by experiments that have shown that PI-4Kβ colocalizes with the *Arabidopsis* RAB-A4b GTPase and interacts with this small GTPase and with the calcium sensor calcineurin B-like protein, providing a link between Ca^{2+} and phophoinositide signaling (Preuss et al., 2006). How these components are interconnected still remains unclear and constitutes a great challenge for further research.

Root Hair Responses after Rhizobial Infection

Nod factor recognition inhibits polar growth of the root hair and initiates a new growth axis (de Ruijter et al., 1998) in a process that is independent of the calcium spiking in and around the nucleus (Esseling et al., 2003; Miwa et al., 2006). Changes in the cytoskeleton, such as actin remodeling, occur within a few minutes after Nod factor application (Cárdenas et al., 1998; Esseling et al., 2003, 2004), triggering the activation of two different signal transduction pathways in the epidermal cells. One of them is linked to the expression of nodulins, and the other one is associated with root hair deformation through the modulation of components that normally are part of the machinery of the root hair growth. Each one has its own purpose toward the formation of a new organ: differentiation of cortical cells to host the bacteria and entrapment of the rhizobia, respectively. Here, we have found that upon rhizobial infection, reorientation of root hair growth was undetectable in RNAi plants (Figure 7), indicating that both inhibition and reinitiation of root hair tip growth associated with Nod factor perception require RABA2. Considering that polar growth seems to be compromised in RabA2 RNAi roots, it is possible that reorientation is not observed because

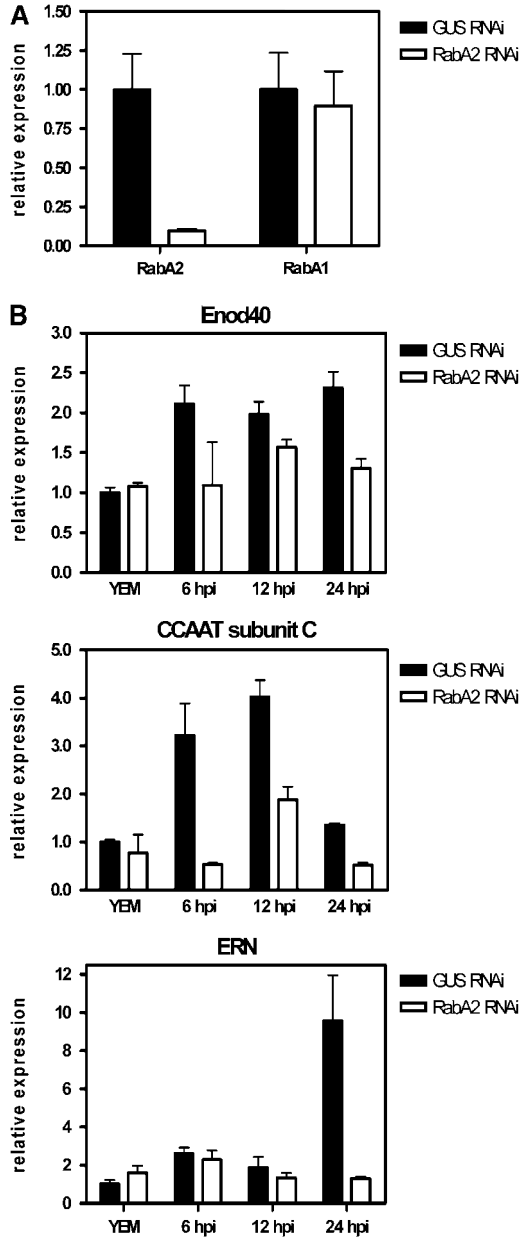

Figure 8. Early Nodulin Expression in RabA2-Silenced Plants.

Composite plants carrying the GUS RNAi (black bars) or the RabA2 RNAi construct (white bars) were inoculated with *R. etli* strain SC15 or with YEM (control). For rhizobia-inoculated plants, root tissue was harvested at the indicated times. Roots from plants inoculated with YEM were harvested at 6, 12, and 24 HAI and then pooled. Total RNA was extracted and transcript levels measured by qRT-PCR. Expression values were normalized to EF1α values. Means ± SD of three technical replicates are presented. Results are representative of three independent biological samples.

(A) RabA2 and RabA1 mRNA levels were determined in YEM-inoculated samples.

(B) Transcript levels of ENOD40, the C subunit of CCAAT binding factor and ERN were determined in RNA samples collected at each time point after inoculation and presented as relative to the values obtained from the YEM-inoculated GUS RNAi sample.

growth is too slow compared with control plants. However, since no curling of root hairs was observed even 72 HAI, it is possible to assume that RABA2 is one component, among others, of the signaling events associated with root hair deformation. As mentioned above, proteins from the Rab family have been shown to play a role in vesicle trafficking. Recently, it has been shown by Peleg-Grossman et al. (2007) that vesicle trafficking genes, such as phosphatydilinositol 3-kinase (PI3K) and small GTPases of the Rab and ADP-ribosylation factor families, are highly expressed in *M. truncatula* roots at early time points after *Sinorhizobium meliloti* infection. In addition, the authors showed that inhibition of PI3K activity blocked endocytotic internalization of the host plasma membrane and ROS production, resulting in a reduction in root hair curling and formation of ITs. The involvement of both ROS metabolism and a Ca^{2+} influx in the early stages of this symbiotic interaction was evidenced also by a pharmacological approach, since application of the flavin inhibitor diphenyliodonium and Ca^{2+} channel antagonist suppressed root hair curling and IT formation (Peleg-Grossman et al., 2007). Recent experiments reported by Cárdenas et al. (2008) revealed that a fast and transient increase of ROS levels is induced within seconds after Nod factor application in actively growing tips of common bean root hair cells. Whether Rab acts within or independently of the PI3K-mediated ROS signaling pathway remains to be elucidated. Nevertheless, our results showing that RabA2 is essential for symbiosis give insights into requirements for initiation of IT formation.

RabA2 Is Required for Nod Factor–Induced Expression of ENODs

The first contact between bacteria and the plant takes place at the root epidermis, root hair being the point of bacterial penetration in the majority of rhizobia–legume interactions. Perception of Nod factors occurs primarily in the epidermal cells, where initial induced responses in the plant lead to ENOD induction in the zone of actively growing root hairs (Journet et al., 1994, 2001; Scheres et al., 1990). Expression of ENODs is under the control of several transcription factors (Kalo et al., 2005; Smit et al., 2005; Andriankaja et al., 2007; Middleton et al., 2007). Mutation of the gene encoding the *M. truncatula* transcription factor ERN blocked the initiation and development of ITs and the Nod factor–induced gene expression (Middleton et al., 2007). Here, we have shown that expression of ERN from common bean is induced by the presence of its microsymbiont *R. etli* (Fig. 8B). However, induction of ERN was almost undetectable in RabA2–silenced roots at early and late time points after infection (Figure 8B; see Supplemental Figure 4 online). The lack of this response, which has been shown to be required for activation of ENOD expression, correlates well with the null nodulation phenotype observed in RabA2 RNAi roots. RabA2 was also found to be required for activation of other molecular responses, since no induction of ENODs such as Enod40 and Hap5 was observed at early time points after infection. However, 6 d after rhizobium inoculation, a slight increase in ENOD40 mRNA levels was detected in RabA2 RNAi roots compared with those inoculated with YEM. It is possible that Enod40 can be partially induced by a signaling pathway that is independent of RabA2, or,

alternatively, it might be a consequence of incomplete silencing by RNAi. Enod40 plays a role in cell division in the cortex leading to the formation of the nodule primordium (Charon et al., 1997, 1999), and it was shown that a functional CCAAT transcription factor is also required for normal nodule development (Combier et al., 2006, 2008). Therefore, RabA2 is required at early steps after Nod factor perception prior to early signaling events and root hair deformation. This temporal pattern is consistent with our previous reported data in which RabA2 was identified in a cDNA library obtained from RNA 24 HAI; secondly, its transcripts were found to increase 6 HAI and reach its maximum peak 12 HAI (Peltzer Meschini et al., 2008).

Concluding Remarks

The results presented here and those reported previously by others lead us to suggest that the altered root hair phenotype and lack of nodulation in RabA2 RNAi plants is most likely the consequence of a compromised vesicle trafficking. Polarized growth of root hairs requires, among other factors, traffic of vesicles moving cell wall and membrane materials from the Golgi to the apex of the cell. Rab proteins assist proper targeting and docking of vesicle during active cell expansion of root hairs. On the other hand, contact of epidermis with compatible rhizobia implies the perception of Nod factor by receptors present in the root hair plasma membrane, which in turn induce a signal transduction pathway and root hair deformation. To support redirectioning of tip growth, RabA2 would be recruited to assist delivery of cell wall material to the new point of growth. Furthermore, this process seems to be required for nodulin induction. Certainly, it will be of interest to fully elucidate the proposed function for RabA2 in regulating both root hair growth and preinfection stages of the legume–*Rhizobium* interaction. Although, several aspects of this hypothetical model need to be demonstrated, we believe it offers a novel insight in the biology of symbiosis and opens new avenues to explore in the future.

METHODS

Biological Material

Seeds of common bean (*Phaseolus vulgaris*) cultivar NAG12 (Mesoamerican) were provided by Susana García Medina (Instituto Nacional de Tecnología Agropecuaria Salta, Cerrillos, Argentina). *Rhizobium etli* strains SC15 and 55N1 were previously reported (Aguilar et al., 2004). *Agrobacterium rhizogenes* strain K599 (Bond and Gresshoff, 1993) and the same strain carrying the p35S:GFPGUS+ construct were kindly provided by Federico Sánchez (Universidad Nacional Autónoma de México, Cuernavaca, México). *R. etli* strain 55N1 was transformed with the pDG71 vector (Gage, 2002) by electroporation according to Oshiro et al. (2006) to obtain a strain expressing GFP. Root hairs were prepared as described (Peltzer Meschini et al., 2008).

A. rhizogenes–Mediated Common Bean Transformation

Common bean seeds were surface sterilized and germinated for 2 d as described by Peltzer Meschini et al. (2008). Sprouts were then transferred to small plastic pots containing vermiculite and watered with Fahraeus media (Fahraeus, 1957) supplemented with 0.8 M KNO_3. 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 for 5 d and then transformed with *A. rhizogenes* essentially as described by Estrada-Navarrete et al. (2006) with the following modifications: after hairy roots were developed (~18 d after inoculation with *A. rhizogenes*), the main root was cut 1 cm below the inoculation site, and the plants were transferred to acrylic boxes (17 × 22 × 4.5 cm) containing slanted agar-Fahraeus media or the same media supplemented with 0.8 M KNO_3. Approximately 5 d after transplantation, hairy roots were observed under UV light in a Leica Fluo MZ8 dissection microscope (Leica Microsystems), and untransformed roots, not expressing GFP, were excised with a scalpel. The following day, roots were inoculated with 5 mL of a liquid culture of *R. etli* strains 55N1 or SC15 as described by Peltzer Meschini et al. (2008). Nodule number was recorded every 24 h starting at 3 d after inoculation. Only data from overexpressing plants that showed 10-fold or higher increase in mRNA levels of RabA2 were considered. In the case of RabA2 RNAi, all plants exhibiting >80% of silencing were included.

To assess silencing of RabA2 over time, tissue from globular tumors and developing hairy roots was collected from five biological replicates every 3 d over a period of 27 d. Material was frozen in liquid nitrogen and stored at −80°C.

Plasmid Construction for Reverse Genetics and Localization

To create a construct for RNAi-mediated silencing of RabA2, a fragment corresponding to the coding region of this gene was amplified by PCR using the primers 5′-CACCGGTACCGTGCCATTACCAGT-3′ and 5′-TTGCCTCCAGTGCAGATGTC-3′ and cDNA from bean nodules as template. The PCR product was cloned into the pENTR/D-TOPO entry vector following manufacturer's instructions (Invitrogen) and recombined into the Gateway-compatible destination vector pK7GWIWG2D(II) (Karimi et al., 2002) to produce the RabA2 RNAi construct. pK7GWIWG2D carries a screenable marker containing the *rol*D promoter fused to the coding sequences of the enhanced GFP linked to the endoplasmic reticulum-targeting signal (EgfpER) and 35S terminator for early visualization and selection of transgenic roots. The GUS RNAi plasmid was generated recombining a pENTR-GUS (Invitrogen) into the same destination vector. For overexpression of RabA2, a fragment corresponding to the ORF was amplified by PCR using 5′-CACCATGACGCATCGAGTGGAC-3′ and 5′-CTAAGTGGAGCAGCAGCCTCTC-3′ as primers and cloned into the pENTR/D-TOPO entry vector. This entry clone was recombined with the destination vector pB7WG2D (Karimi et al., 2002) using the Gateway technology (Invitrogen) to generate the p35S:RabA2. pB7WG2D also contains the EgfpER marker for selection of transgenic roots. For subcellular localization experiments, the entry clone containing RabA2 ORF was recombined into the destination vector pMDC43 (Curtis and Grossniklaus, 2003) to produce a translational fusion to the C-terminal end of GFP. Transcription of this fusion was controlled by the CaMV35S promoter. All constructs were introduced into the *A. rhizogenes* strain K599 by electroporation.

Quantitative RT-PCR Assays

RNA extraction, cDNA synthesis, and qRT-PCR assays 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. Absence of contaminant genomic DNA was confirmed in reactions with DNase-treated RNA as template. Amplification of common bean EF1α was used to normalize the amount of template cDNA. At least three biological replicates were performed per condition.

Primers for qRT-PCR assays of RabA2 and Hap5 transcripts were as previously described for Pv-RHS24-03 and Pv-RHS24-27, respectively, by Peltzer Meschini et al. (2008). For qRT-PCR analysis of RabA1, the following primers located in the 5′ UTR region were used:

5′-TCGCAGAAGACAATCCCCAC-3′ and 5′-CTGATCGATTATGACGC-TACACAAA-3′. Primers for Enod40 were 5′-AGTTTTGTTGGCAAGCA-TCC and 5′- TAAGCACAAGCAAACTGTTG and for ERN: 5′-CTTCTC-GAATTCGGAATCTT-3′ and 5′-TGTTGCCATTGCCATTGTT-3′. Enod40 and ERN nucleotide sequence data were retrieved from the DFCI Bean Gene Index database (http://compbio.dfci.harvard.edu/tgi/plant.html) by a BLASTN search using the *M. truncatula* sequences (Crespi et al., 1994; Middleton et al., 2007).

Molecular Phylogenetic Analysis

Amino acid sequences of *Arabidopsis* Rabs were retrieved from public databases based on classification of the Rab family proposed by Rutherford and Moore (2002). Sequences were aligned using ClustalW (Thompson et al., 1994), and the phylogenetic analysis was performed with the MEGA4 package developed by Tamura et al. (2007) using the neighbor-joining method (Saitou and Nei, 1987) with 1000 trials to obtain bootstrap values. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). All positions containing gaps and missing data were eliminated from the data set. The alignment used for the phylogenetic analysis is available as Supplemental Data Set 1 online. Alignment of the common bean amino acid sequence with Rab protein sequences from other species was generated with ClustalW and shaded with the 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).

Quantification of Root Hair Number and Length

Number and length of root hairs was determined on transgenic roots at 7 d after transplantation to agar-Fahraeus acrylic boxes. Roots were observed under a Leica MZ8 dissection microscope. Digital images were captured using a DFC 480 camera and analyzed with Image-Pro Plus 5.1 (Media Cybernetics). To avoid variations linked to the longitudinal root sections, the zone between 3 and 5 mm from the root tip was analyzed. Quantification data are the means of >500 root hairs representing 10 roots from 15 plants. Root hair deformation and IT formation were counted from at least five independent plants in the root hair elongation zone and expressed as percentage of total root hairs showing deformation or associated with an IT ($n \geq 150$).

Microscopy and Imaging

Bright-field and epifluorescence imaging of root hair deformation and IT formation were performed with an Olympus BX51 microscope (Olympus Optical). Images were recorded with an Olympus DP70 camera and processed using Image-Pro Plus 5.1 (Media Cybernetics).

Several independent transgenic roots ($n = 20$) containing the p35S: GFP-RABA2 construct in pMDC43 were analyzed under the confocal microscope. Confocal microscopy was performed on an Olympus FV300 using a 488-nm argon laser for excitation, an Uplan FI 20X objective (numerical aperture of 0.5) or an Uplan FL 10X (numerical aperture of 0.3), a dicroic SDM 570 to split the acquisition channel (Olympus Optical). Emission was collected at 510 nm for GFP. Images were captured with FluoView 3.3 (Olympus Optical) and processed with ImageJ 1.42q (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: RabA2 (GQ475492), RabA1a (At1g06400), RabA1b (At1g16920), RabA1c (At5g45750), RabA1d (At4g18800), RabA1e (At4g18430), RabA1f (At5g60860), RabA1g (At3g15060), RabA1h (At2g33870), RabA1i (At1g28550), RabA2a (At1g09630), RabA2b (At1g07410), RabA2c (At3g46830), RabA2d (At5g59150), RabA3 (At1g01200), RabA4a (At5g65270), RabA4b (At4g39990), RabA4c (At5g47960), RabA4d (At3g12160), RabA5a (At5g47520), RabA5b (At3g07410), RabA5c (At2g43130), RabA5d (At2g31680), RabA5e (At1g05810), RabA6a (At1g73640), and RabA6b (At1g18200). Sequences used in the alignment are *Lotus japonicus* Rab11c (CAA98179), *Glycine max* RabA2 (AF532625), and *Medicago truncatula* RabA2 (DFCI Medicago Gene Index TC114360). RabA1 (CV531039), Enod40 (CV536158), and ERN (CV535404) were obtained from the DFCI Bean Gene Index database.

Supplemental Data

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

Supplemental Figure 1. Sequence Analysis of RabA2.

Supplemental Figure 2. Expression of the RabA2 RNAi Construct in the Root Does Not Produce Systemic Silencing of RabA2 in Shoot.

Supplemental Figure 3. RabA2 Silencing or Overexpression Does Not Affect Shoot Length or Biomass in the Experimental System.

Supplemental Figure 4. Effect of RabA2 Silencing and Overexpression on Root Hairs Is Not Affected by Nitrate.

Supplemental Figure 5. Early Nodulin Expression in RabA2-Silenced Plants 6 d after Inoculation.

Supplemental Table 1. Expression of Common Bean RabA2 and Its Homologs in Selected Species Relative to Root Values.

Supplemental Table 2. Expression of Common Bean RabA2 and Its Homologs during Nodulation, Relative to Values Obtained for Uninoculated Roots.

Supplemental Movie 1. GFP-RABA2 Is Localized in Vesicles inside Root Hairs.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis in Figure 1.

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A Small GTPase of the Rab Family Is Required for Root Hair Formation and Preinfection Stages of the Common Bean–*Rhizobium* Symbiotic Association

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