***Lotus japonicus* NF-YA1 Plays an Essential Role During Nodule Differentiation and Targets Members of the *SHI/STY* Gene Family**

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Legume plants engage in intimate relationships with rhizobial bacteria to form nitrogen-fixing nodules, root-derived organs that accommodate the microsymbiont. Members of the *Nuclear Factor Y (NF-Y)* gene family, which have undergone significant expansion and functional diversification during plant evolution, are essential for this symbiotic liaison. Acting in a partially redundant manner, NF-Y proteins were shown, previously, to regulate bacterial infection, including selection of a superior rhizobial strain, and to mediate nodule structure formation. However, the exact mechanism by which these transcriptional factors exert their symbiotic functions has remained elusive. By carrying out detailed functional analyses of *Lotus japonicus* mutants, we demonstrate that *LjNF-YA1* becomes indispensable downstream from the initial cortical cell divisions but prior to nodule differentiation, including cell enlargement and vascular bundle formation. Three affiliates of the *SHORT INTERNODES/STYLISH* transcription factor gene family, called *STY1*, *STY2*, and *STY3*, are demonstrated to be among likely direct targets of *LjNF-YA1*, and our results point to their involvement in nodule formation.

Transcriptional reprogramming, one of the key drivers of cellular specification (Smith et al. 2016), is integral to root-nodule

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development (Høgslund et al. 2009; Moreau et al. 2011; Soyano and Hayashi 2014). Nodules are lateral organs that differentiate from root cortical and pericycle cells following the symbiotic signaling that occurs between leguminous plants and nitrogen-fixing rhizobial bacteria (Desbrosses and Stougaard 2011; Oldroyd et al. 2011; Suzuki et al. 2015). Several transcription factors have been shown to play an essential role during nodule differentiation (Soyano and Hayashi 2014). Among them, members of the CCAAT-box binding transcriptional factor family, known as Heme Activator or Nuclear Factor Y (NF-Y) proteins, have emerged as important plant regulators of early nodulation events (Battaglia et al. 2014; Combier et al. 2006, 2008; Laloum et al. 2013, 2014; Laporte et al. 2014; Soyano et al. 2013; Zanetti et al. 2010).

NF-Ys are heterotrimeric protein complexes composed of the NF-YA, NF-YB, and NF-YC subunits (Dolfini et al. 2012; Laloum et al. 2013; Mantovani 1999). In animals, NF-Ys are known to regulate genes associated with the cell-cycle progression and those related to various diseases (Dolfini et al. 2016; Ly et al. 2013). Unlike animals and fungi, plants have evolved multiple copies of genes encoding each of the three NF-Y subunits, for which both ubiquitous and more specialized expression patterns are known (Laloum et al. 2013; Petroni et al. 2013; Siefers et al. 2009). Plants in the legume (Leguminosae) family have co-opted some of these genes, such as *Medicago truncatula* *NF-YA1* (*MtNF-YA1*) and *MtNF-YA2* (Baudin et al. 2015; Combier et al. 2006, 2008; Laloum et al. 2014), *Phaseolus vulgaris* *NF-YC1* (*PvNF-YC1*) (Battaglia et al. 2014; Zanetti et al. 2010), and *Lotus japonicus* *NF-YA1* (*LjNF-YA1*) and *LjNF-YB1* (Soyano et al. 2013), to perform specialized functions during the development of nitrogen-fixing root-nodule symbiosis. A role for soybean (*Glycine max*) *GmNF-YA1a* and *GmNF-YA1b* as positive regulators of arbuscular mycorrhizae (AM) (Corradi and Bonfante 2012; Parniske 2008) and as potential targets of the autoregulatory mechanism that restricts the extent of root colonization by AM fungi has also been documented (Schaarschmidt et al. 2013). Together, these findings demonstrate the importance of NF-Y transcription factors in mediating the two major classes of plant-microbe endosymbiosis.

The establishment of nitrogen-fixing root nodules involves two spatially and temporally coordinated processes and NF-Ys are important for both (Combier et al. 2006, 2008; Laporte et al. 2014; Zanetti et al. 2010). At the root surface, symbiotic colonization commences with a mutual recognition (Kawaharada et al. 2015; Lerouge et al. 1990) that initiates the formation of

root hair infection threads (ITs), plant plasma membrane-derived transcellular tunnels that guide rhizobia inside the root (Fournier et al. 2015; Miri et al. 2016). In the subtending inner root, presumed cell nonautonomous root epidermis to cortex signaling that precedes bacterial entry activates mitotic cell divisions for nodule primordia (NP) formation (Held et al. 2014; Madsen et al. 2010). Both of these cellular events commence as a result of a compatible interaction between symbionts, wherein bacterially-encoded lipochito-oligosaccharide signaling molecules, known as nodulation factors (NF), are perceived by the host plant NF receptor complex at the root epidermis (Arrighi et al. 2006; Broghammer et al. 2012; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003, 2007). This triggers the NF-dependent root response pathway (Geurts et al. 2016), with rapid calcium (Ca^{2+}) influx and the perinuclear fluctuation of Ca^{2+} concentrations in the root epidermis, known as Ca^{2+} spiking, among the earliest cellular responses (Charpentier et al. 2016; Morieri et al. 2013; Oldroyd and Downie 2006). Ca^{2+} and calmodulin-dependent receptor kinase (CCaMK) transduces these Ca^{2+} signals to downstream effectors (Lévy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006b), including a nuclear coiled-coil protein, CYCLOPS. CYCLOPS, the direct phosphorylation target of CCaMK (Singh et al. 2014; Yano et al. 2008), participates in the NF/CCaMK-dependent transcriptional reprogramming and its artificial activation in *L. japonicus* is sufficient to stimulate formation of empty root-nodule structures in the absence of any external stimulus, including rhizobia and NF (Limpens and Bisseling 2014; Singh et al. 2014). The regulator NODULE INCEPTION (NIN), a RWP-RK domain-containing protein, which is essential for both rhizobial infection and nodule organogenesis (Marsh et al. 2007; Schauser et al. 1999; Vernié et al. 2015), partakes in the CYCLOPS-dependent specification. CYCLOPS regulates expression of NIN (Singh et al. 2014) and, in *L. japonicus*, NIN was shown to directly activate *LjNF-YA1* and *LjNF-YB1*, which is required for normal nodule organogenesis (Soyano et al. 2013).

MtNF-YA1 was the first nodule-specific NF-Y gene to be identified and functionally characterized in the context of indeterminate, meristem-containing nodule development in *Medicago truncatula* (Combie et al. 2006, 2008). It was shown to be necessary for the regulation of cell proliferation and proper formation of nodule meristem, where it is expressed (Combie et al. 2006, 2008; Xiao et al. 2014). Subsequent research highlighted an additional role for *MtNF-YA1* in the root epidermis, in which it acts partially redundantly with *MtNF-YA2* to control bacterial infection (Laloum et al. 2014; Laporte et al. 2014).

Expression of *MtNF-YA1* is regulated at both transcriptional and posttranscriptional levels, with the latter involving miR169 and an unusual, *trans*-acting peptide derived from the upstream open reading frame (uORF) of an alternatively spliced *MtNF-YA1* mRNA (Combie et al. 2008). These posttranscriptional mechanisms are thought to account for the tight spatial and temporal regulation of the *MtNF-YA1* gene expression domains during indeterminate nodule development (Combie et al. 2006, 2008). RNA interference (RNAi)-mediated downregulation of the *MtNF-YA1* gene expression resulted in attenuation of nodule growth, a defect mimicked by constitutive overexpression of miR169 (Combie et al. 2006). This negative effect was further accentuated in the *Mtnf-ya1-1* loss-of-function mutant, in which the impairment in the rhizobial infection also becomes apparent. *Mtnf-ya1-1* forms ITs with cell walls that are half the thickness of those in wild type, which likely accounts for the observed instability of the infection process in the mutant (Laporte et al. 2014). The symbiotic defects are even more severe when *MtNF-YA1* and *MtNF-YA2* are simultaneously impaired, which leads to few or no nodules and a significant, early block in infection (Laloum et al. 2014).

NF-Ys are also pertinent during the development of determinate, meristem-deprived nodules, including the apparent participation of *PvNF-YC1* in a mechanism by which common bean selects for a more efficient symbiotic partner (Zanetti et al. 2010). Downregulation of *PvNF-YC1* through RNAi impedes nodule development and leads to defects in the infection process (Zanetti et al. 2010). Similarly, downregulation of *LjNF-YA1* in hairy roots was shown to significantly reduce nodule formation events, with the majority of composite plants displaying a non-nodulating phenotype, even though the IT formation was apparently not affected (Soyano et al. 2013). The presence of the *LjNF-YA1* RNAi construct also prevents spontaneous nodule formation caused by a gain-of-function *CCaMK* allele in the absence of rhizobia (Soyano et al. 2013). However, it remains unclear what specific mechanism is regulated by NF-Ys during determinate nodule development and whether this is equivalent to the function of *MtNF-Ys* in indeterminate nodules.

By performing detailed analyses of four independent *L. japonicus* mutant lines, we demonstrate here that *LjNF-YA1* is not required for IT formation and becomes essential only downstream from the initial cell divisions for NP formation. Its function during determinate nodule development in *L. japonicus* can be fully substituted by *MtNF-YA1*. Using transcriptomic data, we have defined several potential downstream targets for the *LjNF-YA1*-dependent regulation, including three members of the *SH1/STY* gene family.

RESULTS

Identification of *L. japonicus nf-ya1* mutants.

A screen for suppressors of the *L. japonicus har1-1* hyper-nodulation phenotype (Murray et al. 2006) identified two allelic lines, *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2*, each carrying a secondary mutation at the *LjNF-YA1* locus. In contrast to the parental *har1-1* line, which, at 21 days after inoculation (dai) with *Mesorhizobium loti*, showed the typical, short and hypermodulated root phenotype (Wopereis et al. 2000), both *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2* had longer roots with many small nodules (Fig. 1A). A closer examination of the double mutant symbiotic phenotypes 10 dai showed that *M. loti* was able to readily penetrate roots; however, these root colonization events were apparently met with a limited cortical response, resulting in the presence of a large number of NP and small nodule bumps (Fig. 1B). At 21 dai, NP formation was enhanced in *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2*, such that they significantly outnumbered the corresponding events in *har1-1* (Fig. 1C). By contrast, the number of mature nodules was significantly diminished in *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2* as compared with *har1-1* and many of them were small in size (Fig. 1C).

Sequencing of the *LjNF-YA1* gene, selected as a candidate locus based on the map-based cloning experiment, showed the presence of the G₂₄₁₈ to A splice-site mutation and the G₃₃₀₉ to A substitution that define the *nf-ya1-1* and *nf-ya1-2* mutant alleles, respectively (Supplementary Figs. S1 and S2). F1 plants derived from four independent allelic crosses between the corresponding *nf-ya1-1* and *nf-ya1-2* single mutants (carrying the wild-type *HAR1* allele), displayed the same mutant nodulation phenotype. Together with the phenotypic complementation of the *nf-ya1-2* nodulation defect with the wild-type *LjNF-YA1* gene and also with *MtNF-YA1* (Supplementary Fig. S3), these data provided unambiguous proof for the causative nature of *nf-ya1-1* and *nf-ya1-2* alleles for the observed mutant symbiotic phenotypes.

The NF-YA1 gene.

The *L. japonicus* genome contains a family of at least nine genes that are predicted to encode LjNF-YA proteins, named here as *LjNF-YA1* to *LjNF-YA9* (Supplementary Fig. S4). The *LjNF-YA1* gene is composed of eight exons encoding a

1,618-nucleotide (nt)-long *LjNF-YA1* mRNA, including 5' and 3' untranslated regions (UTRs). The adenine residue (A) in the predicted ATG initiation codon is located in the fourth exon, 247 nts downstream from the mRNA 5' end. This begins the 996-nt-long ORF for the conceptual *LjNF-YA1* protein. The first and second exons of the *LjNF-YA1* gene encompass a predicted uORF of 93 nt, which is in-frame with the main ORF. The 376-nt-long 3' UTR contains the 5'-AAGGCAACTC ATTCTTGGCTA-3' sequence, a predicted target of miR169.

The *LjNF-YA1* protein (GenBank accession number AB378633) has a high primary sequence homology with NF-YAs from different plant species, including *Glycine max* NF-YA10 (GenBank accession number KHN43704, 77% identity and 84% similarity), *Medicago truncatula* NF-YA1 (GenBank accession number ABP68866.1, 76% identity and 83% similarity), and *Arabidopsis thaliana* NF-YA10 (GenBank accession number NP_196269, 42% identity and 51% similarity). All share a highly conserved CCAAT-binding transcription factor domain (Laloum et al. 2013). This domain includes subunit interaction (SI) and DNA binding (DB) regions that are highly conserved between NF-YA proteins in different species (Fig. 2) (Laloum et al. 2013). Both *nf-ya1-1* and *nf-ya1-2* mutations are predicted to alter the NF-YA domain, leading either to a deletion of a portion of the SI region or a substitution of an invariant glycine residue (G₂₁₃) to aspartic acid (D) within the DB region of the *LjNF-YA1* protein, respectively (Fig. 2).

Additional *nf-ya1* alleles.

Both *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2* were backcrossed to wild-type *L. japonicus* 'Gifu' and the corresponding *nf-ya1-1* and

nf-ya1-2 mutants carrying the wild-type *HAR1* gene were selected. The presence of either of these *nf-ya1* alleles, each characterized by a single nucleotide substitution, leads to similar, mutant nodulation phenotypes, and *nf-ya1-2* was chosen for subsequent detailed analyses. To assess whether a null mutation at the *LjNF-YA1* locus would potentiate the symbiotic defect, additional alleles called *nf-ya1-3* and *nf-ya1-4*, were selected from the *L. japonicus* LORE1 retrotransposon resource (Fukai et al. 2012; Małolepszy et al. 2016; Urbański et al. 2012). In *nf-ya1-3*, LORE1 is inserted immediately after the ATG initiation codon of the main ORF, while *nf-ya1-4* carries the LORE1 insertion in the same exon, 104 bp downstream from the ATG. The presence of the 5.041-kb LORE1 sequence at the beginning of the *LjNF-YA1* coding region introduces multiple premature, translational stop codons. As the entire LORE1 sequence remains and is transcribed within the corresponding *nf-ya1-3* and *nf-ya1-4* mRNAs, we considered these as strong or loss-of-function alleles.

Comparative phenotypic analysis of *nf-ya1-2* with *nf-ya1-3* and *nf-ya1-4* LORE1 insertion lines showed that they all have similar symbiotic defects. When scored 7 dai, all three mutants developed NP (i.e., foci of cortical cell divisions), but mature nodules were rarely formed (e.g., on average 0.26 ± 0.23 nodules per *nf-ya1-2* plant). By contrast, the wild-type control developed several nodules at this early timepoint (Fig. 3A). At 21 dai, the number of NP was significantly higher and the proportion of mature nodules was significantly smaller in *nf-ya1* mutants as compared with wild type (Fig. 3B). As such, the single mutants had nearly double the number of early primordia compared with wild type (Fig. 3B). Importantly, at 7 dai, all three *nf-ya1* mutants had a wild-type number of epidermal ITs

Fig. 1. Plant phenotypes. **A**, The *har1-1* parental line and two independent, suppressor lines, *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2*, are shown. Plants were photographed 21 days after inoculation (dai) with *Mesorhizobium loti*. Note differences in the root length and nodule development (insets show more details). **B**, Longitudinal sections through *Lotus japonicus har1-1*, *har1-1 nf-ya1-1*, and *har1-1 nf-ya1-2* roots that were stained for β -galactosidase activity 10 dai with *M. loti* NZP2235 carrying the *hemA::LacZ* reporter fusion (darker regions within nodules and nodule primordia indicate presence of bacteria). **C**, Scores of various nodulation events 21 dai. Ten individuals were scored for each genotype and averages $\pm 95\%$ confidence intervals are given. Asterisks (*) indicate values that are significantly different (Student's t test, $P \leq 0.05$) from the corresponding *har1-1* parental line.

(eITs) (Fig. 4C). These eITs were observed to descend into the subtending root cortex (Fig. 3D), leading to NP events that, although more numerous, were seemingly wild type in appearance (Fig. 3E).

Taken together, these observations support and extend previously published data by showing that deleterious mutations at the *LjNF-YA1* locus restrict symbiotic development in *L. japonicus* downstream from the initial cell divisions by impacting the cortical program for nodule formation. This was further evaluated by performing detailed analyses of *LjNF-YA1* gene expression and also by examining the effect of *nf-ya1* mutant alleles on the symbiotic development in the context of gain- and loss-of-function alleles of the *L. japonicus* *Lhk1* cytokinin receptor gene.

Expression of *LjNF-YA1* associates with nodule development.

According to the *L. japonicus* gene expression atlas, *LjNF-YA1* is the only member of the *L. japonicus* *NF-YA* gene family that displays a strictly nodule-specific expression pattern (Verdier et al. 2013). Indeed, the *NF-YA1* mRNA can be detected in *M. loti*-inoculated roots but not in any other *L. japonicus* tissues tested. It rapidly accumulates within the first 2 dai, to reach the highest steady-state level at 7 dai. The *NF-YA1* mRNA is also detectable at 12 dai and in mature nodules, although at a comparatively lower level (Supplementary Fig. S5A and B). When examined at various stages following *M. loti* inoculation, only a single species of the *LjNF-YA1* mRNA could be detected in *L. japonicus* roots and nodules, indicating lack of

Fig. 2. *nf-ya1-1* and *nf-ya1-2* mutations affect conserved domains of the NF-YA1 protein. Alignment of NF-YA protein sequences encompassing the predicted subunit interaction (SI) and DNA-binding (DB) domains from different species. Only invariant amino acid residues are highlighted. The *nf-ya1-1* mutation imposes mis-splicing 66 nucleotides downstream from the predicted intron 6-exon 7 junction. As a result, 22 amino acids, including invariant VN and KQ residues within the SI domain, are deleted (as marked by boxed sequence in the *Lotus japonicus* NF-YA1). Note that this leaves the downstream sequence intact and preserves the remainder of the wild-type open reading frame. *nf-ya1-2* leads to substitution of an invariant glycine (G) residue to aspartic acid (D) within the predicted DB domain (arrow). The following protein sequences were used for this alignment: *Glycine max* (accession number XP_003519110.1), *L. japonicus* (BAG50060.1), *Medicago truncatula* (XP_003590326), *Vitis vinifera* (XP_002263793.1), *Arabidopsis thaliana* (NP_196269), *Oryza sativa* (CAA71844.1), *Caenorhabditis elegans* (NP_509999.1), and *Drosophila melanogaster* (NP_648313.1).

Fig. 3. *nf-ya1* mutations affect the cortical program for nodule formation. **A**, and **B**, Scores of nodulation events in wild type and *nf-ya1* single mutant lines 7 and 21 days after inoculation (dai) with *Mesorhizobium loti*. Given the abundance of nodule primordia, roots were evaluated under higher (20×) magnification to capture even the smallest nodulation event, such as a single layer of dividing cortical cells. Ten to 15 individuals were scored for each genotype and averages $\pm$ 95% confidence intervals are given. Asterisks (*) indicate values that are significantly different (Student's *t* test, $P \leq 0.05$) from the wild-type control. **C**, Number of epidermal infection threads (eITs) as formed by a given genotype at 7 dai. **D**, A representative eIT formed on an *nf-ya1-2* root. The eIT has successfully migrated to become a cortical IT (cIT), while descending toward an early nodule primodium (NP). PIT = a preinfection thread that marks one possible route for further progression of the cIT. **E**, A typical nodule primodium formed by the *nf-ya1-2* mutant having a wild-type appearance.

alternative splicing for this mRNA under the conditions tested (Supplementary Fig. S6).

Histochemical localization indicated that the *LjNF-YA1* promoter activity (*LjNF-YA1_{pro}:GUS*) associates with the earliest stages of symbiosis, including the initial cortical cell divisions for NP formation (Fig. 4A and B). The *LjNF-YA1_{pro}:GUS* activity was also detected in the root epidermis, including root hairs (Fig. 4C); however, this was rather intermittent and not

always associated with eITs. Developmentally more advanced NP showed a uniform β -glucuronidase (GUS) activity in all cell types, including the root pericycle (Fig. 4D and E). With further progression in development, *LjNF-YA1_{pro}:GUS* activity gradually became restricted, eventually to be entirely confined to the nodule vasculature and vascular parenchyma in fully mature nodules (Figs. 4F and 8C). Interestingly, in *M. loti*-inoculated transgenic hairy roots formed on wild-type *L. japonicus* shoots, the *Medicago truncatula NF-YA1* promoter conferred a pattern of expression that was very similar to the *LjNF-YA1* promoter (Fig. 4G and H). This is consistent with the fact that the *MtNF-YA1* gene is able to complement the symbiotic defect of the *L. japonicus nf-yal-2* mutant by restoring the wild-type nodulation phenotype.

Expression of *NF-YA1* is controlled by cytokinin.

In *L. japonicus*, expression of *LjNF-YA1* is regulated by *NIN* (Soyano et al. 2013) and both genes respond to exogenous cytokinin (Heckmann et al. 2011; Murray et al. 2007). This is consistent with the observation that, at 50 nM, exogenous cytokinin induced the expression of *LjNF-YA1* in uninoculated *L. japonicus* roots and, within the first 6 h of treatment, this response was both *Lhk1*- and *NIN*-dependent. The *LjNF-YA1* mRNA was also detectable in uninoculated roots of a *L. japonicus* mutant carrying the *snf2* gain-of-function allele of the LHK1 cytokinin receptor gene but not in corresponding wild-type roots, further supporting a regulatory role for cytokinin (Supplementary Fig. 7A and B). Moreover, like *NIN*, the *LjNF-YA1* expression domain was enlarged in the *lhk1-1* mutant. In the wild-type background, at 7 dai, the *LjNIN_{pro}:GUS* and *LjNF-YA1_{pro}:GUS* reporters showed punctuated activity patterns, reflecting a tight association with developing nodules. By contrast, their expression domains were extended in the *lhk1-1* mutant (Supplementary Fig. 8A and B). As evidenced by the intense staining, the *LjNF-YA1_{pro}:GUS* activity appeared also to be more emphasized in the *lhk1-1* root epidermis, which constituted another departure from its expression pattern in wild type. Given these observations, we tested a model wherein *LjNF-YA1* works downstream from and is regulated by *Lhk1*-dependent signaling to mediate nodule differentiation.

nf-yal-2 attenuates the *snf2*-dependent spontaneous nodule formation.

In *L. japonicus*, the presence of *snf2* confers formation of spontaneous nodules (Tirichine et al. 2006a, 2007). This was also observed in our experiments, in which uninoculated *snf2* plants developed large, spontaneous nodules (Fig. 5A and F). Except for the absence of rhizobia, these empty structures displayed all features of genuine nodules, including well-developed peripheral vasculature and the presence of both large and smaller cells, with the latter containing significant starch deposits (Fig. 5B and C). By contrast, the *snf2 nf-yal-2* double mutant formed only small nodule bumps (Fig. 5D and F) that failed to grow to the stage at which the root epidermis becomes dislodged. Deprived of vasculature, these nodule bumps were composed of uniform, small cells, showing no evidence of differentiation (Fig. 5E). In the absence of *M. loti*, *nf-yal-2* roots were devoid of spontaneous nodulation events (Fig. 5F).

These results highlight the indispensable role of *LjNF-YA1* during nodule differentiation, downstream from LHK1 and the initial cell divisions. Thus, the *lhk1-1* mutation would be expected to be epistatic to *nf-yal-2*, which was tested by evaluating the nodulation phenotype of the *lhk1-1 nf-yal-2* double mutant.

lhk1-1 nf-yal-2 reveals a novel infection phenotype.

Both *lhk1-1* (Murray et al. 2007) and *nf-yal-2* (Fig. 3) are able to form NP and nodules. In *lhk1-1*, nodule formation is delayed and depends on prior, local infection of the root cortex by

Fig. 4. Activity of the *Lotus japonicus* and *Medicago truncatula NF-YA1* promoters during *L. japonicus* nodule development. **A to F**, Fully transgenic *L. japonicus* plants showing the *L. japonicus NF-YA1_{pro}:GUS* reporter activity as associated with the progressive stages in nodule development. With the exception of panel F, all panels reflect approximately 35- μ m thick sections of previously stained specimens. Note that, in panel F, the corresponding nodule was sectioned prior to staining for the β -glucuronidase activity. **G and H**, Sections of a nodule primordium and a fully developed nodule, respectively, from transgenic hairy roots formed on *L. japonicus* wild-type shoots and carrying the *Medicago truncatula MtNF-YA1_{pro}:GUS* reporter. All images represent specimens collected at either 14 (A to F) or 21 days after inoculation (G and H) with *Mesorhizobium loti*. VB = vascular bundles.

M. loti (Held et al. 2014). When examined 21 dai, however, the *lhk1-1 nf-ya1-2* double mutant displayed a novel root-infection phenotype in the complete absence of any nodule development.

At 10 and 14 dai, *lhk1-1 nf-ya1-2* had many microcolonies entrapped within curled root hairs, along with partially or fully elongated eITs, (Fig. 6A), resembling the hyperinfected phenotype of *lhk1-1* (Held et al. 2014; Murray et al. 2007). A proportion of eITs terminated at the base of root hair cells with

a hugely exaggerated tip swelling, and some infrequent incidents of bacteria being released into the epidermis-cortex intercellular space were also apparent at these earlier stages (Fig. 6B). As in *lhk1-1*, eITs were occasionally able to pass the *lhk1-1 nf-ya1-2* epidermis-cortex interface to become cortical ITs (cITs), and this was associated with limited, cortical cell divisions (Fig. 6C). However, sporadic, massive colonization of the root cortex by cITs, which in *lhk1-1* leads to limited nodule

Fig. 5. *nf-ya1-2* restricts spontaneous nodule formation. Spontaneous nodules, as formed by **A**, *Lotus japonicus snf2* and **D**, the *snf2 nf-ya1-2* double mutant. **B** and **E**, Corresponding spontaneous nodule sections. Note that the largest spontaneous nodule has been selected from among ten independent *snf2 nf-ya1-2* roots to generate the image shown in panel E. **C**, Close-up image of *snf2* nodule cells containing large deposits of starch granules. **F**, Scores of spontaneous nodulation events 8 weeks after sowing; ten individuals were scored for each genotype and averages $\pm$ 95% confidence intervals are given. Asterisk (*) indicates statistically significant difference (Student's *t* test, $P \leq 0.05$) from *snf2*. NVB = nodule vascular bundle.

Fig. 6. *Mesorhizobium loti* is mainly restricted to the extracellular space within the root cortex in the *lhk1-1 nf-ya1-2* double mutant background. **A** to **C**, *M. loti* infection of *lhk1-1 nf-ya1-2* roots at 10 (**A** and **B**) and 14 days after inoculation (dai) (**C**). **D**, Hyperinfection of the *lhk1-1* root epidermis and massive, local migration of infection threads inside the root cortex at 14 dai. **E** to **G**, Extracellular colonization of the *lhk1-1 nf-ya1-2* root cortex by *M. loti* at 21 dai. **E**, Note an extended *M. loti* infection strand that follows the longitudinal axis of the *lhk1-1 nf-ya1-2* root. **F**, A close-up image of the *lhk1-1 nf-ya1-2* root epidermis showing the presence of *M. loti* inside the epidermal cell and also within the intercellular spaces (arrows) of the subepidermal cortex. **G**, A close-up image of the *lhk1-1 nf-ya1-2* root cortex showing intercellular infection strands formed by *M. loti*. eIT = epidermal infection thread and c = root cortical cell.

formation events (Fig. 6D), was not observed in *lhk1-1 nf-ya1-2*. Nonetheless, at 21 dai, the *lhk1-1 nf-ya1-2* root cortex was colonized by *M. loti*, which instead formed intercellular infection strands (i.e., loose bacteria present in between cells) that primarily followed the longitudinal axis of the root (Fig. 6E), a phenotype rarely observed in wild type or the *lhk1-1* and *nf-ya1-1* single mutants. Although cortical cells that were abutted by these extracellular bacteria underwent at least one anticlinal cell division, incipient NP and, hence, nodules were not formed and *M. loti* failed to penetrate inside them (Fig. 6F and G).

Downstream targets of LjNF-YA1.

To gain further insight into the role of LjNF-YA1 during determinate nodule differentiation, a microarray experiment aimed at identifying downstream targets for this presumed

Fig. 7. A Venn diagram showing a subset of differentially expressed probes identified by a microarray approach. Numbers reflect probe sets that show significant (≥ 2 -fold at $P \leq 0.05$) expression changes between the indicated genotypic contrasts. The 20 probe sets that show significant expression changes in all four comparisons correspond to 17 predicted *Lotus japonicus* genes.

transcriptional factor was carried out using Affymetrix *L. japonicus* GeneChip. The gene expression levels in roots of wild type and *har1-1* parental lines, at 4 dai with *M. loti*, were compared with either *nf-ya1-1* and *nf-ya1-2* single mutants or *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2* double mutants, respectively. We reasoned that a subset of genes for which expression is affected by *nf-ya1* mutations at this relatively early stage after inoculation should emerge as common across all four comparisons, and the presence of the *har1-1* allele could accentuate more subtle differences. Assuming at least two fold changes in expression levels at $P \leq 0.05$, genes corresponding to 549 and 36 probe sets could be attributed to the *nf-ya1* mutations in the *har1-1* and wild-type backgrounds, respectively (Fig. 7; Supplementary Table S1). However, concurrent with the analysis of the microarray data, an RNAseq experiment performed in our laboratory revealed that the steady-state level of the *LjNF-YA1* mRNA was significantly upregulated in uninoculated roots of *har1-1* in comparison with the equivalent wild-type samples (E. Zimmerman, S. U. Anderson, J. Stougaard, and K. Szczygłowski, manuscript in preparation). Being expressed ectopically in *har1-1* roots in the absence of *M. loti*, LjNF-YA1 is likely to target genes that are not associated with nodule formation, which complicates the interpretation of the *har1-1*-associated microarray data. Therefore, for the purpose of this work, only 17 genes (corresponding to 20 probe sets) that were deemed as being differentially expressed in wild type versus single mutant comparisons and showed similar expression changes in the double mutants were further considered (Table 1). The gene corresponding to probe chr4.TM0851.2.1_at was included in this common (core) set because changes in expression of this gene were significant for the P value criterion in all four comparisons while falling only marginally outside the twofold threshold in one of the two double mutants (i.e., *har1-1* versus *har1-1 nf-ya1-1*, for which a 1.84-fold decrease in the expression level was recorded). These 17 genes, which were expressed at lower levels

Table 1. The core, target gene set for NF-YA1, as defined by the microarray experiment

Probe ID ^a	Gene ID (v. 3.0)	Presumed function
Signaling		
CM0584.15.1_at	Lj6g3v0959410.1	SHI/STY-like protein 1
Ljwgs_008021.2_at*	Lj0g3v0059359.1	SHI/STY-like protein 2
Ljwgs_022749.1_s_at*	Lj2g3v1728900.1	SHI/STY-like protein 3
Ljwgs_011361.1_at	Lj2g3v1455120.1	AP2-like ethylene-responsive transcription factor
Ljwgs_029578.1_s_at	Lj3g3v2888290.1	S-locus lectin protein kinase family protein
chr3.CM0152.8_at*	Lj3g3v2888290.1	S-locus lectin protein kinase family protein ^b
chr1.CM0295.68_at*	Lj1g3v4515410.1	G1L4-like probable transcriptional regulator; short hypocotyl 4-like
TC15698_at*	Lj1g3v4515410.1?	G1L4-like probable transcriptional regulator; short hypocotyl 4-like ^c
chr1.TM0805.9.1_at*	Lj1g3v3729250.1	BAG-family-like, ubiquitin domain containing protein
chr4.TM0851.2.1_at *	Lj4g3v1237990.1	Auxin canalization-like/VAN3-binding protein (VAB)
Cell growth/divisions		
chr1.CM0121.32_s_at	Lj1g3v2264900.1, Lj1g3v2264910.1	Ornithine decarboxylase
gi34495196_at	nf ^e	Ornithine decarboxylase
chr2.CM0060.76_at	Lj0g3v0047169.1	AMN1-like protein
Cell-wall modification		
Ljwgs_010358.2_at*	Lj5g3v0670070., Lj5g3v0670070.2	Pectate lyase-like protein
Ljwgs_016679.1_at*	Lj5g3v0670070.1, Lj5g3v0670070.2	Pectate lyase-like protein ^d
TM1040.6.1_at	Lj0g3v0019969.1, Lj0g3v0019969.2	Glucan endo-1,3- β -glucosidase
TM0936.13_at*	Lj0g3v0156149.1	CASP-like protein
Metabolism		
chr5.TM1077.34_at*	Lj5g3v0670650.1, Lj5g3v0670760.1, Lj5g3v0670760.2	Nodulin/glutamate-ammonia ligase-like protein
Ljwgs_037651.1_at*	Lj5g3v2182110.1	Alcohol dehydrogenase
Unknown		
chr4.CM0046.23.1_at	Lj4g3v2616100.1	Unknown protein
chr5.CM0200.5_at	Lj5g3v2133810.1	HMA domain-containing protein

^a Asterisks (*) denote probes with nodule-enhanced or nodule-specific expression patterns, as reported in the *Lotus japonicus* Gene Expression Atlas.

^b Same as Ljwgs_029578.1_s_at.

^c Likely the same as chr1.CM0295.68_at.

^d Same as Ljwgs_010358.2_at.

^e nf = no corresponding gene in v.3.0.

in the mutants than in the wild type, fell into five functional classes, including signaling, cell growth and division, cell-wall modification, metabolism, and unknown categories (Table 1). Interestingly, three of eight genes in the signaling category were found to represent members of the predicted *L. japonicus* *SHI/STY* gene family, which, in *Arabidopsis*, regulates development of various lateral organs (Ståldal et al. 2012). We chose to analyze these three genes further in order to validate the

microarray data and also to advance the understanding of the functional significance of *LjNF-YA1* during nodule formation.

***STY* genes are likely direct targets of NF-YA1 and are essential for nodule development.**

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) comparisons of *STY1*, *STY2*, and *STY3* mRNA levels in wild-type and *nf-ya1-2* roots, at 4 dai with *M. loti*, showed

Fig. 8. NF-YA1 mediates *STY* gene expression during nodule development. **A**, Quantitative reverse transcription-polymerase chain reaction analysis of the *STY1*, *STY2*, and *STY3* mRNAs in *Lotus japonicus* wild-type and *nf-ya1-2* mutant roots. UNR = uninoculated roots harvested 7 days after sowing; 4DAI = roots harvested 4 days after inoculation (dai) with *Mesorhizobium loti*. Average values for three biological replicates $\pm$ standard error are given. Asterisks indicate statistically significant differences (one asterisk [*], $P \leq 0.05$; two asterisks [**], $P \leq 0.01$) in expression between wild type and mutants. **B**, Electrophoretic mobility shift assays show specific binding of NF-YA1 to *STY* gene promoters in vitro. The migration of biotinylated *STY1*, *STY2*, and *STY3* promoter fragments in the absence (-) or the presence (+) of 1 μ g of purified FLAG-NF-YA1 wild type or FLAG-NF-YA1^{G213D} mutant DNA-binding domain is shown. The corresponding, nonlabeled, wild-type probe in 100-fold molar excess (100 $\times$) relative to the biotinylated probes have been added as competitors. Arrows indicate the specific binding of the wild-type FLAG-NF-YA1 protein. **C**, Transgenic hairy roots showing GUS reporter activity, as driven by the *STY1*, *STY2*, and *STY3* promoters. Images of nodules were taken 21 dai with *M. loti*. The activity of the *NF-YA1* promoter is shown for comparison.

significant upregulation of the three genes in the wild type that was absent in the mutant (Fig. 8A). This is consistent with the microarray data, confirming that elevated expression levels of the *STY* genes during early symbiotic events depend on the presence of intact *NF-YA1*. We subsequently tested whether *NF-YA1* could interact with *STY1*, *STY2*, and *STY3* promoters by performing electrophoretic mobility shift assays (EMSA). Such interaction would be expected if *STYs* constitute direct targets of *NF-YA1*-dependent regulation. The DNA-binding domain of the *LjNF-YA1* protein was able to specifically bind to promoters of the three *STY* genes in vitro. By contrast, the *NF-YA1*^{G213D} mutant protein, carrying the *nf-ya1-2* version of the DNA-binding domain (Fig. 2), could not do so (Fig. 8B). While supporting the specificity of the interaction in vitro, this also provided a plausible explanation for the *nf-ya1-2* mutant symbiotic phenotype. We further reasoned that if *NF-YA1* binds to *STY1*, *STY2*, and *STY3* promoters in vivo, their expression domains would be expected to overlap in nodules. Indeed, when examined in hairy roots 21 dai with *M. loti*, the three *STY* promoters directed the GUS reporter activity to nodule vascular bundles, which mirrors the *NF-YA1* expression domain in *L. japonicus* nodules (Fig. 8C).

To begin testing the hypothesis that *STY* proteins mediate, at least in part, the effect of *NF-YA1* on nodule development, we made a chimeric construct composed of the *L. japonicus STY3* coding region translationally fused with a DNA fragment encoding the 12-amino acid-long ERF-associated amphiphilic repression (SRDX) domain. The SRDX domain would be expected to convert the presumed *STY3* transcriptional activator into a dominant transcriptional repressor, as previously documented for several different transcriptional factors (Hiratsu et al. 2003).

The *STY3::SRDX* mRNA was expressed in transgenic hairy roots formed on wild-type *L. japonicus* shoots under the *Ubiquitin (UBI_{pro})* and *LjNF-YA1* promoters. The green fluorescent protein (GFP) fluorescent marker was used to identify cotransformation events. In the presence of *UBI_{pro}::STY3::SRDX*, only nonfluorescing hairy roots were formed, indicating a detrimental effect of the construct. GFP-positive hairy roots were recovered from the empty vector control and *LjNF-YA1_{pro}::STY3::SRDX* transformations and their nodulation phenotypes were evaluated 21 dai with *M. loti*. While the empty vector-transformed hairy roots showed the wild-type nodulation ($\bar{x} = 8.6 \pm 2.1$; $n = 28$), the presence of *LjNF-YA1_{pro}::STY3::SRDX* led to a low nodulation phenotype ($\bar{x} = 1.5 \pm 0.8$). Of 28 independent hairy root systems transformed with *LjNF-YA1_{pro}::STY3::SRDX*, 13 did not form nodules at all, resembling the prevalent symbiotic phenotype of hairy roots formed on *nf-ya1-2* shoots, while the remaining 15 showed a low-nodulation phenotype that included small and regular-size nodules.

DISCUSSION

Here, we show that the *L. japonicus* genome contains a family of at least nine *LjNF-YA* genes, with *LjNF-YA1* displaying a *M. loti* inoculation-dependent expression pattern and being essential for the normal development of functional, nitrogen-fixing root-nodule symbiosis. Our observations confirm that deleterious mutations at the *LjNF-YA1* locus attenuate nodule development in *L. japonicus*. Refining the previously published RNAi-silencing data (Soyano et al. 2013), we demonstrate that the developmental block imposed by *nf-ya1* mutations occurs primarily at the NP stage, after the first cell divisions but prior to nodule differentiation, including cell enlargement and vascular bundle formation. Our transcriptomic data suggest that this negative effect of *nf-ya1* mutations on symbiosis is, at least in part, due to impaired regulation of *STY1*, *STY2*, and *STY3* genes identified by this work as likely direct targets of the *NF-YA1*-dependent regulation.

NF-YA1 is likely to work partially redundantly to mediate nodule formation in *L. japonicus*.

When analyzed, at 21 dai with *M. loti*, all four *nf-ya1* mutant lines had many small NP, but a few wild type-looking nodules were also formed, which indicates that a block imposed by the corresponding mutations is sometimes overcome. Considering the similar symbiotic defects in four independent *nf-ya1* allelic mutant lines analyzed, including the two LORE1 insertion lines that carry premature stop codons and *nf-ya1-2*, which encodes the *NF-YA1*^{G213D} protein that has apparently lost its DNA-binding activity (Fig. 8B), this is likely due to the presence of genes that operate in a partially redundant manner. The *L. japonicus* gene atlas data show that several *NF-YA* genes, including *LjNF-YA4* (LjGEA probe ID: chr1.CM0361.18_at), the closest paralogue of *LjNF-YA1* and a likely ortholog of *MtNF-YA2*, are expressed in uninoculated roots and, also, to some degree in nodules, although the steady-state levels of their corresponding mRNAs appear to be, at most, only slightly affected by *M. loti* infection (Verdier et al. 2013). Work is in progress to define whether *LjNF-YA4*, singly or in combination with other *LjNF-YA* genes, contribute to the presumed, partial redundancy of *LjNF-YA1* functions during nodule development and if this is similar to what has been described for *MtNF-YA1* and *MtNF-YA2* (Laloum et al. 2014). Our data indicate that *MtNF-YA1* is able to rescue the symbiotic defect of *nf-ya1-2*. Hence, in spite of forming different types of nodules, *MtNF-YA1* and *LjNF-YA1* must be functionally equivalent in *L. japonicus*.

The NF-YA1 infection puzzle?

Although activity of the *LjNF-YA1* promoter is detectable at the initial stages of the interaction (Fig. 4), no early symbiotic defects were apparent in any of the *L. japonicus nf-ya1* single mutants and NP formation was initiated in a timely manner. Furthermore, neither eIT development nor the ability to form cITs appeared to be perturbed by *nf-ya1* mutations. The wild-type infection phenotype of *nf-ya1* mutants is consistent with the results of Soyano et al. (2013), who showed that RNAi of *LjNF-YA1* in *L. japonicus* hairy roots had no effect on eIT formation, although the status of cITs was not reported. Development of cITs in *L. japonicus* requires at least some level of cytokinin and NIN-dependent activation of the subtending root cortex (Held et al. 2014; Miri et al. 2016; Yoro et al. 2014). Our phenotypic observations, in particular the presence of cortical cell divisions along with cIT formation, indicate that this signaling process must be operational in *L. japonicus nf-ya1* mutants.

By contrast, the *Mtnf-ya1-1* mutation in *Medicago truncatula* was associated with misshapen and enlarged eITs that were prematurely arrested within root hairs and had twofold thinner cell walls (Laporte et al. 2014). Furthermore, unlike *L. japonicus nf-ya1* mutants, *Mtnf-ya1-1* was found to develop six times more eITs, as compared with the wild-type control. Interestingly, silencing of *MtNF-YA2* in the *Mtnf-ya1-1* mutant background restricted this presumed compensatory effect, with the composite plants (i.e., *Medicago truncatula nf-ya1-1* plants with transgenic hairy roots expressing the *MtNF-YA2* silencing construct) forming only a few ITs that were almost all arrested within root hairs (Laloum et al. 2014). As previously stated by others, these results show that, while functioning in a partially redundant manner, the *MtNF-YA1*-dependent regulation is important for the symbiotic infection in *Medicago truncatula* (Laloum et al. 2014; Laporte et al. 2014). In contrast, we show here that, in *L. japonicus*, *LjNF-YA1* is dispensable for this process (Fig. 3). This could reflect different requirements for symbiotic infection of determinate versus indeterminate nodules in *L. japonicus* and *Medicago truncatula*, respectively. However, in common bean, which like *L. japonicus* forms determinate nodules, RNAi silencing of *PvNF-YC1* significantly reduced the number of eITs and affected their progression inside the root cortex (Zanetti et al.

2010). Thus, an NF-Y transcription factor containing the PvNF-YC1 subunit is likely important for rhizobial infection during determinate nodule development in common bean.

If LjNF-YA1 participates in the regulation of *M. loti* infection, this would have to be through a mostly or fully redundant function. The unusual infection phenotype seen in the *lhk1-1 nf-ya1-2* double mutant (Fig. 6) might be indicative of the former. The *lhk1-1* mutation imposes a significant block that halts the majority of eITs from progressing into the root cortex (Miri et al. 2016; Murray et al. 2007). This is manifested in both *lhk1-1* and the *lhk1-1 nf-ya1-2* double mutant as the presence of swollen IT tips at the root epidermis-cortex interface (Fig. 6B), although not all eITs whose growth has been stalled in either of the mutants display this feature. In the *lhk1-1 nf-ya1-2* double mutant, release of *M. loti* from ITs into the intercellular root cortical space was apparent at 21 dai (Fig. 6E to G). This is likely the result of eITs that had managed to traverse root hair cells but failed to transit to cITs and became disintegrated. Such relatively wide-spread release and subsequent migration of *M. loti* within the root intercellular compartment has not been observed in the *nf-ya1-2* single mutant, nor is it a feature of *lhk1-1*. The latter shows that the role of LjNF-YA1 during nodulation is not completely dependent on LHK1. It is, therefore, conceivable that the *nf-ya1-2* mutation directly or indirectly weakens the integrity of ITs and that this is exacerbated by hindered IT growth in the *lhk1-1* background, resulting in the release of bacteria. Whether this is related to diminished integrity of the IT cell wall, as reported for *Mtnf-ya1-1* (Laporte et al. 2014), remains to be determined.

Importantly, the phenotype of *lhk1-1 nf-ya1-2* demonstrates that, once beyond the root epidermis, *M. loti* can be an avid extracellular endophyte, with the ability to colonize the root interior by propagating through infection strands within the root intercellular space. This *M. loti* trait becomes apparent in the *lhk1-1 nf-ya1-2* double mutant, in which the ability to form eITs appears to be intact but entry into cortical cells has been compromised. The fact that the *lhk1-1 nf-ya1-2* double mutant does not form nodules, in contrast to the corresponding single mutants, extends our previous observations and further demonstrates the requirement for a local, massive entry of *M. loti* inside root cortical cells in order for *lhk1-1* to develop nodules (Held et al. 2014). Apparently, formation of extracellular, cortical infection strands (Fig. 6G) is not sufficient for nodule formation in the absence of functional LHK1 and NF-YA1.

***nf-ya1* mutations attenuate the cortical program for nodule formation prior to differentiation.**

Evaluation of symbiotic phenotypes of hairy roots associated with the RNAi of PvNF-YC1 or its interacting partner, *Scarecrow-like13 Involved in Nodulation (SINI)*, led to the conclusion that the corresponding proteins might act in common bean to activate cortical cell divisions for NP formation (Battaglia et al. 2014; Zanetti et al. 2010). Similarly, RNAi-mediated downregulation of LjNF-YA1 gene expression in hairy roots of both the *L. japonicus* wild type and a gain-of-function CCaMK^{T265D} mutant demonstrated an early inhibition of cortical cell divisions for NP formation, with the majority of composite plants developing no nodules (Soyano et al. 2013). The mainly non-nodulating phenotype of *nf-ya1-2* hairy roots is consistent with these earlier observations. However, all *L. japonicus nf-ya1* mutants tested in this study developed a large number of small NP and the *snf2 nf-ya1-2* double mutant was capable of forming underdeveloped nodule bumps in the absence of *M. loti*, indicating that, in *L. japonicus*, hairy roots exaggerate the *nf-ya1-2* mutant symbiotic phenotype.

In comparison with mostly very small NP of *M. loti*-inoculated *nf-ya1* single mutants, spontaneously formed nodule

bumps of *snf2 nf-ya1-2* appeared to be more advanced in terms of the extent of cell divisions. It may be that the constitutive activity of *snf2* accounts for this phenotypic difference. Regardless, even the largest spontaneous nodulation event in *snf2 nf-ya1-2* lacked some key features of nodule differentiation, including the presence of enlarged cells and nodule vasculature (Fig. 5). We conclude, therefore, that, during determinate nodule development in *L. japonicus*, LjNF-YA1 becomes indispensable either downstream from the initial cortical cell divisions at the stage of cell division maintenance (Suzaki et al. 2012), during the transition of young NP cells from divisions to differentiation, or both. Given the activity pattern of the LjNF-YA1 promoter, it is also likely that LjNF-YA1 partakes in mediating earlier symbiotic events, prior to or concomitant with the initiation of cell divisions, but this must be through one or more redundantly operating functions. In *Medicago truncatula*, the induction of MtNF-YA1 and MtNF-YA2 in root hairs of rhizobially inoculated plants is accompanied by the activation of auxin and cell-cycle markers, suggesting that infection may require engagement of NF-Ys and cell-cycle machinery (Breakspear et al. 2014).

NF-YA1 regulates STY gene expression.

Among 17 genes defined by this work as likely being regulated by NF-YA1 (Table 1), three members of the *L. japonicus* SHI/STY zinc-finger transcription factor gene family, i.e., STY1, STY2, and STY3, were characterized as potential direct targets. We show that their expression is significantly up-regulated in *L. japonicus* roots early on upon *M. loti* infection and associates with vascular bundles in mature nodules. In *Arabidopsis thaliana* and *Physcomitrella patens*, STY proteins have been shown to regulate various developmental processes in a redundant manner, including formation of lateral roots and flowers and leaf venation (Baylis et al. 2013; Kuusk et al. 2002, 2006; Smith and Fedoroff 1995). Our data suggest that they might also play an essential role during symbiotic root nodule formation in *L. japonicus*.

Downstream targets of *L. japonicus* STY1, STY2, and STY3 are currently unknown but in *Arabidopsis*, STY1 was shown to regulate different transcription factors and genes associated with cell expansion and auxin biosynthesis (Eklund et al. 2010a and b; Sohlberg et al. 2006). The latter has been demonstrated to be carried out by STYs through direct transcriptional activation of YUC genes (Eklund et al. 2010a and b; Sohlberg et al. 2006), which encode the indole-3-pyruvic acid (IPyA) decarboxylase, a rate-limiting enzyme that works downstream from tryptophan aminotransferases in the IPyA auxin biosynthesis pathway (Cheng et al. 2006; Zhao 2012).

Several independent studies have shown that, during nodule formation, auxin response maxima are generated downstream from NF perception and the resulting cytokinin signaling (Deinum et al. 2015; Mathesius 2008; Mathesius et al. 2000; Plet et al. 2011; Suzaki et al. 2012). In *L. japonicus*, initiation of auxin activity accompanies the initial cortical cell divisions for NP formation but has also been linked with their maintenance and with the development of the nodule vasculature and lentils (Suzaki et al. 2012; Takanashi et al. 2011). A model has been proposed in which local accumulation of auxin within *M. loti*-inoculated *L. japonicus* roots originates as a result of the LHK1-dependent NIN activation; however, the mechanism by which NIN regulates auxin is unknown (Suzaki et al. 2012). One possibility is that NF-YA1, which is transcriptionally activated by NIN in the root cortex (Soyano et al. 2013) partakes in the generation of local auxin maxima by mediating the STY1, STY2, and STY3 gene expression, which aids in the differentiation of the determinate nodule structure, including cell expansion and formation of nodule vascular bundles. However,

due to the lack of a clear response of the *YUC* genes in our microarray experiment, this idea needs to be further examined.

Interestingly, downregulation of another gene, *Lj4g3v1237990*, in both *nf-ya1-1* and *nf-ya1-2* mutants (Table 1) might also be of relevance in the context of auxin signaling. Predicted as legume-specific in the *Lotus japonicus* gene expression atlas database, *Lj4g3v1237990* encodes a protein that contains the auxin canalization domain (pfam05073) and plant pleckstrin-homology region (pfam08458). These features are shared by the *Arabidopsis* FORKED1/VAB (Hou et al. 2010) and other VAN3-binding (VAB)-like proteins from various species, including *Glycine max* (XP_014630987) and *Medicago truncatula* (Medtr5g012870). VAN3 is an adenosine diphosphate-ribosylation factor guanosine triphosphatase activating protein (Koizumi et al. 2005). Together with the COTYLEDON VASCULAR PATTERN2 inositol polyphosphate 5'-phosphatase and VAB, VAN3 functions in *Arabidopsis* in vascular pattern formation by influencing localization of the PIN1 auxin efflux carrier (Caño-Delgado et al. 2010; Hou et al. 2010; Koizumi et al. 2000; Naramoto et al. 2009). A similar mechanism might be relevant during differentiation of nodule vasculature in *L. japonicus* and our data suggest that NF-YA1 could be a regulator of this process. In *Arabidopsis*, expression of FORKED1/VAB was shown to be responsive to auxin levels (Hou et al. 2010). Future research will have to establish whether the reduced expression level of *Lj4g3v1237990*, which we named *LjVAB-like1* or *LjVABLI*, correlates with a local deficiency in auxin transport in *L. japonicus* NP deprived of NF-YA1 and if this, indeed, contributes to the attenuated development of *nf-ya1* nodules.

MATERIALS AND METHODS

Plant materials, growth conditions, and assessment of symbiotic phenotypes.

The *L. japonicus nf-ya1* mutants were identified from a screen for genetic suppressors of the *har1-1* hypernodulation phenotype, with the originally selected LjS47-A and LjS95-E lines (Murray et al. 2006) corresponding to *har1-1 nf-ya1-1* and *har1-1 nf-ya1-2*, respectively. Three additional mutant alleles, namely *nf-ya1-3*, *nf-ya1-4*, and *nf-ya1-5*, were identified from the *L. japonicus* LORE1 retrotransposon mutant resource (line numbers 30004771, 30003811, and 30003445, respectively) (Fukai et al. 2012; Urbanski et al. 2012). Although not analyzed further, *nf-ya1-5* carries the insertion within exon 7, 1,520 bp downstream from the NF-YA1 initiation codon, and showed the same defective symbiotic phenotype as other *nf-ya1* mutant plants described in this work.

Seed germination, soil growth conditions, and evaluation of symbiotic phenotypes were as previously described (Wopereis et al. 2000), except that microscopic observations were made under higher, 20× magnification in order to capture even the smallest nodule formation events.

For studying the response of *L. japonicus* wild type, *lhk1-1*, and *nin-8* plants to exogenous cytokinin, roots were collected from 10-day-old plants that had been incubated for 6 h in the presence of 50 nM 6-benzylaminopurine, as described in Murray et al. (2007).

Map-based cloning of the NF-YA1 locus.

The rough map position of the causative locus was initially determined using two sets of 30 mutants, each selected from the two independent F2 segregating populations obtained by crossing LjS47-A (*har1-1 nf-ya1-1*) and LjS95-E (*har1-1 nf-ya1-2*) with the polymorphic MG20_ *har1-1* introgression line (Murray et al. 2006). In both cases, the mutated locus was determined to be located on chromosome 5 between markers TM0494 and TM0471 in the *L. japonicus* genome. Allelic tests between *nf-ya1-1* and *nf-ya1-2* confirmed that the same locus was affected in

both mutant lines. To further delineate the flanking region, 960 selected F2 *har1-1 nf-ya1-2* mutant individuals were used to analyze recombination events. The simple sequence repeat markers, as selected from the publicly available *L. japonicus* genome website, were used for the linkage analysis.

Characterization of the NF-YA1 mRNA.

The *L. japonicus* 'Gifu' NF-YA1 cDNA was amplified from the total RNA derived from 21-day-old nodules, using the ThermoScript RT-PCR system (Invitrogen). To obtain the full length NF-YA1 mRNA sequence, 5' and 3' rapid amplification of cDNA ends was performed, using the FirstChoice RLM-RACE kit (Ambion). The PCR conditions for amplifying the full-length NF-YA1 mRNA using the NF-YA1-cDNA full F1 and NF-YA1-cDNA full R1 primers were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 4 min, with a final extension for 7 min at 68°C. To determine the extent of the *nf-ya1-1* specific sequence deletion (Fig. 3), the *nf-ya1-1del-F1* and *nf-ya1-1del-R1* primers were used. Sequences of primers used are listed in Supplementary Table S2.

In planta complementation assays.

The *L. japonicus* and *Medicago truncatula* NF-YA1 genes (including their respective promoter, coding, and 3' UTRs) were amplified using the gene-specific primers. The resulting DNA fragments were recombined into the pKGWD,0 destination vector (University of Ghent Gateway Vectors website) (Karimi et al. 2002). The resulting binary vectors were introduced by *Agrobacterium rhizogenes*-mediated transformation to generate transgenic hairy roots on nontransgenic *L. japonicus nf-ya1-2* mutant shoots, following the previously established procedure (Murray et al. 2007). The same *A. rhizogenes* strain carrying an empty vector was used as a negative control. Wild-type and *nf-ya1-2* shoots that developed hairy roots were transplanted into a sterilized sand and vermiculite mixture and were grown for 5 days prior to *M. loti* inoculation. For each construct and genotype, at least 10 individual plants from two independent experiments were evaluated 21 dai with *M. loti*.

Gene expression analysis using quantitative RT-PCR.

Total RNA was extracted from various *L. japonicus* 'Gifu' tissues using the Plant/fungi total RNA purification kit (Norgen Biotek). The analysis of RNA quality, cDNA synthesis, and qRT-PCR of the NF-YA1 mRNA were performed as described by Held et al. (2014), using NF-YA1-qPCR-F and NF-YA1-qPCR-R primers. qRT-PCR of *STY1*, *STY2*, and *STY3* mRNA was carried out using the SsoFast EvaGreen supermix (Bio-Rad) under the following conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. Expression levels were normalized against three reference genes including *UBC*, *PP2A*, and *ATPs* (Tirichine et al. 2007).

Gene expression analysis using GUS histochemical assay.

To develop the gene expression localization constructs, the 3.045 and 2.4 kb *LjNF-YA1* and *MtNF-YA1* promoter fragments, respectively, were amplified. In addition, 4-kb fragments encompassing the *STY1*, *STY2*, and *STY3* promoter regions (position -1 to -4,000 in the corresponding genomic sequences, where -1 denotes the first base upstream from the predicted ATG initiation codon) were prepared by gene synthesis (Bio Basic Inc.). These were recombined in the pKGWFS7 destination vector containing GFP/GUS reporter fusion (University of Ghent Gateway vector website), using the GatewayTM technology (Invitrogen). After validation by sequencing, the corresponding vectors were transferred to either *Agrobacterium tumefaciens* LBA4404 (*LjNF-YA1_{pro}::GUS*) or to *A. rhizogenes*

AR10 (*MtNF-YA1_{Pro}:GUS*, *STY1_{Pro}:GUS*, *STY2_{Pro}:GUS*, and *STY3_{Pro}:GUS*). Standard transformation protocols were employed to generate either fully transgenic *L. japonicus* plants carrying the *LjNF-YA1:GUS* promoter gene fusion (Lombardi et al. 2005) or nontransgenic *L. japonicus* shoots with hairy roots expressing other constructs (Murray et al. 2007). At least seven independent fully transgenic *L. japonicus* plants or 10 independent hairy root systems were carefully inspected to define expression patterns. The staining procedure for detection of the GUS reporter activity was as described previously (Held et al. 2014), except for the nodule section shown in Figure 4F. In the latter case, fully mature nodules derived from transgenic *L. japonicus* plants carrying *LjNF-YA1_{Pro}:GUS* were embedded in agar and were sectioned to a thickness of 30 μ m, prior to being stained for GUS activity for approximately 30 min, in order to improve the penetrance of the GUS histochemical substrate.

Evaluation of *nf-ya1-2* phenotype in the presence of cytokinin loss and gain-of-function alleles.

An *A. rhizogenes* strain carrying the *snf2* allele integrated within its T-DNA (a gift from J. Stougaard, Aarhus University, Denmark) (Tirichine et al. 2007) was used to induce hairy roots on the *L. japonicus* wild-type and *har1-1*, *nf-ya1-1*, *nf-ya1-2*, *har1-1 nf-ya1-1*, and *har1-1 nf-ya1-2* mutant shoots. The resulting composite plants were grown under sterile conditions in a sand and vermiculate mixture supplemented with the 1 $\times$ B&D nutrient solution (Broughton and Dilworth 1971) containing 0.5 mM KNO₃. The number of spontaneous nodules was determined 8 weeks after planting. Similar growth conditions were employed to evaluate spontaneous nodule formation in *snf2*, *nf-ya1-2*, and *snf2 nf-ya1-2* mutant plants (Fig. 7) and also to determine steady-state levels of the *NF-YA1* mRNA in uninoculated wild-type and *snf2* roots (Fig. 6), except that the latter two root samples were collected 9 days after sowing.

The *snf2 nf-ya1-2* and *lhk1-1 nf-ya1-2* double mutant lines were developed through genetic crosses between *nf-ya1-2* and *snf2* or *lhk1-1*, respectively. Homozygous double mutants were selected from the corresponding F₂ populations, using allele-specific markers for *snf2* (*snf2* dCAPS) (Madsen et al. 2010) *nf-ya1-2*, and *lhk1-1* (*nf-ya1-2*-CAPS-*Mbo*I and *lhk1-1*-CAPS-*Bcc*I, respectively).

Development of *NF-YA1_{Pro}:GUS* and *NIN_{Pro}:GUS* lines in *lhk1-1* background.

To evaluate the activity of the *LjNF-YA1* promoter in the *lhk1-1* genetic background, a transgenic line carrying the *LjNF-YA1_{Pro}:GUS* reporter (discussed above) was crossed with the homozygous *lhk1-1*. Individual plants from a segregating F₂ population were screened for GUS activity, and GUS-positive plants were genotyped for the presence of the *lhk1-1* allele. *LjNF-YA1_{Pro}:GUS* plants carrying the homozygous *lhk1-1* mutation were selected and F₃ plants were used for promoter localization studies. A similar procedure was used to develop a *L. japonicus* line expressing *NIN_{Pro}:GUS* (provided by J. Stougaard, Aarhus University, Denmark) (Radutoiu et al. 2003) in the *lhk1-1* background.

Microarray gene expression analysis.

Three biological replicates were generated for each genotype, and root samples were harvested 4 dai with *M. loti*. Total RNA extractions were carried out using TRIZOL reagent (Invitrogen) (Chomczynski and Mackey 1995), followed by genomic DNA removal by DNaseI (Ambion), with an additional column purification with RNeasy MinElute cleanup kit (Qiagen). RNA was quantified using a NanoDrop Spectrophotometer ND-100 (NanoDrop Technologies) and was evaluated for purity with a

Bioanalyzer 2100 (Agilent). The Affymetrix GeneChip Lotus Genome Array (Affymetrix) was used for expression analysis. Probe labeling, using 4 μ g RNA, array hybridization, and scanning were performed according to the manufacturer's instructions (Affymetrix) for eukaryotic RNA, using a one-cycle protocol for cDNA synthesis. Raw data were normalized by robust multichip averaging, as previously described (Irizarry et al. 2003). Presence and absence calls for probe sets were obtained using the dCHIP algorithm (Li and Wong 2001). Differentially expressed genes in mutants were identified using the associative analysis (Dozmorov and Centola 2003). Type I family-wise error rate was reduced by using a Bonferroni corrected *P* value (threshold 0.05). False discovery rate was controlled by calculating the *q* value using extraction of differential gene expression (Leek et al. 2006). To identify differentially regulated probe sets, we used a *P* value threshold of 95% and at least a twofold difference in transcript expression level. All microarray data were deposited in ArrayExpress under accession number E-MTAB-4970.

EMSA.

A C-terminal portion of the *NF-YA1* mRNA encompassing the DB domain (i.e., amino acid residues 184 to 332) was amplified, using the *NF-YA1_DB-F* and *NF-YA1_DB-R* primers. Restriction enzyme sites for *Hind*III and *Bgl*II along with three random nucleotides were introduced at 5' ends of forward and reverse primers, respectively, in order to aid cloning into the pT7-FLAG-2 expression vector (Sigma). The integrity of the resulting *NF-YA1DB-FLAG* expression plasmid was confirmed by sequencing. To generate the *nf-ya1-2* version of the same construct, a site-directed mutagenesis was carried out using *NF-YA1DB-FLAG* (above) as the template and the 5'-phosphorylated forward (5'-AAGGGGATGTGGTGATCGGT TCTTGAACA-3') and reverse (5'-GGCCGCGCATTCATGTAGATGGC-3') primers. The resulting vector was sequenced to confirm the presence of the *nf-ya1-2* mutation (i.e., A to G substitution, shown in bold italics in the forward primer). The recombinant NF-YA1 and NF-YA1^{G213D} DB domains with C-terminal FLAG tag were produced and purified using ANTI-FLAG M2 affinity gel (Sigma), following the manufacturer's instructions.

EMSA assays were carried out using the LightShift chemiluminescent EMSA kit (ThermoFisher). For *STY1*, the region spanning nucleotide positions from -1,615 to +88 (where +1 reflects A in the predicted ATG translation initiation codon) was amplified as three overlapping fragments (-1,615 to -1,079; -1,101 to -539; and -558 to +88). These fragments were biotin end-labeled using the Biotin 3' end DNA labeling kit (ThermoFisher) and were tested for the binding of NF-YA1DB-FLAG in vitro. The middle fragment, -1,101 to -539, which showed binding, was subsequently divided into three additional sub-fragments, with only the -953 to -729 region demonstrating the specific binding activity. The same -953 to -729 *STY1* promoter fragment was used to test for the binding activity of the mutant NF-YA1DB^{G213D}-FLAG. A similar procedure was used to select the *STY2* (-357 to -104) and *STY3* (-293 to -82) promoter fragments, which were specifically recognized by NF-YA1DB-FLAG in vitro. The corresponding nonlabeled promoter fragments were used as cold competitors.

A dominant negative *STY3::SRDX*.

The ubiquitin promoter (Maekawa et al. 2008) was synthesized (Bio Basic Inc.), with attL1 and attR5 recombination sites at its 5' and 3' ends, respectively. A DNA fragment encompassing the entire *STY3* coding region (gene ID Lj2g3v1728900.1 [Kazusa Research Institute's *L. japonicus* database]), translationally fused with the 36-bp sequence for the ERF-associated amphiphilic

repression domain (superman repression domain X [SRDX]) (Hiratsu et al. 2003), and the attL5 and attL2 recombination sites at the 5' and 3' ends, respectively, was also prepared by gene synthesis (Bio Basic Inc.). Similarly, another DNA fragment encompassing the *L. japonicus* *NF-YA1* promoter (position -2766 to -1, gene ID Lj5g3v0841080.1) transcriptionally linked to the *STY3::SRDX* coding region (as above) and followed by the *NF-YA1 3' UTR* gene, was synthesized with attL1 and attL2 sites (Bio Basic Inc.). These DNA fragments were recombined directly into the pKGWD,0 destination vector, using either the standard or MultiSite Gateway technologies (Invitrogen), giving rise to *UBI_{Pro}::STY3::SRDX* and *NF-YA1_{Pro}::STY3::SRDX::NF-YA1 3' UTR* vectors. Upon validating the integrity of the vectors by sequencing, they were transferred along with the empty pKGWD,0, used as a negative control, to *A. rhizogenes* AR1193. The resulting *Agrobacterium* strains were used to generate transgenic hairy roots on nontransgenic *L. japonicus* wild-type shoots, as described above. The symbiotic phenotype of the composite plants was evaluated 21 dai with *M. loti* by counting nodulation events on GFP-fluorescing hairy roots.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ArrayExpress database: <https://www.ebi.ac.uk/arrayexpress>
 Centre for Carbohydrate Recognition and Signalling *Lotus japonicus* retrotransposon resource: <http://users-mb.au.dk/pmgrp>
 University of Ghent Gateway vector website: <https://gateway.psb.ugent.be/search>
 Kazusa Research Institute's *L. japonicus* genome clone list: <http://www.kazusa.or.jp/lotus/clonelist.html>
 Kazusa Research Institute's *Lotus japonicus* database: <http://www.kazusa.or.jp/lotus/index.html>
L. japonicus LORE1 retrotransposon mutant resource: <https://lotus.au.dk>
 The Noble Foundation's *Lotus japonicus* gene expression atlas database: <http://ljgea.noble.org/v2>