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RESEARCH PAPER

Transcriptional and functional variation of *NF-YC1* in genetically diverse accessions of *Phaseolus vulgaris* during the symbiotic association with *Rhizobium etli*

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ABSTRACT

***Phaseolus vulgaris* (common bean) is an agronomic important legume crop native to America, where two centres of genetic diversification (GD) are recognised, one in Mesoamerica and the other in the south Andes. Mesoamerican bean accessions have preferential and more efficient nodulation with *Rhizobium etli* strains carrying the allele *nod*C type-α, which is predominant in soils of Mesoamerica. It was previously demonstrated that the host nuclear factor NF-YC1, which is involved in nodule formation and rhizobial infection, contributes to this preferential selection and enhances nodulation in the domesticated accession NAG12 from Mesoamerica. Here, we show that both domesticated and wild Mesoamerican beans exhibit higher nodulation performance with a *nod*C type-α than with a *nod*C type-δ strain. Transcripts of *NF-YC1* significantly increased in roots of these accessions 24 h post-inoculation (hpi) with the *nod*C type-α strain. On the other hand, accessions from the Andean GD centre formed a higher number of nodules with a strain carrying the *nod*C type-δ, which is predominant in Andean soils. However, *NF-YC1* transcript levels did not exhibit significant changes in Andean accessions upon inoculation with the *nod*C type-δ strain, at least at 24 hpi. RNA interference (RNAi)-mediated gene silencing of *NF-YC1* in the domesticated Andean accession Alubia showed that *NF-YC1* or a closely related member of this family is required for nodule formation and bacterial infection, in agreement with observations in Mesoamerican common beans. Isolation and sequencing of the full-length ORF of *NF-YC1* from Alubia revealed that it was identical to the sequence previously identified in the Mesoamerican accession NAG12. Interestingly, overexpression of *NF-YC1* had a negative impact on nodule formation in the Alubia accession, independently of the *R. etli* lineage. Our findings suggest that transcriptional and functional variation of *NF-YC1* occurs among genetically diverse bean accessions, which might positively or negatively contribute to the fine-tuning mechanisms that regulate nodule formation in the common bean–*R. etli* symbiosis.**

INTRODUCTION

Most legume plants establish a nitrogen-fixing association with soil bacteria collectively known as rhizobia. This symbiotic interaction results in the formation of a new root organ, the nodule, where bacteria are allocated, differentiate into bacteroids and fix atmospheric dinitrogen into reduced forms that are readily available for the plant. In exchange, bacteria obtain a source of carbon. The development of functional N-fixing nodules relies on two independent, but coordinated programmes: the re-initiation of cell division in the root cortex to form the nodule primordium and infection by the bacteria that will colonise the nodule (Madsen *et al.* 2010). In most interactions, rhizobial infection occurs predominantly through root hairs, which swell and curl to entrap the bacteria into infection foci. A tubular structure, called the infection thread (IT), is formed by invagination of the plant plasma membrane. Within it, bacteria multiply and progress toward the actively dividing cortical cells, where they are released and switch to an intracellular lifestyle (Oldroyd *et al.* 2011).

At the root-soil interface, the interaction is initiated by exchange of signals between the plant and the bacterium. Nod factors are lipochito-oligosaccharide molecules produced by rhizobia in response to flavonoids exuded by the plant, which are subsequently perceived by receptor-like kinases (RLK) with LysM motifs in the extracellular domain (Limpens *et al.* 2003; Radutoiu *et al.* 2003). Perception of Nod factors triggers a signalling pathway that ultimately activates the expression of a number of genes known as early nodulation genes or 'early nodulins' (ENODs) in both epidermal and cortical tissues (Oldroyd & Downie 2008). Several transcriptional regulators required for nodule formation and/or bacterial infection have been characterised through forward or reverse genetic studies. Nodule inception (NIN) is a transcriptional regulator required for bacterial infection and development of both determinate and indeterminate types of nodule (Schauser *et al.* 1999; Marsh *et al.* 2007). Two members of the GRAS domain transcriptional regulators, NSP1 and NSP2, and ERN (Ethylene response factor Required for Nodulation) were identified as transcription factors required for nodulation and transcriptional activation of

the early nodulation gene *ENOD11* in epidermal cells (Kalo *et al.* 2005; Smit *et al.* 2005; Andriankaja *et al.* 2007; Middleton *et al.* 2007; Hirsch *et al.* 2009). The list of transcriptional regulators also includes two subunits of the nuclear factor Y (NF-Y) family of transcription factors, NF-YA and NF-YC (also known as HAP2 and HAP5, respectively), which are required for both nodule organogenesis and rhizobial infection (Combier *et al.* 2006, 2008; Zanetti *et al.* 2010). NF-Ys are evolutionary conserved transcription factors composed of three subunits (NF-YA, NF-YB and NF-YC), which bind with high affinity and specificity to the CCAAT box, a *cis*-element present in many eukaryotic promoters (Mantovani 1999).

Phaseolus vulgaris (common bean) is a widely distributed legume crop of significant importance for many countries, and represents a major source of protein input in the diets of developing nations in Africa and the Americas (Broughton *et al.* 2003). It is generally accepted that *P. vulgaris* is original from the American continent (Bitocchi *et al.* 2012) where two major centres of genetic diversification (GD) have been proposed: the Mesoamerican centre, distributed from northern Mexico to Colombia, and the Andean centre, from southern Peru to northwestern Argentina (Gepts & Bliss 1988). The analysis of both an alpha-amylase inhibitor and internal transcribed spacer (ITS) sequences suggested that divergence of Mesoamerican and Andean gene pools took place about 0.5 million years ago (Kwak & Gepts 2009). Domestication of *P. vulgaris* took place independently at each centre from these already diverged gene pools. Five species have been recognised as microsymbionts of *P. vulgaris* forming nodules in nature: *Rhizobium etli* bv. *phaseoli*, *R. leguminosarum* bv. *phaseoli*, *R. tropici*, *R. gallicum* and *R. giardini* (Amarger 2001). Among them, *R. etli* bv. *phaseoli* is the dominant species found associated with both wild and cultivated common beans from Mexico, Colombia and the southern Andes (Amarger 2001). The characterisation of polymorphisms in the nodulation gene *nod*C among *R. etli* strains from a wide range of geographic origins identified three different alleles designated type-α, -γ and -δ. Aguilar *et al.* (2004) analysed the distribution of *nod*C alleles in rhizobial populations from American soils and found that strains isolated from Mesoamerica predominantly show the alleles *nod*C type-α and -γ, whereas the allele type-δ was prevalent in strains isolated from Andean soils. Moreover, the authors showed that nodules of wild and cultivated Mesoamerican beans were almost exclusively occupied by strains carrying the allele *nod*C type-α, suggesting co-evolution between the macro- and micro-symbionts at this centre of genetic diversification. A subsequent study revealed that the cultivated Mesoamerican accession NAG12 formed more nodules with strain *nod*C type-α SC15 as compared with strain *nod*C type-δ 55N1 (Peltzer Meschini *et al.* 2008). In that study, a subtractive hybridisation approach was applied to identify host genes involved in this preferential and more efficient nodulation. This led to isolation of a number of cDNA clones, whose mRNAs accumulated at higher levels in roots inoculated with strain SC15 than in those inoculated with strain 55N1. Among others, one of these clones encoded the above-mentioned NF-YC1 transcription factor required for nodule organogenesis and bacterial infection. We have previously shown that overexpression of NF-YC1 in roots of the Mesoamerican accession NAG12 was sufficient to increase nodule formation and improve the symbiotic outcome of *R. etli* strains carrying the allele *nod*C-δ (Zanetti *et al.* 2010). These findings led us to investigate whether the increase of *NF-YC1* transcript levels in response to specific strains of rhizobia was a common mechanism present in both wild and domesticated accessions of common bean from the Andean and Mesoamerican GD centres.

In this study, we confirmed that both domesticated and wild Mesoamerican beans form more nodules with a strain that belongs to the *nod*C-α genotype (SC15) than with one that belongs to the *nod*C-δ genotype of *R. etli* (55N1). At early time points after inoculation, Mesoamerican accessions exhibited higher accumulation of *NF-YC1* transcript in response to strain SC15 than to strain 55N1. On the other hand, domesticated and wild Andean beans developed a higher number of nodules with strain 55N1 than with SC15. However, no significant increase in *NF-YC1* mRNAs in response to strain 55N1 was detected in these accessions, at least at the time point analysed here [24 h post-inoculation (hpi)]. We also investigated whether overexpression of this transcription factor in an Andean accession would lead to an increase in nodule formation with strains that display low efficiency in Andean beans. Interestingly, overexpression of *NF-YC1* did not increase nodulation in the Andean accession, but rather decreased nodule formation independent of the *R. etli* genotype. However, infection thread formation and progression was not affected in *NF-YC1* overexpressing roots. Knockdown of *NF-YC1* by RNAi in the Andean accession revealed a similar phenotype to that previously observed in the Mesoamerican accession NAG12, which is characterised by a reduced number of nodules and defects in the formation and progression of infection events.

MATERIAL AND METHODS

Biological material

Phaseolus vulgaris (common bean) accessions NAG12, Camilo, Alubia and Aborigineus were obtained from the Instituto Nacional de Tecnología Agropecuaria-Salta, Argentina. Accession G24591 was obtained from Centro Internacional de Agricultura Tropical, Colombia. A complete list of accessions and their origins is provided in Table 1. *Agrobacterium rhizogenes* strain K599 (Bond & Gresshoff 1993) and the same strain carrying the p35S:GFPGUS+ construct were obtained from Federico Sánchez (Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico). *Rhizobium etli* strain SC15 and 55N1

Table 1. Cultivated and wild common bean accessions used in this study.

Accession	GD Centre	Domesticated or wild	Reference
NAG12[a]	Mesoamerican	Domesticated	Galván *et al.* (2001)
Camilo[b]	Mesoamerican	Domesticated	Galván *et al.* (2001)
G24591	Mesoamerican	Wild	Lioi *et al.* (2003)
Alubia Cerrillos	Andean	Domesticated	Galván *et al.* (2001)
Aborigineus	Andean	Wild	Burkart (1943)

[a]Introgression of [(G03664 × G02045) × (G04792 × G05694)] × [(G04495 × G05431) × (G03645 × G05481)].
[b]Introgression XAN 19 × ICA PIJAO.

were previously described (Aguilar *et al.* 2004). *R. etli* strain 55N1 expressing GFP was reported in Blanco *et al.* (2009).

Plant growth and rhizobia inoculation

Common bean seeds were surface sterilised as previously described and germinated on wet paper for 2–3 days at 26 °C in the dark. Germinated seedlings were transferred to acrylic boxes containing slanted agar-Fahraeus media free of nitrogen (Fahraeus 1957). Seedlings were grown in a MLR-350HT growth chamber (Sanyo Electric, Sanyo, Osaka, Japan) maintained at 26 °C with a 16 h/8 h day/night cycle and 80% humidity. Five days after transplantation to acrylic boxes, root seedlings were inoculated with 5 ml *R. etli* culture grown in liquid YEM media to OD_{600} of 0.8. For RNA extraction, root tissue was harvested 24 h after inoculation, frozen in liquid N_2 and stored at −80 °C. For the time-course of nodule formation in wild-type plants, the number of nodules per plant was recorded from a minimum of five plants for each *P. vulgaris* accession at 5, 7, 10 and 14 days post-inoculation (dpi).

Constructs, hairy root transformation and phenotype analysis

The p35S:FLAG-NF-YC1 construct (Figure S1A) used for overexpression in Alubia accession is the same as used for NAG12 (Zanetti *et al.* 2010). The *GUS* RNAi construct is described in Blanco *et al.* (2009). The *NF-YC* RNAi construct was obtained by PCR amplification of a 210-bp fragment using the primers NF-YC RNAi F and NF-YC RNAi R, as described in Zanetti *et al.* (2010), cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA), followed by recombination into the destination vector pK7GWIWG2D (II), which carries EgfpER as a reporter, for early visualisation and selection of transgenic roots (Karimi *et al.* 2007). RNAi cassettes of both constructs are illustrated in Figure S2. All constructs were introduced into *A. rhizogenes* strain K599 by electroporation. Transformation of common bean roots was performed essentially as described previously (Blanco *et al.* 2009). Composite plants, consisting of wild-type shoots and transgenic roots, were transferred to acrylic boxes containing slanted agar-Fahraeus media. Five days after transplantation to acrylic boxes, transgenic roots were inoculated with *R. etli* strain SC15 or 55N1 as described above. The number of nodules per root formed by each strain in control (35S:GFPGUS) or 35S:FLAG-NF-YC1 roots was recorded at 5, 7, 10, 12 and 14 dpi. The number of nodules per root formed in *GUS* RNAi and *NF-YC1* RNAi roots by strains SC15 and 55N1 was recorded at 6, 9 and 14 dpi. When roots were inoculated with the *R. etli* strain expressing GFP, the number of nodules per root was quantified at 15 dpi. Nodule occupancy in these roots was visualised by epifluorescence using a Leica MZ8stereo microscope (Leica, Wetzlar, Germany). Digital images were captured using a DFC 480 camera and analysed with Image-Pro Plus 5.1 (Media Cybernetics, Bethesda, MD, USA). Nodule diameter was recorded for over 30 nodules using the measuring tool of Adobe Photoshop v. 7.0. Infection threads were visualised with epifluorescence using a Nikon Eclipse Ti inverted microscope (Nikon Instruments, Melville, NY, USA) under white and UV light with appropriate GFP filters. Images were captured using a Nikon digital high-resolution DS-Ri1 camera (Nikon Instruments) and NIS-Elements Imaging software F3.0 (Nikon Instruments).

RNA extraction and expression analysis

For expression analysis, root tissue from at least three plants was collected and pooled. Total RNA extraction was performed with Trizol following manufacturer's instructions (Invitrogen). RNA concentration was determined by measuring absorbance at 260 nm in a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and quality was evaluated by electrophoresis in ethidium bromide-stained agarose gels. One microgram of total RNA was treated with RNAse free-DNAse I and used in a reverse transcription reaction with 0.5 μg of oligo dT_{15} primer and 200 U of M-MLV-RT following instructions provided by the manufacturer (Promega, Madison, WI, USA). Individual cDNA samples were used in quantitative PCR (qPCR) reactions as previously reported (Zanetti *et al.* 2010). Primers used for measurement of common bean *ERN*, *ENOD40*, *NF-YC1*, *NF-YC2* and *NF-YC3* transcripts were described previously (Zanetti *et al.* 2010). PCR products amplified from cDNA of the different accessions were cloned into the TOPO-TA vector (Invitrogen) and sequenced to confirm their identity. Amplification of common bean elongation factor 1α (*EF1α*) was used to normalise the amount of template cDNA following Peltzer Meschini *et al.* (2008). Statistical significance of mean differences between samples was determined with unpaired two tailed *t*-tests using at least two biological replicates. Each biological replicate was performed using at least three technical measurements. NF-YC1 forward (5′GCTCAGCAGTCTCACCCCTACA-3′) and OCS reverse (5′-CATGCGATCATAGGCGTCTCG-3′) primers were used in semiquantitative PCR amplification reactions for detection of the *FLAG-NF-YC1-OCS* transcript.

Sequence analysis and accession numbers

The ORF of *NF-YC1* from the Alubia accession was PCR amplified from root cDNA using the NF-YC1 OE F and NF-YC OE R primers described in Zanetti *et al.* (2010). The PCR product was cloned into the pENTR/D-TOPO (Invitrogen) and sequenced from both ends. The nucleotide sequence of Alubia *NF-YC1* ORF was compared against the GenBank non-redundant database using BLASTN (Altschul *et al.* 1997). Sequence data used in this article can be found at the EMBL/GeneBank or DFCI gene index data libraries under the following accession numbers: *NF-YC1* (GQ913690), *NF-YC2* (TC9603 composed of ESTs CV532047, CV531056, CV544140, FE698328 and FE707163), *NF-YC3* (TC10494 composed of ESTs FE681706 and FE681701), *ENOD40* (CV536158) and *ERN* (CV535404).

RESULTS

Wild and domesticated common bean accessions formed more nodules with *R. etli* strains that are predominant in soils of the same GD centre

In a previous study, we have shown that one domesticated accession of *P. vulgaris* from Mesoamerica (NAG12) and one from the Andes (Alubia) were more efficiently nodulated by *R. etli* strains that predominate in soils of the corresponding GD centres (Peltzer Meschini *et al.* 2008). To investigate whether this behaviour could be extended to other Mesoamerican and

Andean bean accessions, we examined the time course of nodule formation of both domesticated and wild accessions upon inoculation with either strain SC15 or strain 55N1. Three accessions from Mesoamerica and two from the Andean GD centres were selected, including the previously mentioned domesticated accessions NAG12 and Alubia (Table 1). Roots of 7-day-old plants grown in the absence of nitrogen were inoculated and the number of nodules formed by each strain recorded (Fig. 1). As expected, the number of nodules formed by NAG12 plants with strain SC15 was significantly higher than those formed with 55N1 at all times analysed (*t*-test, $P < 0.001$). The wild accession G24591 also exhibited a significantly higher number of nodules when inoculated with SC15 than with 55N1 at all the time points (*t*-test, $P < 0.01$), whereas in the domesticated accession Camilo, although the number of nodules was also higher with strain SC15 than with 55N1, the differences were not significant ($P > 0.1$). On the other hand, the number of nodules developed in the Andean accession Alubia was significantly higher when roots were inoculated with strain 55N1 than with SC15 at 7, 10 and 14 dpi (*t*-test, $P < 0.05$). Differences between the two strains were less pronounced in the wild Andean accession Aborigineus than in the domesticated Alubia; however, strain 55N1 still formed more nodules than strain SC15 (*t*-test, $0.05 < P < 0.1$) at all the time points analysed (Fig. 1). These results confirmed that common beans from Mesoamerica are more efficiently nodulated by strains carrying the *nod*C allele that is predominant in Mesoamerican soils, whereas Andean beans exhibit more efficient nodulation with strains predominant in soils of the Andean centre. It is noteworthy that wild accessions developed fewer nodules than domesticated ones. In the case of the Andean cultivars, this is particularly notable with the more efficient strain 55N1, which formed ~40 nodules per plant at 14 dpi in the wild accession Aborigineus compared to ~70 nodules per plant in the domesticated Alubia. On the other hand, the Mesoamerican wild bean accession G24591 developed ~45% less nodules than the domesticated NAG12 with either strain SC15 or stain 55N1 (Fig. 1).

The *NF-YC1* transcripts accumulated in Mesoamerican and wild Andean accessions in response to *R. etli* strain SC15, but not to strain 55N1

In order to evaluate whether *NF-YC1* transcript levels were up-regulated by each strain in domesticated and wild accessions, we analysed accumulation of *NF-YC1* transcripts at an early time point after inoculation. Levels of *NF-YC1* mRNAs were first evaluated in non-inoculated roots of the different accessions, revealing that roots of domesticated accessions NAG12, Camilo and Alubia had similar NF-YC1 transcript levels, whereas the wild accessions G24591 and Aborigineus had lower levels (~65% and 75%, respectively) as compared to the domesticated accessions corresponding to the same GD centre (see Figure S3). Upon inoculation, NF-YC1 accumulated to higher levels in roots of NAG12, Camilo and G24591 at 24 hpi with SC15 than in control roots, with increases of 7-, 2.5- and 4-fold, respectively. On the other hand, *NF-YC1* transcript levels did not change in accessions NAG12 and G24591 and decreased in Camilo upon inoculation with strain 55N1 as compared to control roots (Fig. 2A). These results indicate that *NF-YC1* is specifically up-regulated in wild and domesticated Mesoamerican common beans by the strain carrying the *nod*C-α allele. However, in roots of the Andean accession Alubia, *NF-YC1* transcript levels showed only a slight increase that was

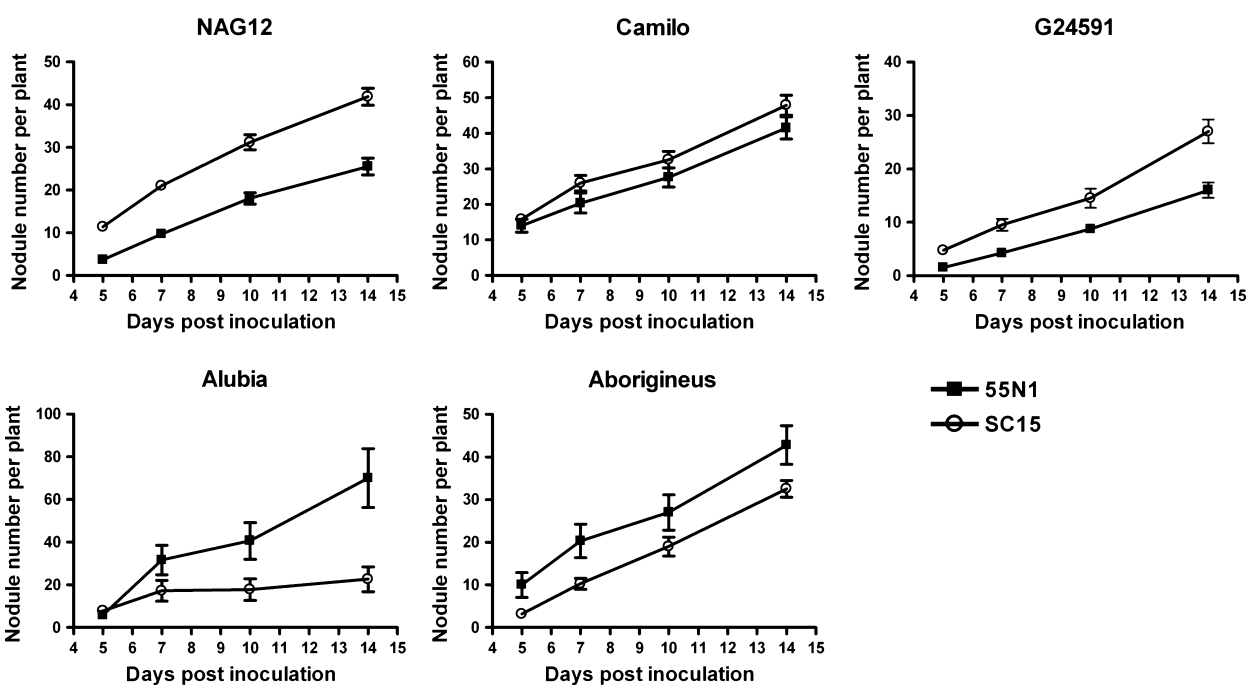

Fig. 1. Time course of the number of nodules per plant in Mesoamerican and Andean *Phaseolus vulgaris* accessions upon inoculation with *Rhizobium etli* SC15 or 55N1. Error bars represent SE. The number of nodules formed at 14 dpi by SC15 was significantly different than that of strain 55N1 in an unpaired two-tailed *t*-test for NAG12 ($P < 0.0001$), G24591 ($P < 0.01$), Alubia ($P < 0.05$) and Aborigineus ($P < 0.1$), but not for Camilo ($P > 0.1$).

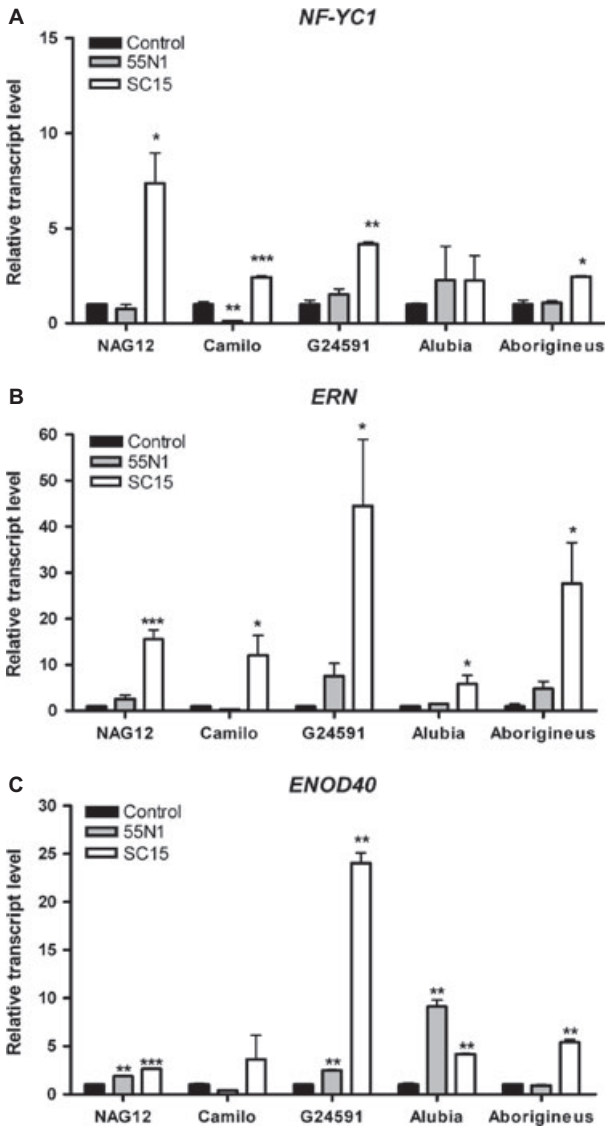

Fig. 2. qRT-PCR analysis of *NF-YC1* (A), *ERN* (B) and *ENOD40* (C) mRNA levels in Mesoamerican and Andean *P. vulgaris* accessions inoculated with *R. etli* SC15 (white bars), 55N1 (grey bars) or YEM (control, black bars). Tissue from roots 24 hpi was harvested and pooled. Transcript levels were quantified and normalised to *EF1α*. Error bars represent SD of at least two biological replicates. Single, double and triple asterisks indicate that expression values in 55N1 or SC15 inoculated roots are significantly different from control roots (inoculated with YEM) in an unpaired two-tailed *t*-test with $0.01 < P < 0.05$, $0.001 < P < 0.01$ and $P < 0.001$, respectively.

not statistically significant upon inoculation with strain SC15 or 55N1 as compared to uninoculated roots; whereas, in the wild accession Aborigineus, *NF-YC1* levels did not change in response to strain 55N1 and increased about 2.4-fold in response to strain SC15 (Fig. 2A).

In order to test whether the strain-specific up-regulation observed in *NF-YC1* also occurs in other genes involved in the symbiotic interaction, we investigated the expression of two early nodulation genes known to accumulate in roots of the NAG12 accession at 24 hpi with *R. etli* (Blanco *et al.* 2009; Zanetti *et al.* 2010). These are *ERN*, a transcription factor required for the formation of infection threads and nodule invasion, and *ENOD40*, RNA involved in the activation of cortical cell divisions necessary for nodule formation (Crespi *et al.* 1994; Middleton *et al.* 2007). Analysis of *ERN* in different *P. vulgaris* accessions revealed that transcripts of this gene accumulated more in response to strain SC15 than to strain 55N1, independently of the origin of the accession (Fig. 2B). In Mesoamerican accessions, *ERN* mRNA levels increased 12- to 45-fold with strain SC15 with respect to control roots, whereas only two and sevenfold induction were observed in NAG12 and G24591 upon inoculation with strain 55N1, respectively, and no increases were detected in the Camilo accession with strain 55N1. In contrast, levels of *ERN* increased 6- and 20-fold in response to strain SC15 in roots of Andean accessions Alubia and Aborigineus, respectively, but were not significantly modified in response to strain 55N1. Analysis of *ENOD40* mRNA revealed a distinct transcriptional response to each strain in the domesticated and wild accessions, either from the Mesoamerican or the Andean GD centre (Fig. 2C). In NAG12, *ENOD40* transcript level increased about twofold in response to either strain SC15 or 55N1, in agreement with our previous report (Zanetti *et al.* 2010). In accession Camilo, mRNA levels of *ENOD40* increased in response to strain SC15 but not 55N1, whereas in the Mesoamerican wild G24591, *ENOD40* transcripts accumulated 24-fold in response to strain SC15 and only two and half fold in response to 55N1 (Fig. 2C). In the Andean accessions, accumulation of *ENOD40* transcripts differed between the domesticated Alubia and the wild Aborigineus. In Alubia, transcripts accumulated at higher levels in response to strain 55N1 than SC15 as compared to control roots (nine *versus* fourfold), whereas in Aborigineus, mRNAs accumulated fivefold in roots inoculated with SC15 with respect to control roots and did not change significantly in response to strain 55N1 (Fig. 2C). Together, these results suggest that the induction of *ERN*, a gene related to the bacterial infection process in epidermal cell layers, is a common response of both Mesoamerican and Andean beans to strain SC15; while *ENOD40*, a gene related to cortical cell division, showed more variable transcriptional response to the two strain among the Mesoamerican and Andean accessions analysed here. Interestingly, *ERN* and *NF-YC1* genes, but not *ENOD40*, were up-regulated in response to SC15 in all three Mesoamerican accessions and in the wild Andean Aborigineus.

Both overexpression and knock-down of *NF-YC1* resulted in reduced nodule number in the Andean accession Alubia

Overexpression of NF-YC1 protein in the Mesoamerican accession NAG12 increased the efficiency of nodule formation and nitrogen fixation when roots were inoculated with strains that are less efficient and competitive (Zanetti *et al.* 2010), revealing that this transcription factor might influence strain selection by the plant. To evaluate whether overexpression of NF-YC1 had a positive impact on nodule formation in the Andean common beans, we generated composite plants, with a wild-type shoot and transgenic roots, expressing the NF-YC1 protein fused to the C-terminus of the FLAG epitope (FLAG-NF-YC1) under control of the near-constitutive promoter cauliflower mosaic virus 35S (see Figure S1A). The Alubia accession was selected because it is an agronomically important cultivated variety of common bean and showed a significantly higher number of nodules upon inoculation with the *nod*C-δ strain than with

*nod*C-α. The full ORF of *NF-YC1* was PCR amplified from cDNA of the Alubia accession, cloned and sequenced. The sequence obtained was 100% identical at nucleotide level to that of the *NF-YC1* cDNA previously isolated from NAG12; therefore, we transformed Alubia roots with the 35S:FLAG-NF-YC1 construct previously used in experiments with NAG12 (Zanetti *et al.* 2010). Control roots were transformed with the p35S:GFPGUS+ vector, which expresses GFP and GUS proteins. In Alubia, the percentage of plants that produced hairy roots ranged from 88% to 91% ($n > 40$). Each composite plant produced an average of five hairy roots independent of the construct used for transformation (5.3 ± 0.45 and 5.2 ± 0.24 hairy roots for p35S:GFPGUS+ and p35S:FLAG-NFYC1, respectively, $n > 20$). Fluorescence microscopy revealed that expression of GFP was detected in over 87% of the hairy roots evaluated ($n > 80$). Expression of *NF-YC1* was examined in both 35S:FLAG-NF-YC1 roots and control roots transformed with the p35S:GFPGUS+ vector with qRT-PCR using primers located in the coding region of *NF-YC1*. Levels of *NF-YC1* mRNA were about twofold higher in NF-YC1 overexpressing roots than in control roots (Fig. 3A). In addition, expression of the transgene was confirmed by semiquantitative RT-PCR using a primer located in the coding region of *NF-YC1* and a primer located in the 3′ untranslated region (UTR) of the octopine synthase (*ocs*) gene present in the 35S:FLAG-NF-YC1 construct (Figure S1B). Roots transformed with p35SGFPGUS+ were more efficiently nodulated by strain 55N1 than strain SC15, showing that composite plants behave as wild type in terms of strain efficiency (*t*-test, $P < 0.001$ at 14 dpi; Fig. 3B). Surprisingly, overexpression of NF-YC1 in Alubia resulted in a significant reduction in the number of nodules formed with 55N1 or SC15 as compared to those formed in 35S:GFPGUS control roots (57% or 76% of reduction at 14 dpi, respectively, *t*-test $P < 0.0001$; Fig. 3B). This result, obtained in three independent experiments performed on different days, contrasts with that previously observed in the Mesoamerican accession NAG12 and suggests that *NF-YC1* might negatively regulate nodulation in Andean beans. If this speculation is correct, roots of Alubia with reduced levels of *NF-YC1* might therefore exhibit an increased nodulation phenotype. To test this hypothesis, an RNAi-mediated gene silencing approach was used. The introduction of a *NF-YC1* RNAi construct in Alubia roots produced an over 98% reduction in *NF-YC1* transcript levels with respect to *GUS* RNAi control roots (Fig. 4A). The number of nodules developed in *NF-YC1* RNAi roots was reduced by 77.5% at 14 dpi with strain 55N1 as compared to control roots (*t*-test $P < 0.001$; Fig. 4B). Upon inoculation with strain SC15, the number of nodules formed in *NF-YC1* RNAi roots was also lower than that of *GUS* RNAi roots (57% reduction). These results indicate that *NF-YC1* or a closely related member of this gene family is required for nodule formation in the Andean accession Alubia. Since overexpression of NF-YC1 also led to a reduction in nodulation of Alubia, it can be speculated that a fine-tuned mechanism regulating expression of the *NF-YC1* gene is operating in this accession, and that alteration of *NF-YC1* transcript levels negatively affects nodule formation.

We have previously shown that two members of the NF-YC family (*NF-YC2* and *NF-YC3*) of the Mesoamerican cultivar NAG12 show little or no increase, respectively, in response to strains SC15 or 55N1 (Zanetti *et al.* 2010). Since we found that *NF-YC1* mRNA levels did not significantly change in Alubia upon rhizobial inoculation (Fig. 2A), we questioned whether *NF-YC2* or *NF-YC3* could be up-regulated in the Andean accession Alubia in response to *R. etli*. qRT-PCR analysis of Alubia roots revealed that neither *NF-YC2* nor *NF-YC3* mRNA levels significantly increased in response to inoculation with *R. etli* strains SC15 or 55N1 (Figure S4). Moreover, inoculation with SC15 produced a significant reduction of *NF-YC3* mRNA levels in Alubia roots. These results, together with those in Fig. 2, indicate that none of the *NF-YC* members analysed in this study are significantly up-regulated in response to *R. etli*, at least at the time points analysed here.

RNAi, but not overexpression of NF-YC1, reduced nodule size and impaired initiation and progression of infection threads in Alubia

Nodule size and occupancy was also evaluated in *NF-YC1* RNAi and overexpressing Alubia roots. In these experiments, a *R. etli* strain 55N1 expressing GFP protein was used for visualisation of the bacteria; the reduction in number of nodules

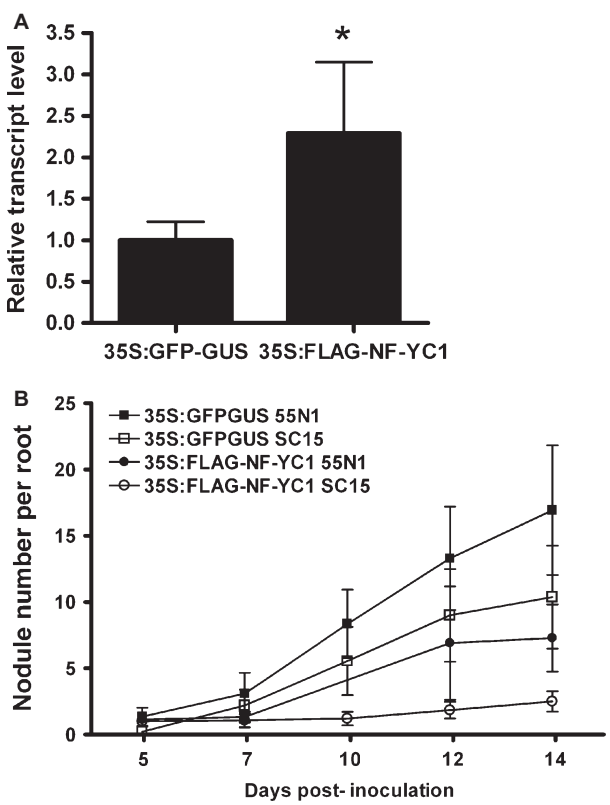

Fig. 3. Overexpression of NF-YC1 in the Andean accession Alubia. A: qRT-PCR analysis of *NF-YC1* mRNA levels in 35S:GFPGUS and 35S:FLAG-NF-YC1 transgenic roots. Transcript levels were normalised to *EF1α*. Root tissue of three independent composite plants was pooled. Error bars represent SD of at least three technical replicates. Asterisk indicates that values are significantly different in an unpaired two-tailed *t*-test with $P < 0.05$. B: Time course of nodule number per root. Error bars represent SE. Data are representative of three independent biological experiments. The number of nodules formed at 14 dpi in 35S:FLAG-NF-YC1 roots was significantly different from that formed in 35S:GFPGUS roots, either with strain SC15 or 55N1 in an unpaired two-tailed *t*-test with $P < 0.0001$.

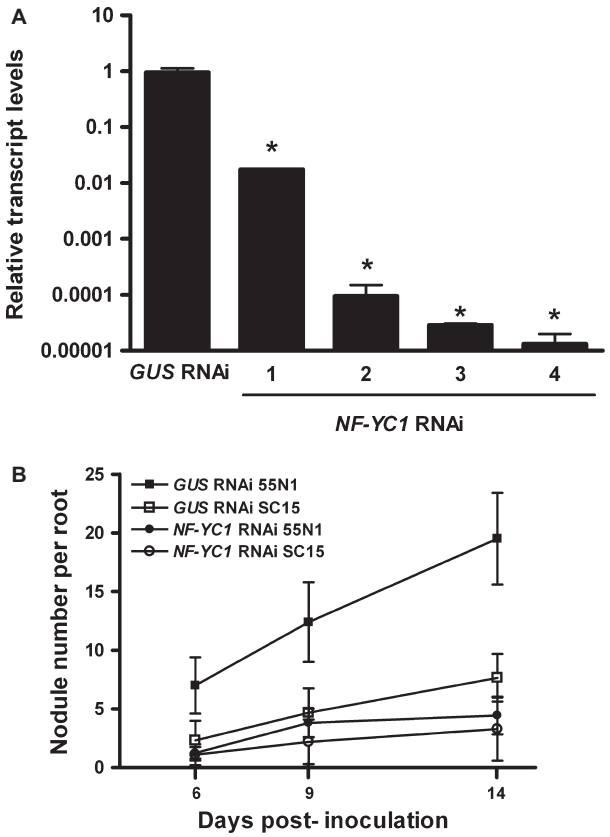

Fig. 4. Silencing of *NF-YC1* by RNAi in the Andean accession Alubia. A: qRT-PCR analysis of *NF-YC1* mRNA levels in transgenic roots from a *GUS* RNAi and four *NF-YC1* RNAi composite plants. Transcript levels were normalised to *EF1α*. Error bars represent SD of at least two technical replicates. Note that y-axis scale is $\log_{10}$. Asterisks indicates that values are significantly lower than those of *GUS* RNAi roots in an unpaired two-tailed *t*-test with $P < 0.05$. B: Quantification of the number of nodules per root at 6, 9 and 14 dpi with *R. etli* strain 55N1 or SC15. The number of nodules in *NF-YC1* RNAi roots was significantly different from that of *GUS* RNAi in an unpaired two-tailed *t*-test at 14 dpi with strain 55N1 ($P < 0.001$), but not with SC15 ($P < 0.05$).

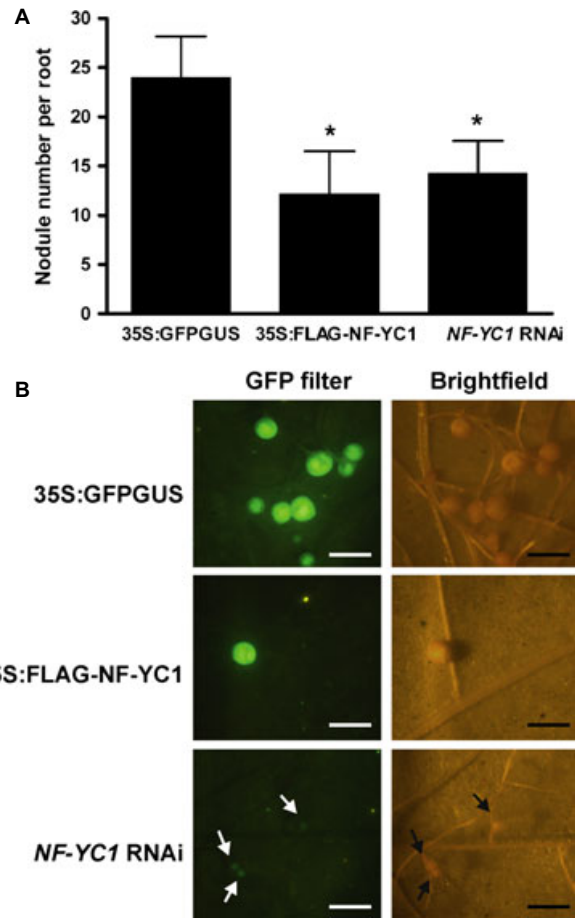

Fig. 5. Effect of overexpression and RNAi of *NF-YC1* on nodule number, size and occupancy in the Andean accession Alubia upon inoculation with *R. etli* strain 55N1 expressing GFP. A: Quantification of the number of nodules per root at 15 dpi with a 55N1 *R. etli* strain expressing GFP. Asterisks indicate that values of 35S:FLAG-NF-YC1 or *NF-YC1* RNAi roots were significantly different from control roots (35S:GFPGUS) in an unpaired two-tailed *t*-test with $P < 0.05$. B: Photographs illustrating the size of the nodules formed in 35S:GFPGUS, 35S:FLAG-NF-YC1 or *NF-YC1* RNAi roots and their occupancy at 15 dpi with 55N1 strain expressing GFP. Arrows point to small nodules formed in the NF-YC1 RNAi roots. Scale bar: 3 mm.

observed in both *NF-YC1* RNAi and overexpressing roots was also verified for this strain (*t*-test $P < 0.01$; Fig. 5A). RNAi of *NF-YC1* reduced nodule diameter in ~45% (1.6 ± 0.09 mm for 35S:GFPGUS *versus* 0.88 ± 0.03 mm for *NF-YC1* RNAi; *t*-test $P < 0.0001$, $n > 30$), whereas overexpression of NF-YC1 had no effect on nodule size or occupancy (1.6 ± 0.09 mm for 35S:GFPGUS *versus* 1.53 ± 0.11 mm for 35S:FLAG-NF-YC1; *t*-test $P > 0.1$, $n > 30$). Photographs illustrating the nodule size and occupancy by strain 55N1 expressing GFP in control, *NF-YC1* overexpressing and RNAi roots are shown Fig. 5B. We previously observed a reduction in nodule size of NAG12 plants caused by defects in infection thread formation and progression, which produced empty nodules (Zanetti *et al.* 2010). Therefore, the number of infection events was quantified at 5 dpi with the GFP-expressing *R. etli* strain. As shown in Fig. 6A and B, the number of infection threads or their progression was not significantly affected by overexpression of NF-YC1, as compared to control 35S:GFPGUS roots. However, the number of infection threads was significantly reduced in *NF-YC1*-RNAi roots with respect to controls (35S:GFPGUS or GUS RNAi) and overexpressing roots (Fig. 6A). In addition, the majority (92%, $n > 15$) of the infection threads formed in the *NF-YC1* RNAi roots ended in the root hair or ramified into the epidermal cell layer. In contrast, most of the infection threads observed in the controls (35S:GFPGUS or GUS RNAi) or overexpressing roots progressed and ramified into the dividing cortical cell of the nodule primordia (Fig. 6B), with only 20–21% of infection threads aborting in the epidermal cells. These results indicate that *NF-YC1* is required not only for nodule development, but also for bacterial infection in the Andean accession Alubia. The reduced number and missguided progression of infection threads resemble the phenotype previously observed in NAG12. Nevertheless, overexpression of *NF-YC1* negatively affected nodule formation in Alubia, but did not have a significant effect on initiation or progression of infection threads.

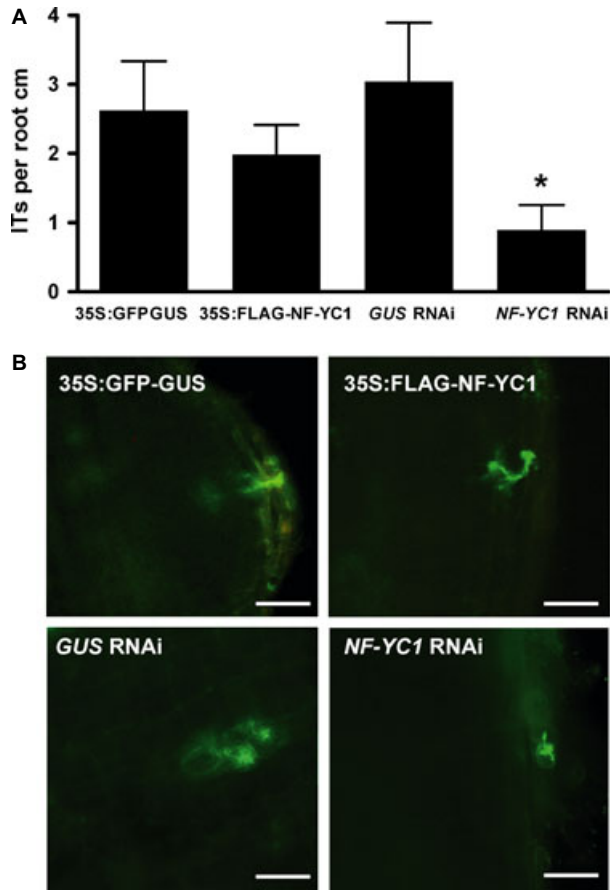

Fig. 6. Effect of overexpression and RNAi of *NF-YC1* on initiation and progression of infection threads (ITs) in the Alubia accession. A: Quantification of IT formation. Density of ITs was scored on more than 10 independent 35S:GFPGUS, 35S:FLAG-NF-YC1, *GUS* RNAi or *NF-YC1* RNAi roots at 5 dpi with 55N1 strain expressing GFP. Asterisk indicates that the value in *NF-YC1* RNAi roots was significantly different from that of *GUS* RNAi roots in an unpaired two-tailed *t*-test with $P < 0.05$. B: IT progression in 35S:GFPGUS, 35S:FLAG-NF-YC1, *GUS* RNAi and *NF-YC1* RNAi roots. Bars = 50 μm.

DISCUSSION

Symbiosis between legumes and rhizobia is a highly specific interaction, where only a few host–rhizobium combinations can lead to successful root colonisation. Recognition of specific rhizobium strains by a plant has a significant impact on nodulation performance and field competitiveness. This can vary according to the species involved in the symbiotic association, but also intrinsic genetic diversity of the micro- and macrosymbiont species has a large impact on nodulation and, ultimately, nitrogen-fixing efficiency. Furthermore, the presence of particular alleles in the host plant can restrict nodule formation with some rhizobial strains. For example, the wild pea *(Pisum sativum)* variety Afghanistan, which contains the $sym2^A$ allele, is nodulated by *R. leguminosarum* bv. *viciae* strains, isolated from Middle East and Central Asian soil, carrying the *nod*X gene, but is resistant to nodulation by European *R. leguminosarum* bv. *viciae* (Lie 1984). Further investigations demonstrated that introduction of *nod*X genes into the European strain was sufficient to overcome the *sym2* controlled nodulation resistance and introgression of the $sym2^A$ allele into cultivated peas restricted nodulation by strains that lacked the *nod*X gene (Kozik *et al.* 1995; Geurts *et al.* 1997). Interestingly, a recent report has shown that different pea genotypes exhibit different nodulation efficiencies when inoculated with a *nod*E mutant of *R. leguminosarum* bv. *viciae*, and that the major variation in nodulation is associated with the haplotype of the SYM37 LysM-type receptor-like kinase (Li *et al.* 2011b). Natural variation in symbiotic specificity has been also observed in soybean, where several dominant genes that restrict nodulation with specific strains of rhizobia have been identified by genetic analysis (Devine & Kuykendall 1996). Recently, positional cloning revealed that two of these dominant genes, *Rj2* and *Rfg1*, are allelic and encode a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance proteins (Yang *et al.* 2010). This finding demonstrated that symbiotic and pathogenic plant–bacteria interactions share common recognition mechanisms, and suggest that evasion of plant defence response is required for successful nodule formation and colonisation.

In this study, we showed that two domesticated and one wild accession of Mesoamerican common bean are more efficiently nodulated by a strain carrying the *nod*C-α allele, which is predominant in Mesoamerican soils; whereas Andean beans formed more nodules with their cognate *R. etli* partner carrying the *nod*C-δ allele, which is more abundant in the Andean GD centre. In previous works we had shown that several strains that belong to *nod*C-α produce similar molecular and physiological responses in Mesoamerican accession NAG12 (Zanetti *et al.* 2010), indicating that these strains might share molecular determinants associated with recognition by the plant. Despite the difference in the number of nodules formed by each *R. etli* lineage in each bean accession, strains belonging to each of the lineages were able to nodulate beans from the different GD centres. This contrasts with the situation of restricted nodulation found in certain soybean and pea varieties (Kozik *et al.* 1995; Devine & Kuykendall 1996). In common bean, co-inoculation experiments have shown that Mesoamerican accessions are almost exclusively nodulated by *R. etli* strains carrying the *nod*C-α allele, whereas about 37% of the nodules formed in Andean accessions were occupied by *R. etli* strains carrying the *nod*C-δ allele (Aguilar *et al.* 2004). The strong sympatric competition effect observed in Mesoamerican beans and their native *R. etli* strains suggests that common beans from this GD centre have optimised mechanisms that allow recognition and selection of more efficient strains, and our previous studies in the Mesoamerican accession NAG12 showed that *NF-YC1* might be part of such a mechanism (Zanetti *et al.* 2010).

Accumulation of *NF-YC1* transcripts in roots at early time points after inoculation with *nod*C-α strains of *R. etli* seems to be a common feature present in Mesoamerican bean accessions, but also – although to a lesser extent – in the wild accession Aborigineus, isolated from northwestern Argentina. Interestingly, this strain-specific accumulation pattern of *NF-YC1* was not observed in the domesticated Andean accession Alubia (Fig. 2; Peltzer Meschini *et al.* 2008). Based on these results, it is possible to hypothesise that this feature was lost during domestication of the Andean genetic pool. However, this must be considered only as speculation and needs a deeper inspection of other wild and domesticated accessions in the Andean germplasm to further the hypothesis.

The distinct pattern of *NF-YC1* transcript accumulation observed in the Mesoamerican accessions *versus* the domesticated Alubia might be due to different mechanisms that regulate expression of this gene in the different accessions, which could act at transcriptional or post-transcriptional levels or at both levels. Since no variation at nucleotide level were observed in the ORF of NAG12 and Alubia accessions, the future isolation and sequence comparison of *NF-YC1* genomic clones from Mesoamerican and Andean accessions, including promoter and untranslated regions, would help to elucidate such regulatory mechanisms.

Genes encoding NF-Y transcription factors have been found in all eukaryotes (Mantovani 1999). In plants, they are involved in developmental processes such as embryogenesis, control of flowering time and persistence of the nodule meristem, as well as in response to diverse environmental stimuli (Kwong *et al.* 2003; Ben-Naim *et al.* 2006; Combier *et al.* 2006; Nelson *et al.* 2007; Li *et al.* 2008). Compared to yeast and mammals, *NF-Y* genes have largely diversified in the plant lineage, each subunit being encoded by gene families with more than 10 members (Gusmaroli *et al.* 2002; Siefers *et al.* 2009). This expansion appears to be consistent in all sequenced plant genomes and has led to proposals that there might be overlapping functionality among different members, and also some degree of heterogeneity in NF-Y complexes (Yang *et al.* 2005; Kumimoto *et al.* 2010). Even in mammals, where each NF-Y subunit is encoded by a single-copy gene, alternative splicing and alternative promoter use contribute to heterogeneity of NF-Y transcription factors (Li *et al.* 1992; Ceribelli *et al.* 2009). In the case of human NF-YC, two protein isoforms, 37 and the 50 kDa, are produced through alternative splicing of the transcript. The accumulation pattern of each isoform is almost mutually exclusive in different human cell lines, *i.e.* cell lines that accumulate the 37 kDa isoform do not accumulate the 50 kDa isoform, and *vice versa* (Ceribelli *et al.* 2009). A detailed study in *Arabidopsis thaliana* revealed that NF-YC genes exhibit a highly variable, tissue-specific expression pattern (Siefers *et al.* 2009). In *P. vulgaris* accession NAG12, we have identified at least three members of the NF-YC family, but only *NF-YC1* showed strain-specific expression response to *R. etli* (Zanetti *et al.* 2010). In this study, the strain-specific response was also verified in other Mesoamerican accessions, such as Camilo and G24591. Interestingly, transcript levels of *NF-YC1, NF-YC2* or *NF-YC3* did not significantly increase in the Andean accession Alubia in response to either strain SC15 or strain 55N1, suggesting that up-regulation of these specific members of the NF-YC family is not required for efficient nodule formation in this accession. Moreover, overexpression of *NF-YC1* in Alubia resulted in a reduced nodulation phenotype with both strains.

Nuclear factor (NF-Y) heterotrimers have been largely implicated in transcriptional activation, but recent studies in both mammals and plants suggest that NF-Y complexes might act as bi-functional transcription factors, directly activating or repressing transcription, depending on the cell type, subunit heterogeneity and/or chromatin context of the CCAAT box (Testa *et al.* 2005; Ceribelli *et al.* 2008, 2009). Moreover, it has been proposed that NF-Y might be part of positive and negative transcriptional complexes that compete for regulation of the same promoter (Kumimoto *et al.* 2010). In addition, interaction of NF-Y subunits with partners that belong to different families of transcription factor (*e.g.* bZIP or MADS) introduce additional complexity to the scenario, since these interactions might provide a flexible combinatorial system to integrate multiple developmental and environmental signals, as observed during flowering in wheat or the unfolded protein response in *Arabidopsis* (Liu & Howell 2010; Li *et al.* 2011a). Based on the observation that either overexpression or knock-down by RNAi of *NF-YC1* in the Alubia accession led to a reduction in number of nodules as compared with control composite plants (35S:GFPGUS or GUS RNAi), we conclude that alteration of *NF-YC1* levels has a negative impact on nodule formation in this genetic background. In this context, it is tempting to speculate that ectopic expression of *NF-YC1* might lead to a dominant negative phenotype, in which NF-YC1 competes with other members of the NF-YC family for assembling of the heterotrimeric complex and subsequent DNA binding. This hypothesis is further sustained by results that showed that *NF-YC1* is not significantly up-regulated in Alubia roots upon inoculation with *R. etli,* in contrast to Mesoamerican accessions. On the other hand, 35S:FLAG-NF-YC1 Alubia roots were not affected in the number of infection threads formed, which might indicate that overexpression of NF-YC1 affects cortex-related process, such as activation of cell division, rather than epidermal responses. This is in agreement with our previous observation that NF-YC1 regulates directly or indirectly the expression of G2/M cell cycle genes (Zanetti *et al.* 2010).

In conclusion, this study provides evidence that there are differences in the mRNA expression pattern, as well as functional variation in the *NF-YC1* transcription factor among common bean accessions belonging to different genetic diversification centres during symbiosis with *R. etli*. The current ongoing sequencing of genomes of both Andean and Mesoamerican beans would certainly help to determine whether allele variations occur in the *NF-YC1* locus, and will elucidate the transcriptional or post-transcriptional mechanisms underlying regulation of this gene in both genetic backgrounds. Elucidation of molecular mechanisms associated with recognition of the most efficient symbionts of the plant is crucial, since this specificity preserves the mutualism in nature. In the absence of such recognition, interaction could easily shift to parasitic relationships, where bacteria will benefit from the plant carbohydrates without the reciprocal supply of nitrogen to the plant. Within this context, strain-specific responses associated with the efficiency of the interaction will shed light on the strain preference phenomenon and mechanisms of competition in the soil, leading to more rational selection of the combination of legumes and rhizobia that are more efficient in terms of biological nitrogen fixation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. A: Schematic representation of the 35S:FLAG-NF-YC1 construct for overexpression of NF-YC1. B: Semi-quantitative RT-PCR analysis of *FLAG-NF-YC1-OCS* mRNA levels in transgenic roots from 35S:GFPGUS and 35S:FLAG-NF-YC1 plants.

Figure S2. Schematic representation of (A) *GUS* RNAi and (B) *NF-YC1* RNAi constructs.

Figure S3. qRT-PCR analysis of *NF-YC1* mRNA levels in un-inoculated roots of Mesoamerican and Andean accessions of *P. vulgaris*.

Figure S4. qRT-PCR analysis of *NF-YC2* and *NF-YC3* mRNA levels in Alubia roots inoculated with *R. etli* SC15 or 55N1.

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REFERENCES

Aguilar O.M., Riva O., Peltzer E. (2004) Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification. *Proceedings of the National Academy of Sciences USA*, **101**, 13548–13553.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

Amarger N. (2001) Rhizobia in the field. *Advances in Agronomy*, **73**, 109–168.

Andriankaja A., Boisson-Dernier A., Frances L., Sauviac L., Jauneau A., Barker D.G., de Carvalho-Niebel F. (2007) AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. *The Plant Cell*, **19**, 2866–2885.

Ben-Naim O., Eshed R., Parnis A., Teper-Bamnolker P., Shalit A., Coupland G., Samach A., Lifschitz E. (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *The Plant Journal*, **46**, 462–476.

Bitocchi E., Nanni L., Bellucci E., Rossi M., Giardini A., Zeuli P.S., Logozzo G., Stougaard J., McClean P., Attene G., Papa R. (2012) Mesoamerican origin of the common bean (*Phaseolus vulgaris* L.) is revealed by sequence data. *Proceedings of the National Academy of Sciences USA*, doi: 10.1073/pnas.1108973109.

Blanco F.A., Meschini E.P., Zanetti M.E., Aguilar O.M. (2009) A small GTPase of the Rab family is required for root hair formation and preinfection stages of the common bean–*Rhizobium* symbiotic association. *The Plant Cell*, **21**, 2797–2810.

Bond J.E., Gresshoff P.M. (1993) Soybean transformation to study molecular physiology. In: Gresshoff P. M. (Ed.), *Plant responses to the environment*. CRC Press, Boca Raton, FL, pp 25–44.

Broughton W.J., Hernández G., Blair M., Beebe S., Gepts P., Vanderleyden J. (2003) Beans (*Phaseolus* spp.) – model food legumes. *Plant and Soil*, **252**, 55–128.

Burkart A. (1943) *Las leguminosas argentinas silvestres y cultivadas*. Acme, Buenos Aires.

Ceribelli M., Dolfini D., Merico D., Gatta R., Vigano A.M., Pavesi G., Mantovani R. (2008) The histone-like NF-Y is a bifunctional transcription factor. *Molecular and Cellular Biology*, **28**, 2047–2058.

Ceribelli M., Benatti P., Imbriano C., Mantovani R. (2009) NF-YC complexity is generated by dual promoters and alternative splicing. *Journal of Biological Chemistry*, **284**, 34189–34200.

Combier J.P., Frugier F., de Billy F., Boualem A., El-Yahyaoui F., Moreau S., Vernie T., Ott T., Gamas P., Crespi M., Niebel A. (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes and Development*, **20**, 3084–3088.

Combier J.P., de Billy F., Gamas P., Niebel A., Rivas S. (2008) Trans-regulation of the expression of the transcription factor MtHAP2-1 by a uORF controls root nodule development. *Genes and Development*, **22**, 1549–1559.

Crespi M.D., Jurkevitch E., Poiret M., d'Aubenton-Carafa Y., Petrovics G., Kondorosi E., Kondorosi A. (1994) *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *The EMBO Journal*, **13**, 5099–5112.

Devine T.E., Kuykendall L.D. (1996) Host genetic control of symbiosis in soybean (*Glycine max* L.). *Plant and Soil*, **186**, 173–187.

Fahraeus G. (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Journal of General Microbiology*, **16**, 374–381.

Galván M.Z., Aulicino M.B., García Medina S., Balatti P.A. (2001) Genetic diversity among Northwestern Argentinian cultivars of common bean (*Phaseolus vulgaris L.*) as revealed by RAPD markers. *Genetic Resources and Crop Evolution*, **48**, 251–260.

Gepts P., Bliss F.A. (1988) Dissemination pathways of common bean (*Phaseolus vulgaris, Fabaceae*) deduced from phaseolin electrophoretic variability. II. Europe and Africa. *Economic Botany*, **42**, 86–104.

Geurts R., Heidstra R., Hadri A.E., Downie J.A., Franssen H., Van Kammen A., Bisseling T. (1997) Sym2 of pea is involved in a nodulation factor-perception mechanism that controls the infection process in the epidermis. *Plant Physiology*, **115**, 351–359.

Gusmaroli G., Tonelli C., Mantovani R. (2002) Regulation of novel members of the *Arabidopsis thaliana* CCAAT-binding nuclear factor Y subunits. *Gene*, **283**, 41–48.

Hirsch S., Kim J., Munoz A., Heckmann A.B., Downie J.A., Oldroyd G.E. (2009) GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. *The Plant Cell*, **21**, 545–557.

Kalo P., Gleason C., Edwards A., Marsh J., Mitra R.M., Hirsch S., Jakab J., Sims S., Long S.R., Rogers J., Kiss G.B., Downie J.A., Oldroyd G.E. (2005) Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science*, **308**, 1786–1789.

Karimi M., Depicker A., Hilson P. (2007) Recombinational cloning with plant gateway vectors. *Plant Physiology*, **145**, 1144–1154.

Kozik A., Heiddstra R., Horvath B., Kulikova O., Tikhonovich I., Ellis T.H.N., von Kammen A., Lie T.A., Bisseling T. (1995) Pea lines carrying syml or sym2 can be nodulated by Rhizobium strains containing nodX; sym1 and sym2 are allelic. *Plant Science*, **108**, 41–49.

Kumimoto R.W., Zhang Y., Siefers N., Holt B.F. 3rd (2010) NF-YC3, NF-YC4 and NF-YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in *Arabidopsis thaliana*. *The Plant Journal*, **63**, 379–391.

Kwak M., Gepts P. (2009) Structure of genetic diversity in the two major gene pools of common bean (*Phaseolus vulgaris* L., Fabaceae). *Theoretical and Applied Genetics*, **118**, 979–992.

Kwong R.W., Bui A.Q., Lee H., Kwong L.W., Fischer R.L., Goldberg R.B., Harada J.J. (2003) LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *The Plant Cell*, **15**, 5–18.

Li X.Y., Mantovani R., Hooft van Huijsduijnen R., Andre I., Benoist C., Mathis D. (1992) Evolutionary variation of the CCAAT-binding transcription factor NF-Y. *Nucleic Acids Research*, **20**, 1087–1091.

Li W.X., Oono Y., Zhu J., He X.J., Wu J.M., Iida K., Lu X.Y., Cui X., Jin H., Zhu J.K. (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *The Plant Cell*, **20**, 2238–2251.

Li C., Distelfeld A., Comis A., Dubcovsky J. (2011a) Wheat flowering repressor VRN2 and promoter CO2 compete for interactions with NUCLEAR FACTOR-Y complexes. *The Plant Journal*, **67**, 763–773.

Li R., Knox M.R., Edwards A., Hogg B., Ellis T.H.N., Wei G., Downie J.A. (2011b) Natural variation in host-specific nodulation of pea is associated with a haplotype of the SYM37 LysM-type receptor-like kinase. *Molecular Plant-Microbe Interactions*, **24**, 1396–1403.

Lie T.A. (1984) Host genes in *Pisum sativum* L. conferring resistance to European *Rhizobium leguminosarum* strains. *Plant and Soil*, **82**, 415–425.

Limpens E., Franken C., Smit P., Willemse J., Bisseling T., Geurts R. (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science*, **302**, 630–633.

Lioi L., Sparvoli F., Galasso I., Lanave C., Bollini R. (2003) Lectin-related resistance factors against bruchids evolved through a number of duplication events. *Theoretical and Applied Genetics*, **107**, 814–822.

Liu J.X., Howell S.H. (2010) bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in Arabidopsis. *The Plant Cell*, **22**, 782–796.

Madsen L.H., Tirichine L., Jurkiewicz A., Sullivan J.T., Heckmann A.B., Bek A.S., Ronson C.W., James E.K., Stougaard J. (2010) The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. *Nature Communications*, **1**, 10.

Mantovani R. (1999) The molecular biology of the CCAAT-binding factor NF-Y. *Gene*, **239**, 15–27.

Marsh J.F., Rakocevic A., Mitra R.M., Brocard L., Sun J., Eschstruth A., Long S.R., Schultze M., Ratet P., Oldroyd G.E. (2007) *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiology*, **144**, 324–335.

Middleton P.H., Jakab J., Penmetsa R.V., Starker C.G., Doll J., Kalo P., Prabhu R., Marsh J.F., Mitra R.M., Kereszt A., Dudas B., VandenBosch K., Long S.R., Cook D.R., Kiss G.B., Oldroyd G.E. (2007) An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. *The Plant Cell*, **19**, 1221–1234.

Nelson D.E., Repetti P.P., Adams T.R., Creelman R.A., Wu J., Warner D.C., Anstrom D.C., Bensen R.J., Castiglioni P.P., Donnarummo M.G., Hinchey B.S., Kumimoto R.W., Maszle D.R., Canales R.D., Krolikowski K.A., Dotson S.B., Gutterson N., Ratcliffe O. J., Heard J.E. (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. *Proceedings of the National Academy of Sciences USA*, **104**, 16450–16455.

Oldroyd G.E., Downie J.A. (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annual Review of Plant Biology*, **59**, 519–546.

Oldroyde G.E., Murray J.D., Poole P.S., Downie J.A. (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics*, **45**, 119–144.

Peltzer Meschini E.P., Blanco F.A., Zanetti M.E., Beker M.P., Kuster H., Puhler A., Aguilar O.M. (2008) Host genes involved in nodulation preference in common bean (*Phaseolus vulgaris*)–*Rhizobium etli* symbiosis revealed by suppressive subtractive hybridization. *Molecular Plant-Microbe Interactions*, **21**, 459–468.

Radutoiu S., Madsen L.H., Madsen E.B., Felle H.H., Umehara Y., Gronlund M., Sato S., Nakamura Y., Tabata S., Sandal N., Stougaard J. (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature*, **425**, 585–592.

Schauser L., Roussis A., Stiller J., Stougaard J. (1999) A plant regulator controlling development of symbiotic root nodules. *Nature*, **402**, 191–195.

Siefers N., Dang K.K., Kumimoto R.W., Bynum W.E. IV, Tayrose G., Holt B.F. III (2009) Tissue-specific expression patterns of Arabidopsis NF-Y transcription factors suggest potential for extensive combinatorial complexity. *Plant Physiology*, **149**, 625–641.

Smit P., Raedts J., Portyanko V., Debelle F., Gough C., Bisseling T., Geurts R. (2005) NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. *Science*, **308**, 1789–1791.

Testa A., Donati G., Yan P., Romani F., Huang T.H., Vigano M.A., Mantovani R. (2005) Chromatin immunoprecipitation (ChIP) on chip experiments uncover a widespread distribution of NF-Y binding CCAAT sites outside of core promoters. *Journal of Biological Chemistry*, **280**, 13606–13615.

Yang J., Xie Z., Glover B.J. (2005) Asymmetric evolution of duplicate genes encoding the CCAAT-binding factor NF-Y in plant genomes. *New Phytologist*, **165**, 623–631.

Yang S., Tang F., Gao M., Krishnan H.B., Zhu H. (2010) R gene-controlled host specificity in the legume–rhizobia symbiosis. *Proceedings of the National Academy of Sciences USA*, **107**, 18735–18740.

Zanetti M.E., Blanco F.A., Beker M.P., Battaglia M., Aguilar O.M. (2010) A C subunit of the plant nuclear factor NF-Y required for rhizobial infection and nodule development affects partner selection in the common bean–*Rhizobium etli* symbiosis. *The Plant Cell*, **22**, 4142–4157.