NIN Acts as a Network Hub Controlling a Growth Module Required for Rhizobial Infection[1][OPEN]

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The symbiotic infection of root cells by nitrogen-fixing rhizobia during nodulation requires the transcription factor Nodule Inception (NIN). Our root hair transcriptomic study extends NIN's regulon to include *Rhizobium Polar Growth* and genes involved in cell wall modification, gibberellin biosynthesis, and a comprehensive group of nutrient (N, P, and S) uptake and assimilation genes, suggesting that NIN's recruitment to nodulation was based on its role as a growth module, a role shared with other NIN-Like Proteins. The expression of jasmonic acid genes in *nin* suggests the involvement of NIN in the resolution of growth versus defense outcomes. We find that the regulation of the growth module component Nodulation Pectate Lyase by NIN, and its function in rhizobial infection, are conserved in hologalegina legumes, highlighting its recruitment as a major event in the evolution of nodulation. We find that Nodulation Pectate Lyase is secreted to the infection chamber and the lumen of the infection thread. Gene network analysis using the transcription factor mutants for *ERF Required for Nodulation1* and *Nuclear Factor-Y Subunit A1* confirms hierarchical control of NIN over *Nuclear Factor-Y Subunit A1* and shows that ERF Required for Nodulation1 acts independently to control infection. We conclude that while NIN shares functions with other NIN-Like Proteins, the conscription of key infection genes to NIN's control has made it a central regulatory hub for rhizobial infection.

Most legumes can interact with nitrogen-fixing bacteria, called rhizobia, which are taken up into cells of specialized root organs called nodules. Nodulation involves two processes, rhizobial infection and nodule formation. Rhizobial infection is preceded by attachment of the bacteria to elongating root hairs, which redirects growth to entrap the rhizobia within a curl, forming an infection chamber. From this chamber, a tubular structure called the infection thread forms as an invagination of the plasma membrane and cell wall. As the infection thread grows, it becomes colonized by dividing rhizobia, directing them through a predefined route to the developing nodule (Fournier et al., 2008, 2015). Within the nodule, the rhizobia are endocytosed from the infection thread into specialized host membrane-enclosed structures called symbiosomes where nitrogen fixation takes place (Ivanov et al., 2010). Thus, nodulation depends on the successful coordination of infection and nodule formation. Legumes such as *Lotus japonicus* form determinate nodules, which are spherical, lack an apical meristem, and harbor some infection threads when mature (Malolepszy et al., 2018), while indeterminate-type nodules formed on legumes

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such as *Medicago truncatula*, are elongated, and feature a persistent apical meristem and an adjacent infection zone containing dense and highly branched infection threads (Guan et al., 2013; Chen et al., 2015).

Nodulation requires host perception of lipochitooligosaccharide signals called Nod factors, which are produced by rhizobia in response to plant flavonoids (Fliegmann and Bono, 2015; Liu and Murray, 2016). Several transcription factors orchestrate the downstream responses to Nod factors, leading to rhizobial infection and nodule formation, including Nodule Inception (NIN; Schauser et al., 1999; Marsh et al., 2007), Interacting Protein of DMI3 (IPD3; Horváth et al., 2011; Ovchinnikova et al., 2011) andIPD3-Like (IPD3L), ERF Required for Nodulation (ERN1/ERN2; Andriankaja et al., 2007; Middleton et al., 2007), Nuclear Factor-Y Subunit A1 (NF-YA1; Laloum et al., 2014), the GIBBERELLIC-ACID INSENSITIVE, REPRESSOR of GIBBERELLIC-ACID INSENSITIVE, and SCARECROW (GRAS) transcription factors Nodulation Signaling Pathway1 (NSP1) and NSP2 (Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006), and the DELLAs (Fonouni-Farde et al., 2016; Jin et al., 2016). Transcript profiling of *M. truncatula* root hairs on rhizobia-inoculated plants revealed several hundred genes with up-regulated expression (Breakspear et al., 2014), including many hormone-related genes and genes with roles in the cell cycle. Along with these genes, numerous transcription factors were induced in this root hair transcriptome, including *NIN*, *NF-YA1*, and *ERN1*, which are prime candidates for mediating a subset of the transcriptional changes that occur during rhizobial infection.

NIN is the founding member of the NIN-Like Protein (NLP) family of transcription factors (Schauser et al., 1999; Chardin et al., 2014). *nin* mutants are unable to form proper infection chambers and form abnormally hypercurled root hair tips. Although the *nin* mutant does entrap some rhizobia within curled root hairs, rhizobia cell division is limited and infection threads fail to initiate (Schauser et al., 1999; Borisov et al., 2003; Marsh et al., 2007; Fournier et al., 2015). Both *nf-ya1-1* and *ern1* are defective in the development of infection threads but show weaker phenotypes than *nin*, as they are able to form infection chambers and microcolonies (Middleton et al., 2007; Laporte et al., 2014). Similar to the *nf-ya1-1* mutant, silencing of both *NF-YA1* and its close homolog *NF-YA2* leads to defective infection, with the strongest phenotypes showing entrapped bacteria, which form microcolonies but with no initiation of infection threads (Laloum et al., 2014; Laporte et al., 2014). *NF-YA1* encodes one of the three subunits (denoted as A, B, and C) of the CCAAT-box complex, a heterotrimeric transcription factor complex named after the CCAAT-box motif, which is bound by the NF-YA subunit. The CCAAT-box complex is found across eukaryotes, and in plants each subunit is encoded by multiple genes (Zanetti et al., 2017). NF-YA1-interacting subunits B/C involved in nodulation were discovered in *M. truncatula*, *L. japonicus*, and *Phaseolus vulgaris* (Zanetti et al., 2010; Soyano et al., 2013; Baudin et al., 2015). *ERN1* encodes an AP2/ERF family transcription factor, expressed in root hairs undergoing rhizobial infection and in developing nodules of both *M. truncatula* and *L. japonicus* (Cerri et al., 2012, 2017; Kawaharada et al., 2017; Yano et al., 2017). This expression is in part mirrored by *ERN1*'s close homolog, *ERN2*, which has relatively lower but partially overlapping expression in root tissues undergoing rhizobial infection (Cerri et al., 2012; Roux et al., 2014). Although unable to form mature nodules, *M. truncatula ern1* can initiate root hair infection and nodule organogenesis (Middleton et al., 2007), which are totally abolished in the *ern1 ern2* double mutant (Cerri et al., 2016). The *L. japonicus Ljern1* mutant develops very few infection threads, but nodule primordia can still form (Cerri et al., 2017; Kawaharada et al., 2017; Yano et al., 2017).

In addition to their important roles during rhizobial infection, *NIN*, *NFY-A1*, and *ERN1* are also required for proper nodule development in the root cortex (Marsh et al., 2007; Cerri et al., 2012, 2016; Laporte et al., 2014; Xiao et al., 2014; Vernié et al., 2015). Despite being the first nodulation gene cloned in model legumes (Schauser et al., 1999), just a few of NIN's targets have been identified, including *NODULATION PECTATE LYASE1* (*NPL1*), *NF-YA1*, *NF-YB1*, *CLE-Root Signal1* (*CLE-RS1*), and *CLE-RS2*, by work in *L. japonicus* (Xie et al., 2012; Soyano et al., 2013, 2014). It was shown that NF-YA1 and NF-YA2 may act upstream of ERN1 during the early stages of rhizobial NF signaling/infection (Laloum et al., 2014; Fonouni-Farde et al., 2016). Despite these findings, the genes and the cellular processes regulated by NIN, NF-YA1, and ERN1 are still largely unknown, especially on the genomic level, in part due to the difficulty posed by the use of root samples composed of many cell types and secondary effects on gene expression that occur during later stages of nodule development.

The established relationships between NIN, NF-YA1, and ERN1 provide an opportunity to better understand how complex transcriptional networks operate. To gain insights on NIN's role in infection and to establish the hierarchical relationships between these three regulators, we transcriptionally profiled *nin*, *nf-ya1*, and *ern1* mutants using root hairs of rhizobia-inoculated plants. By using this single cell-type transcriptional profiling method, which provides greatly improved sensitivity and specificity for detecting genes involved in rhizobial infection, we have generated a more complete model for the gene regulatory network governing this process. This analysis reveals a central role for NIN in the regulation of cell growth and development in infection and identifies new symbiosis-specific genes under the control of ERN1.

RESULTS

A Gene Regulatory Network for Rhizobial Infection

To investigate the regulation of gene expression during rhizobial infection of *M. truncatula*, we transcriptionally

profiled the *ern1-1*, *nf-ya1-1*, and *nin-1* mutants using root hairs isolated from seedlings 5 d post inoculation (dpi) with *Sinorhizobium meliloti* (Rm1021). This time point was chosen because in our system it coincides with the onset of infection thread development and was found to be the best stage to detect gene expression changes (Breakspear et al., 2014). Three biological replicates for each treatment were used, and each replicate consisted of RNA isolated from root hairs of 100 to 200 seedlings. As a reference for comparison with the mutants, we used previously published expression data for root hairs of wild-type plants 5 dpi with rhizobia, which were obtained at the same time, using the same methodology as this study (Breakspear et al., 2014). The gene expression data presented for the wild type were described by Breakspear et al. (2014), the data for *ern1*, *nin*, and *nf-ya1* have not been described elsewhere. The genome-wide expression data for root hairs of *nin-1*, *ern1-1*, and *nf-ya1-1* 5 dpi with *S. meliloti* are provided in Supplemental Data S1. All deregulated genes identified in this study are provided in Supplemental Data S2. In this updated analysis, a total of 1,353 genes (1,124 up-regulated and 229 down-regulated) had altered expression in root hairs of wild-type plants 5 dpi with Rm1021 relative to those inoculated with non-Nod factor-producing controls (SL44; Rm1021 Δ*nodD1ABC*) at the same time point.

To determine the underlying cause for defective infection in the mutants, the gene expression levels were compared between each mutant and the wild type at the same time point (5 dpi). Loss of *NIN* had the largest impact on gene expression, with 5,178 genes down-regulated and 4,281 genes up-regulated relative to the wild type, while relatively fewer genes had changed expression in *ern1* (1,612 down, 1,381 up) and *nf-ya1-1* (526 up and 648 down) mutants (Fig. 1A). In the *nin* mutant, the down-regulated genes included 54% of infection-induced genes. In contrast, in *ern1* and *nf-ya1-1*, only 13% and 5% of infection-induced genes failed to be expressed at wild-type levels, respectively (Fig. 1, B and C). For infection-induced genes with decreased expression in the mutants, ~80% (489 genes) of those in *nin* were specific to *nin*, while only ~25% (37 genes) of those in *ern1* were *ern1* specific (Fig. 1C). All infection genes down-regulated in *nf-ya1* overlapped with *nin* and/or *ern1* (Fig. 1C), and few of them were strongly changed. The overall transcriptional responses of *nin*, *nf-ya1-1*, and *ern1* were in line with their rhizobial infection phenotypes, with loss of *NIN* having the largest impact on gene expression.

To further investigate the relationships between these transcription factors, we carried out a gene network analysis using data from *ern1*, *nf-ya1*, *nin*, and the wild type. The analysis was carried out using NetProphet v2.0, which uses a weighted scoring of coexpression across samples and differential expression in mutants relative to the wild type (Haynes et al., 2013; Kang et al., 2018). This modeling approach assumes

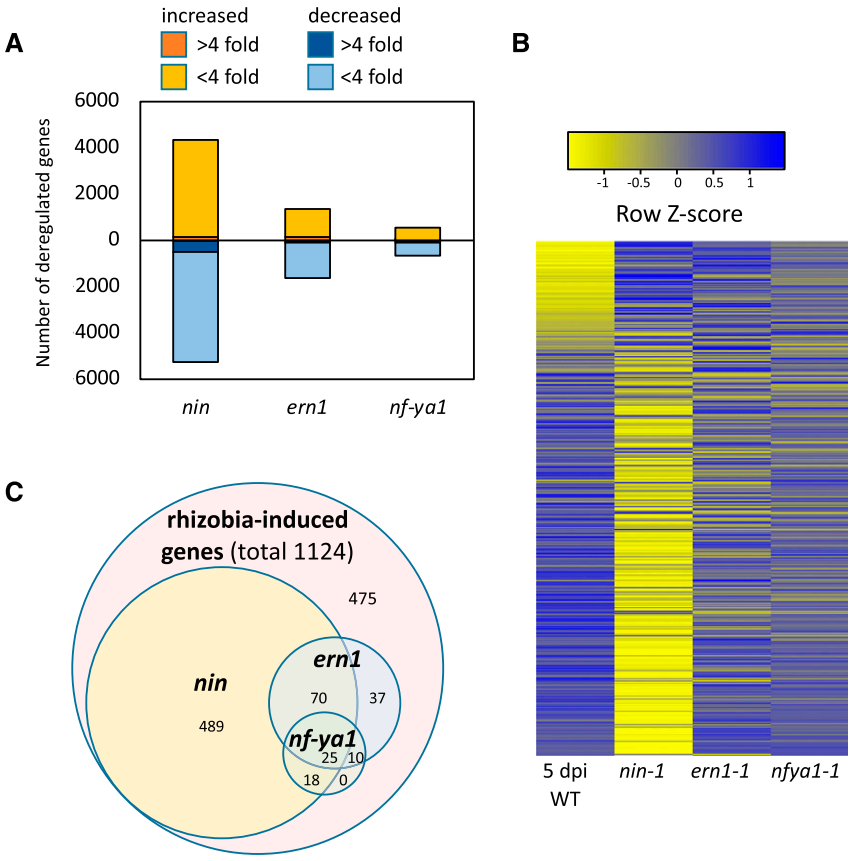

Figure 1. Impact of *nin*, *ern1*, and *nf-ya1* mutations on gene expression in root hairs of *S. meliloti*-inoculated seedlings. A, Genes significantly deregulated in *ern1-1*, *nf-ya1-1*, and *nin-1* relative to the wild type (A17) 5 dpi with *S. meliloti*. B, Heat map showing expression of genes induced in root hairs of the wild type (WT) 5 dpi with *S. meliloti* relative to the Δ*nodD1ABC*-inoculated control (Breakspear et al., 2014) in *nin*, *ern1*, and *nf-ya1*. C, Venn diagram showing the numbers of rhizobia-induced genes down-regulated in the *nin*, *nf-ya1*, and *ern1* mutants.

that (1) transcription factors will be coexpressed with their targets across different samples and (2) expression levels of direct targets of transcription factors will be strongly affected in their mutants. This network analysis, which considers the largest changes in gene expression, indicates that only a small set of genes show strongly ERN1-dependent expression in rhizobia-inoculated root hairs. The induction of these ERN1-dependent genes was independent of NF-YA1 and NIN. Consistent with this, the expression of *ERN1* itself was not decreased in *nin* and *nf-ya1-1*, and *NIN* expression was not decreased in *ern1*, suggesting that *ERN1* acts independently of the NIN and NF-YA1 regulons, which are themselves directly connected by virtue of NF-YA's dependence on NIN (Fig. 2). This analysis highlights a large number of genes as potential targets of NIN and relatively few as targets of ERN1.

We found that expression of *NF-YA1* and *NPL* was dependent on NIN, consistent with the discovery of their orthologs as direct targets of NIN in *L. japonicus* (Xie et al., 2012; Soyano et al., 2013). Their drastically reduced expression in *nin* (1% or less than in the wild type) agrees with a yeast study showing that the most strongly down-regulated genes in transcription factor

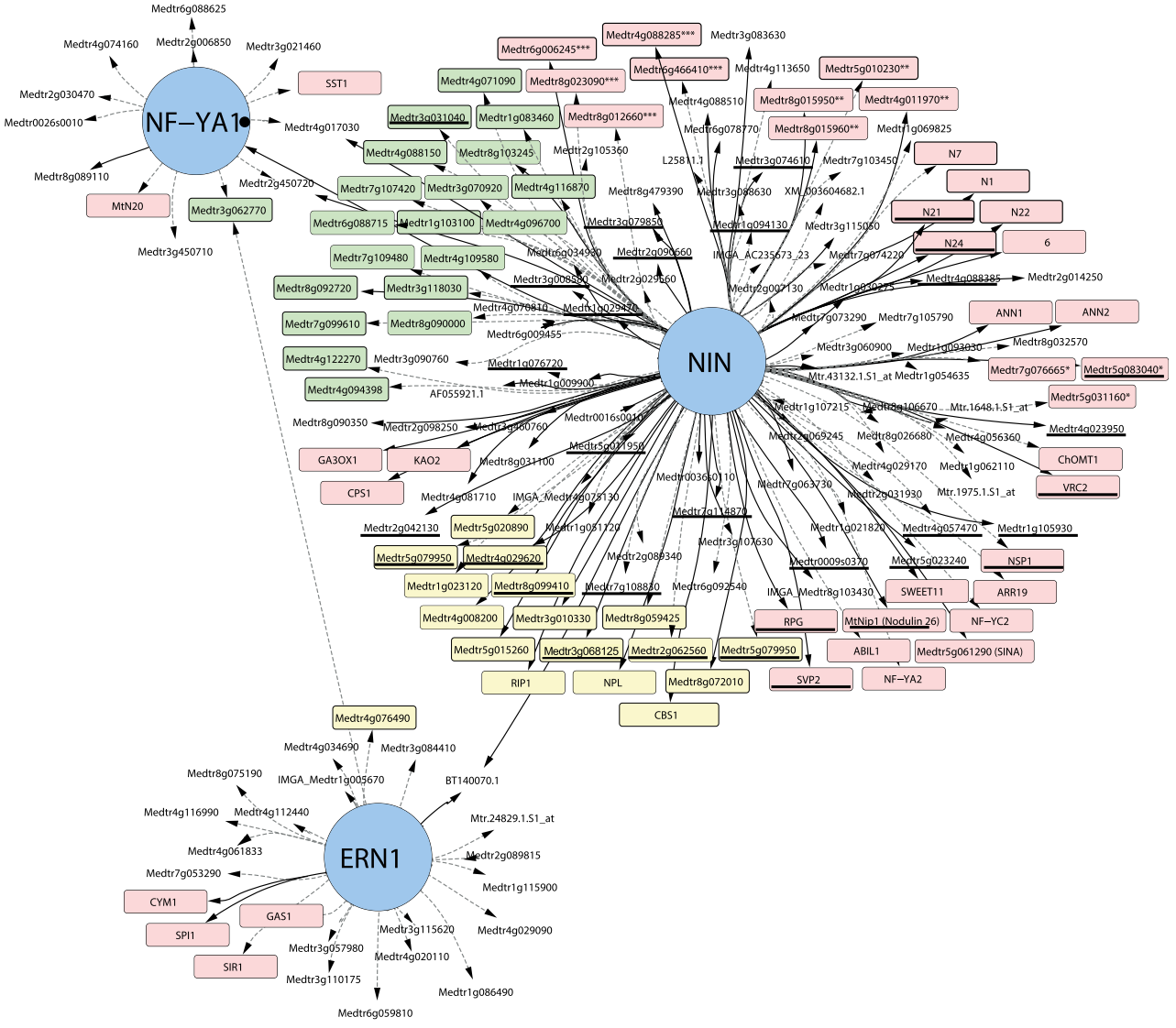

Figure 2. Gene regulatory network for rhizobial infection. Genes induced in root hairs 5 dpi with *S. meliloti* were ranked using NetProphet v2.0 based on differential expression in specific mutants and regression across root hair samples. Only the top 33% scoring genes for each mutant are included for clarity of presentation. Arrows denote directionality of the regulation. Edges for genes strongly repressed (0%–15% of the wild type) for a given mutant are connected by solid lines. Yellow denotes cell wall-related genes, and green denotes genes for DNA replication/translation. Other genes of interest are denoted in red. Genes with homologs enriched for NIN binding using ChIP-seq in *L. japonicus* are underlined (Soyano et al., 2015). *, Infection-specific genes (identified by Breakspear et al., 2014); **, nutrient uptake genes (Table 2); ***, nodule cysteine-rich peptides.

mutants are more likely to be directly controlled (Haynes et al., 2013). Based on this, many potential NIN targets were identified in this study; 145 genes had greatly reduced (less than 15% of the wild type) expression in *nin* (Fig. 1A). This includes *RPG* and *Cystathionine-β-Synthase-like1* (*CBS1*; 15% and 8% of wild-type expression levels, respectively), which are important for successful rhizobial infection in *M. truncatula* (Arrighi et al., 2008; Sinharoy et al., 2016). We confirmed the dependence of *RPG* on NIN using reverse transcription-quantitative PCR (RT-qPCR) in an independent experiment (Supplemental Fig. S1), while *CBS1* was shown previously to be downstream of NIN in early nodulation (Sinharoy et al., 2016). Other infection-induced genes specifically down-regulated at least 4-fold in the *nin* mutant were *LysM receptor-like kinase 10* (*LYK10*), *early nodulin 40* (*ENOD40*; Crespi et al., 1994), *Annexin 1* (de Carvalho-Niebel et al., 2002), an *ABI-1-LIKE1* (*ABIL1*) homolog encoding an actin nucleation protein (Medtr7g116710), and a *SEVEN IN ABSENTIA* gene (Medtr5g061290) that is Nod factor inducible (Jardinaud et al., 2016). A comparison of our findings with an earlier study by Høgslund et al. (2009), which examined gene expression changes in the root susceptible zone of the *L. japonicus nin* mutant after rhizobial inoculation, revealed limited overlap, possibly due to differences in sensitivity between the approaches, but found in common several of the most highly expressed and/or most strongly regulated genes, including *EPR3* (ortholog of *LYK10*), *NPL*, *NF-YA1*, and *CBS1*. While many novel candidate genes were identified as potential direct targets of NIN, it should be noted that their down-regulation in *nin* may be secondary (i.e. mediated by downstream transcription factors) or collateral (i.e. an indirect consequence of the failure to activate specific processes that normally occur during infection).

To investigate what proportion of the genes in the NIN regulon could be direct targets, we compared our transcriptomics data for the *nin* mutant with a chromatin immunoprecipitation sequencing (ChIP-seq) analysis for NIN in *L. japonicus* (Soyano et al., 2014). The authors of the study identified the Clavata3/ESR (CLE) peptide-encoding genes *CLE-RS1* and *CLE-RS2* as direct targets of NIN, but a comparison of the ChIP data with the *nin* mutant transcriptome was not carried out. To make the comparison, we identified the *M. truncatula* homologs/likely orthologs of the *L. japonicus* genes identified by ChIP using BLAST. We found a higher degree of overlap between the genes identified using ChIP and those identified as down-regulated in *nin* in our study than would be expected by chance, at all fold change cutoff levels considered (Supplemental Table S1). The increase in the degree of overlap compared with the randomly sampled controls increased with increasing cutoff stringency, suggesting that the most strongly down-regulated genes are more likely to be direct targets. This analysis suggests that NIN has at least 100 direct targets. A summary of nodulins that have strongly down-regulated expression in NIN, and that had at least 15-fold enrichment in the LjNIN ChIP-seq analysis (Soyano et al., 2014), is provided in Table 1. These include the known targets *CLE13* and *NF-YA1* as well as *RPG*, *symbiotic sulfate transporter 1* (*SST1*), and *symbiotic cysteine-rich receptor-like kinase* (*SymCRK*), which have demonstrated roles in nodulation (Krusell et al., 2005; Arrighi et al., 2008; Berrabah et al., 2014). A complete list of the genes identified by this comparison is provided in Supplemental Data S3.

NIN Is Required for the Expression of Cell Wall Remodeling, Cell Cycle, and Transporter Genes

A total of 63 rhizobia-induced cell wall-related genes were down-regulated in *nin* root hairs after rhizobia inoculation (Fig. 3, A and B), including *NPL*, two cellulases, a pectinacetylesterase, a Gal oxidase, a Gal mutarotase, a galactinol synthase, an α-glucan phosphorylase, an endo-β-mannase, an expansin, and an extensin. Notably, with the exception of *NPL*, none of these genes had symbiosis-specific expression b (Medicago Gene Expression Atlas [MtGEA]v3). Also included in this category were the secreted peroxidases including *rhizobium-induced peroxidase 1* (Wisniewski et al., 2000), which may contribute to cell wall maturation. Of 11 peroxidases previously found to be induced by *S. meliloti* (Breakspear et al., 2014), nine had reduced expression in *nin*, suggesting that *NIN*, either directly or indirectly, is the principal regulator of these genes during infection. All nine of these contained predicted secretion peptides. The NIN regulon also included 100 ribosomal genes and 50 genes predicted to be involved in cell cycle progression, mainly genes encoding components of the DNA replication machinery, such as all of the minichromosome maintenance (MCM) genes and many histones (Fig. 3, A and B). Genes encoding predicted membrane proteins and transporters also represent a relatively large portion of all genes under the control of NIN (Fig. 3, A and C). Many of them showed high percentages of reduced expression in *nin*, including several nodulins, such as the predicted membrane proteins *MtN5*, *MtN19*, and *MtN24*, and the transporters *MtN21*, *MtN26*, and *SWEET11*. The induction of *SWEET11* during nodulation was previously shown to be NIN dependent (Kryvoruchko et al., 2016). The categories of cell wall, DNA replication, MCM complex, histone H4 acetylation, sulfate transport, mitotic cell cycle process, translation, and many related terms were detected as significant using GO term enrichment analysis (Fig. 3; a full list of enriched terms is provided in Supplemental Data S4). The regulation of genes involved in cell cycle-related processes (see discussion in Breakspear et al., 2014), protein translation, and cell wall remodeling by NIN suggests a major role for this transcription factor in engaging the cell cycle and inducing the genes required to support cell growth.

Table 1. *Nodulins with reduced expression in root hairs of inoculated* M. truncatula nin *with close* L. japonicus *homologs identified using ChIP-seq for LjNIN (Soyano et al., 2014).*

ChIP data are from Soyano et al. (2014). CaOMT, Caffeic acid *O*-methytransferase; MIP, major intrinsic protein; na, no gene name yet assigned; PP2C, protein phosphatase2C; TF, transcription factor.

M. truncatula Gene Model	*L. japonicus* Gene Model	Gene Name	Description	Fold Change (*nin*/Wild Type)	Fold Enrichment (ChIP)
Medtr1g056530	chr5.CM0571.340.r2.m	*NF-YA1*	CCAAT-box TF	0.01	68.02
Medtr5g011950	chr2.CM0021.2380.r2.m	na	Lipid transfer protein	0.02	25.82
Medtr3g012420	chr3.CM0005.520.r2.m	*N21*	MtN21 transporter	0.03	11.49
Medtr6g007160	chr2.CM0081.540.r2.d	*N24*	Transmembrane protein	0.07	20.17
Medtr3g079850	chr6.CM0041.530.r2.a	*SymCRK*	Cys-rich RK-like protein	0.09	14.03
Medtr6g086170	chr2.CM0610.70.r2.m	*SST1*	Sulfate transporter	0.09	14.33
Medtr7g114870	LjSGA_056148.1	na	IQ calmodulin protein	0.10	17.89
Medtr8g098815	chr4.CM0004.760.r2.d	na	BEL1-related protein	0.12	18.97
Medtr3g074610	chr4.CM0182.350.r2.m	na	PP2C	0.13	2.87
Medtr2g055940	chr1.LjT11G20.60.r2.d	na	CaOMT	0.16	11.27
Medtr8g087710	chr4.CM0046.1620.r2.m	*Nod26*	MIP family transporter	0.17	30.91
Medtr1g090807	chr5.CM0909.840.r2.a	*RPG*	Myosin-like protein	0.17	8.51
Medtr1g018790	chr4.CM0536.30.r2.m	na	F-box SKIP2-like protein	0.17	13.00
Medtr5g026850	chr2.CM0803.150.r2.m	*IPD3L*	CYCLOPS protein	0.19	26.80
Medtr6g081810	LjSGA_016193.1	na	MATE efflux family protein	0.19	12.33
Medtr1g085680	chr1.CM0017.80.r2.m	na	Cytochrome P450	0.22	11.07
Medtr5g014640	chr2.CM0021.390.r2.m	na	Basic helix-loop-helix TF	0.24	3.67
Medtr4g079610	CM0446.165.r2.a	*CLE13*	Clavata3/ESR peptide	0.25	31.35
Medtr8g069775	chr4.CM0161.180.r2.d	*NRT2.7*	High-affinity NO_3 transporter	0.30	17.25

NIN-Directed *NPL* Expression Is Required for Infection of *M. truncatula*

In *L. japonicus*, *NPL* encodes a pectate lyase with nodulation-enhanced expression required for infection thread formation (Xie et al., 2012). Phylogenetic analysis revealed an *NPL* ortholog in *M. truncatula* (Supplemental Fig. S2), with strongly enhanced expression in response to rhizobia (Breakspear et al., 2014). We studied its expression using promoter-GUS analysis in composite plants in both the wild type and *nin* mutants. In wild-type plants inoculated with *S. meliloti*, *ProMtNPL:GUS* was highly expressed at infection sites, including infected root hairs and underlying cells (Fig. 4A; Supplemental Fig. S3A). *MtNPL* was also expressed throughout young nodules (Fig. 4B) and in the apex of mature nodules (Fig. 4C), which contrasts with *LjNPL*, which is not expressed in mature nodules (Verdier et al., 2013; LjGEA). This difference is consistent with *M. truncatula* nodules having a persistent meristem and infection zone, which are absent in mature *L. japonicus* nodules. *MtNPL* was also found to be expressed in root tips and lateral root primordia (Supplemental Fig. S3, B and C). The wild-type plants showed strong GUS staining in roots of inoculated plants (Fig. 4D) compared with *nin-1*, which exhibited only residual expression (Fig. 4E). *MtNPL* was highly expressed in infected root hairs of wild-type plants (Fig. 4F), but its expression was greatly reduced in curled root hairs of the *nin-1* mutant (Fig. 4G).

To determine if NPL is required in the *M. truncatula-S. meliloti* symbiosis, we identified a homozygous *Tnt1* insertion mutant (line NF18556) that we designated as *Mtnpl*. The *Mtnpl* mutant developed more microcolonies, and most (~70%) of them were enlarged (Fig. 4, H and I), indicating a defect in infection thread initiation leading to increased accumulation of rhizobia within the infection chamber. The mutant had significantly fewer elongating infection threads in root hairs and fewer infection threads ramified in the root cortex (Fig. 4J). The development of most infection threads forming in root hairs on the mutant was abnormal, resulting in large and misshapen infection threads that sometimes ruptured, releasing the rhizobia into the root hair cytoplasm (Fig. 4, J and K; Supplemental Fig. S3, D–F), indicating loss of integrity of the infection thread cell wall. Ruptured infection threads were not observed on the wild type in this experiment. Sometimes bacteria accumulated in intercellular spaces at sites where infections would normally bridge between cells, indicating a difficulty in initiating cell wall invagination in the *Mtnpl* mutant (Supplemental Fig. S3, G and H). The nodulation phenotype of *Mtnpl* was complemented by both a genomic fragment of *MtNPL* and a construct containing the *MtNPL* complementary DNA driven by the *MtNPL* native promoter (Supplemental Fig. S4). We conclude that *NPL* is required for infection and that it has highly specific temporal and spatial expression in rhizobia-infected cells, including in the nodule apex, in the indeterminate nodulator *M. truncatula*.

NPL Is Secreted to the Infection Chamber and the Infection Thread

To further investigate the role of NPL in infection by rhizobia, we investigated its localization in root hairs by

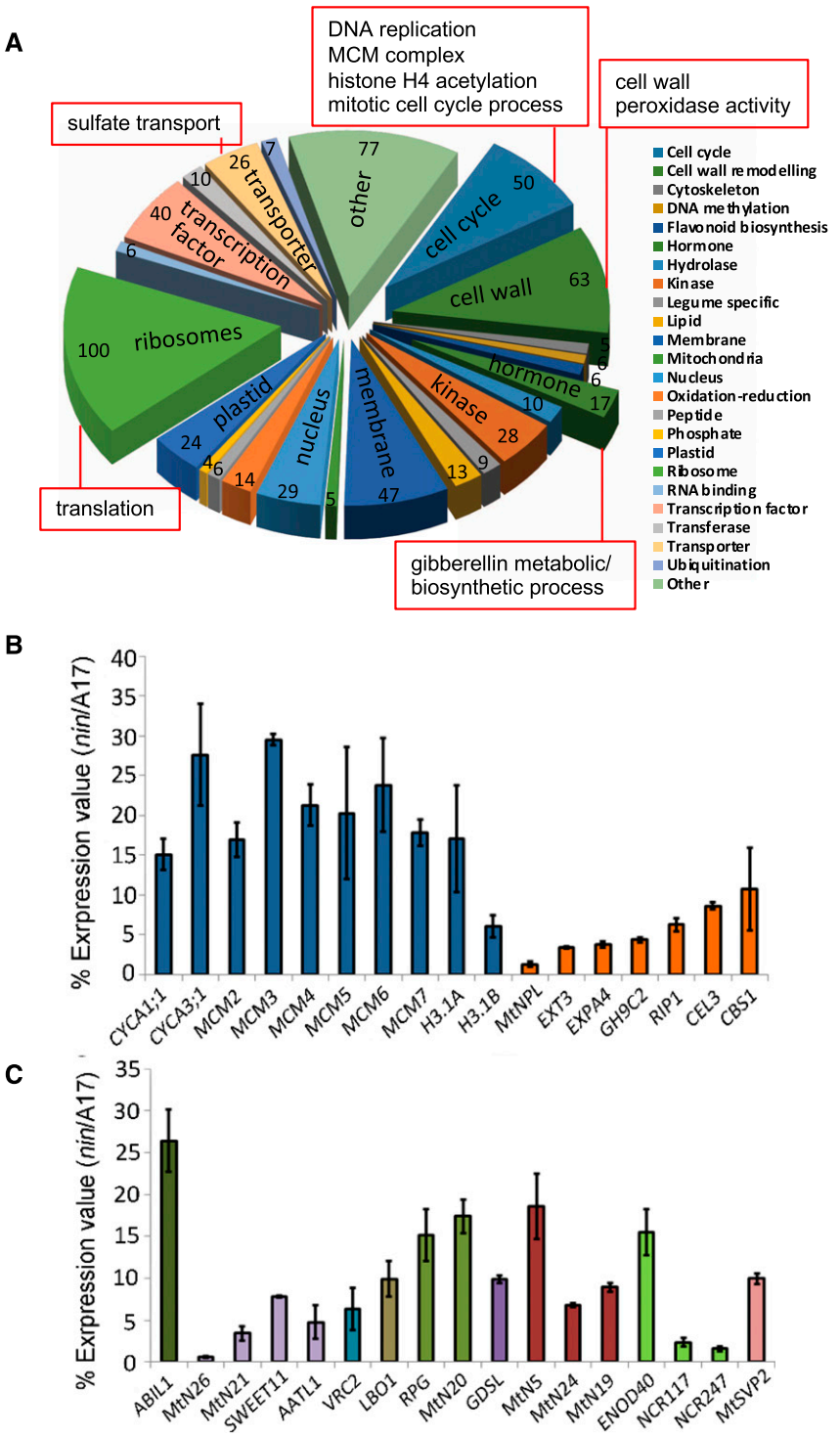

Figure 3. Cellular processes regulated by NIN in root hairs during rhizobial infection. A, Pie chart showing categories of cellular processes with different numbers of infection genes (up-regulated at 5 dpi with Rm1021) that are down-regulated in *nin-1*. Significantly enriched Gene Ontology (GO) terms are indicated by red boxes for the different gene categories. B and C, Percentage of expression values in *nin-1*/the wild type of some representative genes from A. Bars indicate 95% confidence interval. *CYCA, Cyclin A; MCM, MINI-CHROMOSOME MAINTENANCE; H3.1, HISTONE 3.1; NPL, NODULE PECTATE LYASE; EXT3, EXTENSIN 3; EXPA4, EXPANSIN A4; GH9C2, Glycosyl Hydrolase Family 9C; RIP1,* rhizobium-induced peroxidase 1; *CEL3, CELLULASE 3; CBS1, Cystathionine β Synthase-like 1; AATL1, Amino Acid Transporter-Like protein 1; VRC2, Vestitone Reductase Cluster 2; LBO1, LATERAL BRANCHING OXIDOREDUCTASE 1; RPG, Rhizobium-directed Polar Growth; GDSL, GDSL-like lipase; NCR, Nodule Cys-Rich peptide; MtSVP2, Short Vegetative Phase protein 2.*

means of a live-cell imaging system using an MtNPL-GFP (green fluorescent protein) fusion expressed under the control of the native *NPL* promoter (*ProMtNPL:-MtNPL-GFP*). This construct was able to complement the *mtnpl* mutant, demonstrating that the MtNPL-GFP fusion protein is functional (Supplemental Fig. S4, E–G). After inoculation with *S. meliloti*, strong MtNPL-GFP signal was found as small puncta, reminiscent of vesicles, in the cytoplasm of the root hairs before any deformation occurred (Fig. 4L). In the curled root hairs, the punctate pattern was seen in the cytoplasm, including along the cytoplasmic strands (Fig. 4M). Also, at this stage, a large accumulation of MtNPL-GFP was observed in the infection chamber of the root hairs (Fig. 4, M and N), with vesicle-like puncta appearing to converge at the site of the initiating

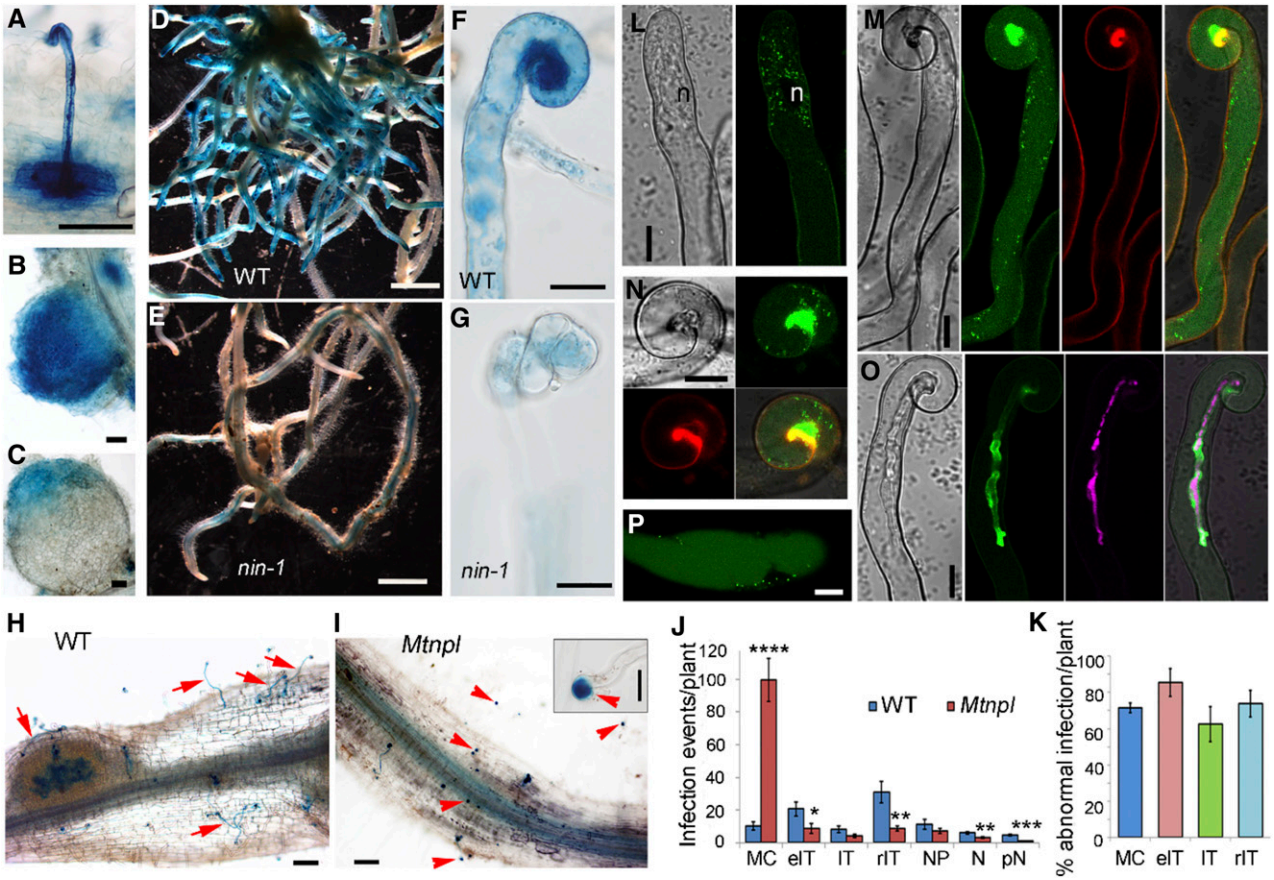

Figure 4. MtNPL's role, regulation, and localization during infection of *M. truncatula*. A to C, *ProMtNPL:GUS* expression in *Agrobacterium rhizogenes*-induced transgenic hairy roots showing expression in a root hair harboring an infection thread (A), a young nodule (B), and an elongated nodule (C). D to G, *ProMtNPL:GUS* expression in the wild type (WT; D and F) and *Mtnin-1* (E and G) 14 dpi with *S. meliloti* 1021. H to K, Rhizobial infection phenotype of *Mtnpl* 14 dpi with *S. meliloti* 1021. H, A root segment from the wild type showing full elongated infection threads (red arrows) and a nodule primordium. I, Infection phenotype of *Mtnpl* showing a typical root segment from *Mtnpl* with many swelled microcolonies (red arrowheads). The inset shows higher magnification of an abnormal microcolony (red arrowhead). J, Quantification of different rhizobial infection events in the wild type and *Mtnpl*. K, Percentage of abnormal infection events 14 dpi in *Mtnpl*. MC, Microcolony; eIT, elongating infection threads; IT, full elongated infection threads in root hair; rIT, ramifying infection threads; NP, nodule primordia; N, nodules; pN, pink nodules. Error bars indicate SE. Significant (Student's *t* test) differences between the wild type and mutants are marked with asterisks (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.0001$). L to P, Subcellular localization of MtNPL-GFP in *A. rhizogenes*-induced transgenic hairy roots with *pMtNPL:MtNPL-GFP* (L–O) or *pLjUBQ1:MtNPL-GFP* (P) 7 dpi with *S. meliloti 1021-CFP*. L, MtNPL-GFP in a root hair before any deformation occurs (left, bright field; right, GFP). M, MtNPL-GFP in a root hair-forming infection pocket (from left to right, bright field, GFP, cell wall autofluorescence, and merge). N, Another root hair showing MtNPL-GFP accumulation around and in the infection pocket (clockwise from top left: bright field, GFP, cell wall autofluorescence, and merge). O, A root hair harboring an infection thread containing rhizobia (magenta) showing MtNPL-GFP (green) in the infection thread lumen. Bars = 100 μm (A–C, H, and I), 200 mm (D and E), 20 μm (F, G, and inset in I), and 10 μm (L–P).

infection thread (Fig. 4N). In extended infection threads, MtNPL-GFP accumulated in the infection thread lumen, particularly at the tip regions (Fig. 4O). The vesicle-like punctate pattern of MtNPL-GFP was also found when driven by a strong constitutive promoter following inoculation with rhizobia (Fig. 4P). These results demonstrate the secretion of MtNPL to the infection chamber and to the infection thread, indicating its role in the cell wall remodeling required for initiation and elongation of infection threads.

Gibberellin Biosynthesis and Jasmonate Biosynthesis/Signaling Are Differentially Regulated in *nin*

We found that the increased expression of several gibberellic acid (GA) biosynthesis and signaling genes following inoculation with *S. meliloti* is dependent on NIN. Specific to the NIN regulon were five genes for gibberellin synthesis, *ENT-COPALYL DIPHOSPHATE SYNTHETASE1* (*CPS1*), *ENT-KAURENE OXIDASE1*, *ENT-KAURENOIC ACID HYDROXYLASE2*, *GIBBERELLIN 20-OXIDASE2*, and *GIBBERELLIN 3-OXIDASE1*,

all of which were strongly reduced in *nin* (Fig. 5, A and B). In particular, *CPS1*, which controls the first committed step in GA biosynthesis, was reduced to baseline (1% of the wild type) levels. *GIBBERELLIN 2-OXIDASE1* and the GA signaling components *GA INSENSITIVE DWARF1B* (*GID1B*) and *GID1-LIKEA/B* were down-regulated in *nin* and were also found to interact with NIN in *L. japonicus* (Fig. 5; Soyano et al., 2014). *DELLA1* also had reduced expression in *nin* (details on these GA-related genes are provided in Supplemental Data S5). This indicates that GA biosynthesis and signaling during infection are dependent on NIN, consistent with a role in growth-related processes.

Unexpectedly, among the 110 genes with significantly higher expression in root hairs of *nin* relative to the wild type after rhizobial inoculation were those with roles in jasmonic acid (JA) biosynthesis *Allene Oxide Synthase* and *Fatty Acid Desaturase 8*, metabolism (*Jasmonic Acid Responsive 3*, *IAA-Alanine Resistant 3*, and *Methy Esterase 1*), signaling (*Jasmonate ZIM-domain Protein 5*, *Protein Phosphatase 2A* [*PP2A*] *β-subunit*, and *Ethylene Responsive Factor Binding Element 4*), and other JA-related genes, including many that are methyl jasmonate inducible (Naoumkina et al., 2007; Fig. 5C; details are provided in Supplemental Data S6). Also up-regulated in *nin* was the gene encoding the ortholog of MYC2, a transcription factor that has been implicated in JA responses. The gene encoding the ortholog of the ubiquitin ligase, Plant U-BOX 10, which negatively regulates MYC2 (Jung et al., 2015), had increased expression in *nin*. In addition, the genes encoding the orthologs of the deubiquitinating enzymes Ubiquitin-Specific Protease 12 (UBP12) and UBP13, which counteract the activity of Plant U-BOX 10 to regulate the stability of MYC2, have decreased expression in *nin* (Jeong et al., 2017). Six of the above-mentioned JA-induced genes are also induced by pathogens (MtGEAv3; Benedito et al., 2008). None of these

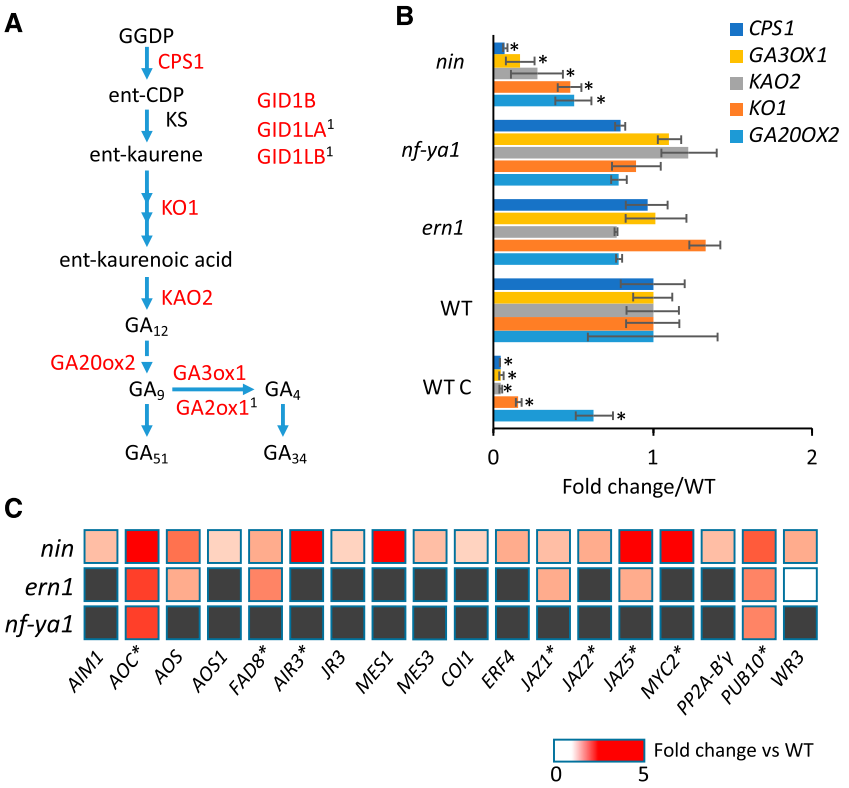

Figure 5. Genes for GA and JA biosynthesis and signaling have decreased and increased expression, respectively, in root hairs of *nin* after rhizobial inoculation. A, The GA biosynthetic pathway in plants. CPS1, ENT-COPALYL DIPHOSPHATE SYNTHETASE1; KS, KAURENE SYNTHASE; KO1, ENT-KAURENE OXIDASE1; KAO2, ENT-KAURENOIC ACID HYDROXYLASE2; GA20ox2, GIBBERELLIN 20-OXIDASE2; GA3ox1, GIBBERELLIN 3-OXIDASE1; GA2ox1, GIBBERELLIN 2-OXIDASE1; GID1B, GA INSENSITIVE DWARF1B; GID1LA/B, GID1-LIKEA/B. Genes encoding the enzymes in red are significantly down-regulated in *nin*. Superscript 1 indicates that the gene was identified using NIN ChIP-seq by Soyano et al. (2014). B, Expression of GA biosynthesis genes in root hairs of *ern1*, *nin*, and *nf-ya1* mutants relative to the wild type (WT; A17) at 5 dpi with *S. meliloti* Rm1021. Bars indicate 95% confidence interval. *, $P \leq 0.05$, Student's *t* test; mutants are compared with the wild type, and the wild type is compared with its control (WT C), which was inoculated with *S. meliloti* Rm1021 Δ*nodD1ABC*. C, Expression of JA-related genes in root hairs of *nin*, *nf-ya1*, and *ern1* mutants relative to the wild type (A17) at 5 dpi with *S. meliloti* Rm1021. Genes reported to be induced by methyl jasmonate are indicated by asterisks (Naoumkina et al., 2007). Only genes with mean expression significantly different from that of the wild type are indicated (gray boxes indicate that expression was not significantly different).

JA-related genes were identified using ChIP-seq for NIN (Soyano et al., 2014), suggesting that their deregulation is indirect, consistent with NIN's role as a transcriptional activator. This indicates that the JA pathway is ectopically activated during infection in root hairs of *nin* plants, which could possibly contribute to the observed infection phenotype.

NIN Is Required for Expression of Nutrient Uptake and Assimilation Genes during Rhizobial Infection

NIN is a legume-specific gene belonging to the NIN-like protein family of transcription factors, which have been shown both in legumes and nonlegumes to play a role in nitrogen and general nutrient homeostasis (Castaings et al., 2009; Konishi and Yanagisawa, 2013; Marchive et al., 2013; Yu et al., 2016). No such role has been attributed to NIN, which instead has been shown to control genes with critical roles in infection, such as *NF-YA1* and *NPL*. Recently, an NLP was shown to positively regulate the expression of *CLE-RS2*, which is involved in autoregulation of nodulation by nitrogen (Nishida et al., 2018), linking NIN, like other NLPs, with the regulation of nutrient homeostasis. To further investigate this possibility, we examined the expression of the *M. truncatula* orthologs of a set of Arabidopsis (*Arabidopsis thaliana*) nutrient homeostasis genes that are down-regulated in *nlp7* (Castaings et al., 2009) and found that many were down-regulated in *nin* (Table 2). We also identified many additional genes with well-established roles in nutrient homeostasis having reduced expression in *nin* (Table 2). Of these 51 genes, 12 were previously identified as enriched by ChIP-seq using NIN in *L. japonicus* (Soyano et al., 2014). In addition, the genes with the GO terms organonitrogen compound biosynthetic process and sulfate transport were overrepresented in the NIN regulon (Supplemental Table S1). We then compared the genes down-regulated in *nin* root hairs with the NLP7 targets identified in Arabidopsis with ChIP-chip (Marchive et al., 2013). We found that 180 of the 850 genes identified by ChIP-chip had close homologs in *M. truncatula* that were also down-regulated in *nin*, an overlap that was higher than expected by chance ($P < 1 \times 10^{-5}$), including genes such as Arabidopsis *LOB-Domain Containing Protein 38*, *CBL-interacting protein kinase 8*, *Nitrate Transporter 1.1* (*NRT1.1*), and *NRT2.5*, which have been implicated in nitrate uptake and signaling (Table 2). These findings suggest that NIN plays a similar role to other NLPs in the regulation of genes involved in nutrient uptake and assimilation.

NIN Is Required for the Expression of a Subset of the Rhizobia-Induced *NF-Ys*

Altogether, 40 transcription factors were found to be NIN dependent for their induction during root hair infection (Fig. 3A). In addition to *NF-YA1*, which was expressed at less than 3% of its wild-type level in *nin*, the expression of *NF-YA2* and *NF-YC2*, whose orthologs were previously implicated in nodulation (Zanetti et al., 2010; Soyano et al., 2013; Laloum et al., 2014; Baudin et al., 2015), and *NF-YB6* was also significantly dependent on NIN. The finding that NIN regulates genes encoding members of all three subunits of the CCAAT-box complex during rhizobial infection reveals that its control of this module is more extensive than previously recognized. However, the CCAAT-box subunit genes encoding NF-YC6 and NF-YB7 were still expressed at wild-type levels or higher in *nin*, indicating a NIN-independent expression of some subunits.

The *ern1-1* Mutant Has Deregulated Expression of Receptor-Like Kinases and Reactive Oxygen Species-Related Genes and Fails to Up-Regulate a Small Set of Symbiotic Genes

Over 3,000 genes had altered expression in *ern1-1* relative to the wild type in root hairs of rhizobia-inoculated seedlings. Remarkably, more than 110 receptor-like kinase (RLK) genes had increased expression in root hairs of *ern1*, and another 30 showed decreased expression. This included many potential TOLL-Interleukin 1-Nucleotide Binding Site-Leucine Rich Repeat resistance genes as well as RLKs involved in development, such as the ortholog of FERONIA and the symbiotic LYSM-receptor kinase *NFP*. Notably, in apparent contrast to findings in *L. japonicus*, which showed that the induction of *EPR3* expression during symbiosis was dependent on *ern1*, we found that the *M. truncatula* ortholog, *LYK10*, is slightly (~2 fold) increased in *ern1* (adjusted $P = 0.07$). This widespread deregulation of RLKs in *ern1* is accompanied by increased expression of nine protein phosphatase-encoding genes (PP2A and PP2B type). We also found that 44 genes involved in reactive oxygen species (ROS) generation and response were altered in *ern1*. This includes the ectopic up-regulation of 20 ROS-producing enzymes (respiratory burst oxidase homolog Hs, peroxidases, and glyoxal oxidases). In addition, we found evidence consistent with increased antioxidant production, in particular increased expression of both flavonoid and carotenoid biosynthesis genes, including those that are normally induced in root hairs by rhizobial inoculation (Supplemental Fig. S5; Breakspear et al., 2014).

In contrast, the gene network analysis, which considers both coexpression and strength of differential expression, indicates only a small number of genes as potential targets of ERN1 during rhizobial infection. Three of these genes are induced by rhizobia and Nod factors (Breakspear et al., 2014; Damiani et al., 2016) and are expressed in the infection zone of nodules in wild-type plants (Supplemental Table S2; Roux et al., 2014). They were strongly down-regulated (greater than 4-fold reduced) in *ern1-1* and had mostly symbiotic-specific expression (MtGEAv3). Based on their homology to

Table 2. *Relative expression of some nutrient uptake and assimilation genes in* S. meliloti*-inoculated root hairs in* nin *versus the wild type*

M. truncatula genes in boldface have *L. japonicus* homologs that are potential NIN targets (Soyano et al., 2014). Arabidopsis genes in boldface were found to be down-regulated in *nlp7* (Castaings et al., 2009). Dashes indicate no data.

N Uptake and Assimilation				S Uptake and Assimilation				P Uptake and Assimilation			
M. truncatula Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)	*M. truncatula* Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)	*M. truncatula* Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)
Medtr7g050870	0.18	**AT1G11580**	Pectin methylesterase	Medtr5g020800	0.38	**AT1G75280**	Isoflavone reductase	Medtr8g015960	0.03[a]	**AT1G17710**	PEP carboxylase (PEPC1)
Medtr5g012290	0.11	**AT1G12110**	Nitrate transporter (NRT1.1)	Medtr5g010230	0.16[a]	**AT4G23100**	Glu-Cys ligase (GSH1)	Medtr8g015950	0.03[a]	**AT1G73010**	PP phosphatase (PPsPase1)
Medtr4g101380	0.56	**AT1G12110**	Nitrate transporter (NRT1.1)	Medtr4g115670	1.55	**AT5G34850**	PAP26	Medtr7g067340	0.55	**AT5G01220**	Sulfoquinovosyltransferase
Medtr8g069775	0.30	**AT1G12940**	Nitrate transporter (NRT2.5)	**Medtr3g070400**	0.46	AT3G55880	S utilization efficiency (SUE4)	Medtr1g043200	0.29	AT5G43360	P transporter (PHT1;3)
Medtr2g030200	0.64	**AT1G30510**	Ferredoxin:NADP (H) oxidoreductase	Medtr4g087520	0.29	AT2G43750	Cys synthase	Medtr3g082700	0.07	AT2G32830	P transporter (PHT1;5)
Medtr1g078030	0.35	**AT1G64720**	Membrane-related protein (CP5)	Medtr6g016325	0.47	AT1G19920	ATP sulfurylase-like (APS2)	Medtr1g074930	0.45	AT3G54700	P transporter (PHT1;7)
contig_63842_1	0.50	**AT3G16560**	Protein phosphatase 2C	**Medtr1g081620**	0.43	AT4G14880	Cys synthase	**Medtr4g083960**	0.70	AT1G20860	P transporter (PHT1;9)
Medtr4g119830	0.71	**AT3G16560**	Protein phosphatase 2C	Medtr5g095470	0.20	AT3G13110	Ser acetyltransferase	Medtr7g102180	0.28	AT5G03430	PAPS reductase
Medtr5g026620	0.18	**AT3G22370**	Alternative oxidase1A (AOX1A)	**Medtr3g074620**	0.44	AT1G34780	APR-LIKE (APRL4)	Medtr8g010590	0.11	AT3G03530	Phospholipase C4
Medtr7g071970	0.55[1]	**AT5G01740**	Nuclear transport factor2 (NTF2)	**Medtr1g102550**	0.44	AT3G22890	ATP sulfurylase (APS1)	–	–	–	–
Medtr5g074500	0.45	**AT5G01740**	Nuclear transport factor2 (NTF2)	Medtr8g098350	0.59	AT4G23100	Glu-Cys ligase (GSH1)	–	–	–	–
Medtr4g077190	0.71	**AT5G04590**	Sulfite reductase (SIR)	Medtr2g008470	0.06	AT1G22150	S transporter (SULTR1;3)	–	–	–	–
Medtr5g026640	0.52	**AT5G45380**	Degradation of Urea3 (DUR3)	Medtr4g011970[b]	0.15	AT3G15990	S transporter (SULTR3;4)	–	–	–	–
Medtr2g021255	0.25	AT5G35630	Gln synthetase2 (GS2)	Medtr2g102243	0.23	AT3G15990	S transporter (SULTR3;4)	–	–	–	–
Medtr5g067150	0.41	AT4G24400	CBL-interacting protein kinase (CIPK8)	**Medtr6g086170**	0.09	AT5G19600	S transporter (SULTR3;5)	–	–	–	–
Medtr1g009200	0.47	AT1G52190	Nitrate transporter (NRT1.11)	Medtr7g022870	0.48	AT5G13550	S transporter (SULTR4;1)	–	–	–	–

(*Table continues on following page.*)

Table 2. (*Continued from previous page.*)

N Uptake and Assimilation				S Uptake and Assimilation				P Uptake and Assimilation			
M. truncatula Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)	*M. truncatula* Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)	*M. truncatula* Gene Model	*nin-1* Versus the Wild Type	Arabidopsis Homolog	Gene Description (Name)
Medtr2g101650	0.59	AT1G52190	Nitrate transporter (NRT1.11)	–	–	–	–	–	–	–	–
Medtr1g045550	0.09	AT4G13510	Ammonium transporter1 (AMT1;1)	–	–	–	–	–	–	–	–
Medtr7g085410	0.35	AT5G66320	GATA transcription factor5 (GATA5)	–	–	–	–	–	–	–	–
Medtr4g095600	0.49	AT3G49940	LOB domain protein (LBD38)	–	–	–	–	–	–	–	–
Medtr2g049790	0.62	AT1G30270	CBL-interacting protein kinase (CIPK23)	–	–	–	–	–	–	–	–
Medtr4g133230	0.44	AT5G47560	Dicarboxylate transporter (TDT)	–	–	–	–	–	–	–	–
Medtr4g125810	0.17	AT5G57050	Protein phosphatase2C (ABI2)	–	–	–	–	–	–	–	–
Medtr4g113505	2.15	AT5G47100	Calcineurin B-like protein (CBL9)	–	–	–	–	–	–	–	–
Medtr5g087490	0.34	AT5G20990	Cofactor for NiR and XD1 (CNX1)	–	–	–	–	–	–	–	–
Medtr2g061710	0.43	AT2G31955	Cofactor for NiR and XD1 (CNX2)	–	–	–	–	–	–	–	–

[a]Induced in wild-type versus control root hairs 5 dpi with *S. meliloti*. [b]Medtr4g011970 = SST1.

Arabidopsis proteins, we named these genes *Cysteine-rich TM module1* (*CYM1*), *Serine Protease Inhibitor1* (*SPI1*), and *Symbiosis Induced Resistance gene1* (*SIR1*). None of these ERN1-regulated genes has been studied.

We checked the expression of *SPI1*, *CYM1*, and *SIR1* using promoter-GUS fusions in transformed roots inoculated with *S. meliloti* and found them to be strongly expressed in infected root hairs, throughout young nodules, and at the apex of mature elongated nodules (Supplemental Fig. S6, A–C). Their ERN1-dependent up-regulation was further confirmed by RT-qPCR in another *ern1* mutant allele (*ern1-3*; NF1390) that we isolated from the Noble Institute *Tnt1* insertion mutant population (Cheng et al., 2011, 2017; R108 background). This *ern1-3* mutant showed similar phenotypes to *ern1-1*, with strongly blocked infections and retarded nodule development (Supplemental Fig. S7; Middleton et al., 2007). Results from RT-qPCR analysis showed that *SPI1*, *CYM1*, and *SIR1* were all significantly up-regulated in the wild type but not in *ern1-3* after rhizobial inoculation (Supplemental Fig. S6, D–F).

Since *ERN1* expression is up-regulated in root hairs responding to Nod factors (Andriankaja et al., 2007; Cerri et al., 2012), we tested whether complementation with *ERN1* could rescue the Nod factor-induced expression of *SPI1* and *CYM1* in *ern1* roots. ERN1 expressed from its own promoter in transgenic hairy roots restored Nod factor-induced expression of *SPI1* and *CYM1* in *ern1-1* (Supplemental Fig. S6, G and H). Together, our data suggest that *SPI1*, *CYM1*, and possibly *SIR1* induction by rhizobia depends on ERN1.

DISCUSSION

Taking advantage of a powerful approach using isolated root hairs, the cell type involved in the initial host-rhizobia interaction, we have transcriptionally profiled three *M. truncatula* infection mutants, *nin*, *ern1*, and *nf-ya1*, providing insight into the roles of these transcriptional regulators in accommodating root hair infection. Here, we reveal a central and specific role for NIN in the reprogramming of root hairs into infection cells, with a particular function in the regulation of genes for cell growth.

Research on NLPs in Arabidopsis has revealed a requirement for NLPs in supporting growth. NLP6/7 are required for root development (Castaings et al., 2009; Marchive et al., 2013), while loss of NLP8 inhibits nitrate-induced seed germination (Yan et al., 2016). Recent work suggests that NLP6 and NLP7 directly interact with TCP-Domain Family Protein 20 to directly control root growth (Guan et al., 2017). Our work shows that NIN similarly supports growth-related processes during infection. First, our work has shown that NIN has a similar function to other NLPs in promoting the expression of genes involved in nutrient uptake and assimilation. One of these genes, the sulfate transporter *SST1*, has been shown to be important for nodulation in *L. japonicus* (Tables 1 and 2; Krusell et al., 2005). Another, *CLE13*, confirms earlier reports in *M. truncatula* and *L. japonicus* for NIN (Mortier et al., 2010, 2012; Soyano et al., 2014, 2015; Nishida et al., 2016) and has recently been implicated in nitrate inhibition of nodulation (Nishida et al., 2018). Recently, it was shown that the Arabidopsis *nlp7* mutant has changed expression of several CLE peptide-encoding genes (Zhao et al., 2018), suggesting that this regulation may predate the divergence of legumes. The importance of the other N/P/S-related genes in NIN's regulon requires further study, but it seems plausible that this reflects the needs of a rapidly growing cell. The up-regulation of over 100 ribosomal genes, which is NIN dependent, suggests that infection requires a large investment in de novo protein biosynthesis. Similarly, S plays a central role in redox homeostasis via its incorporation into glutathione, and its increased uptake may support remodeling of the cell wall during growth. Finally, P uptake may be required for the apparent up-regulation of DNA synthesis, which is also heavily NIN dependent.

Our second line of evidence supporting NIN's function in growth is its regulation of GA signaling and biosynthetic genes. GA-related gene expression was reported in root hairs following Nod factor treatment and inoculation with *S. meliloti* (Breakspear et al., 2014; Jardinaud et al., 2016). Despite the universally accepted role for GA in cell expansion and growth, some studies indicate a negative role for GA in infection: mutations in *DELLA* genes, and plants treated with exogenous GA have reduced density of infection threads and nodule number, and the expression of a GA-insensitive *della1-Δ18* variant increases the number of infection events (Lievens et al., 2005; Maekawa et al., 2009; Fonouni-Farde et al., 2016; Jin et al., 2016). A similar negative role was also reported for cytokinin (Murray et al., 2007; Held et al., 2014). On the other hand, GA biosynthetic mutants have decreased nodulation, suggesting a positive role for GA in nodule growth (Ferguson et al., 2005; McAdam et al., 2018). Further investigation is required to fully elucidate the role of GA at the different stages of nodulation. The concomitant activation of JA biosynthetic genes could be due to intrinsic mechanisms regulating a tradeoff between growth and defense that have been well documented for GA-JA in Arabidopsis and other plant species (Danisman et al., 2012; Yang et al., 2012; Li et al., 2015; Smakowska et al., 2016), an area that needs further study in the context of nodulation.

Finally, in addition to the acquisition of base nutrients, successful rhizobia infection requires extensive remodeling and de novo generation of cell walls and membranes. We show that an enzyme encoded by one of these genes, NPL, is secreted to the infection chamber and is required for infection thread initiation in *M. truncatula*. This localization is similar to that reported for ENOD11 (Fournier et al., 2015). The localization of NPL to the infection chamber suggests that loosening of the extracellular pectin matrix, which is increasingly recognized for its importance in cell wall extensibility (Saffer, 2018), is required for infection thread initiation.

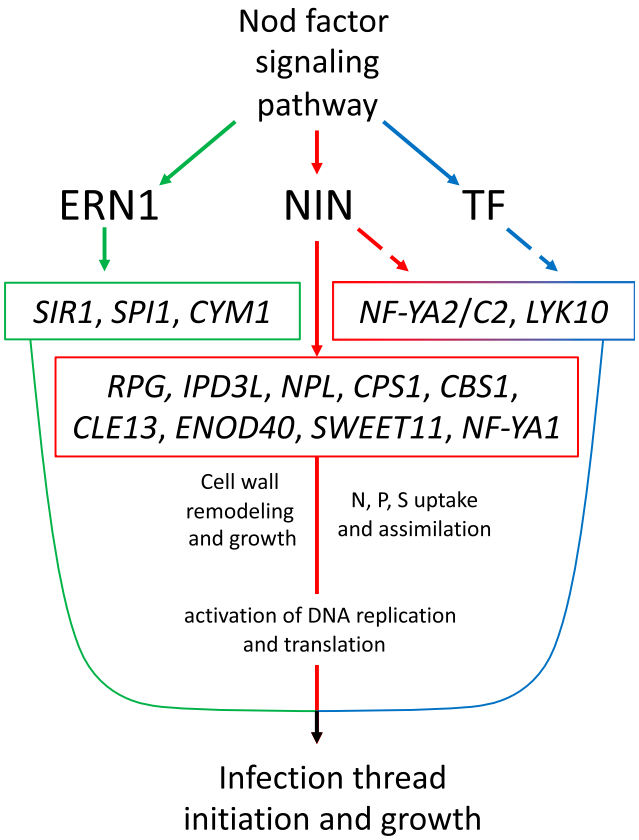

Figure 6. Model for the gene regulatory network underlying rhizobial infection. Nod factor signaling induces the expression of NIN (red), ERN1 (green), and other transcription factors (TF; blue). ERN1 is required for up-regulation of a few genes with symbiotic specific expression, while NIN controls a diversity of functions, including cell wall modification via *NPL*, GA biosynthesis by genes like *CPS1*, and nutrient uptake and DNA synthesis either directly or through subordinate transcription factors such as NF-YA1/A2 and NF-YC2, components of the CCAAT-box transcription factor complex. Other genes, like *RPG*, require NIN for their expression during infection, but their exact roles in the symbiosis are unknown. Many genes are partly dependent on NIN but require another unidentified transcription factor or factors for their full expression during infection (broken lines).

The expression of *NIN* and *NPL* is induced in all epidermal root hair cells when roots are treated with purified Nod factors, whereas after inoculation by rhizobia the expression of *NPL* is limited to infected tissues (Xie et al., 2012; Yoro et al., 2014; Vernié et al., 2015; this study). This highly localized expression could therefore reflect localized accumulation of Nod factors within the infection chamber and infection thread, resulting in the cell-specific activation of the NIN regulon. Notably, NPL localization was restricted to the infection chamber and infection thread cell wall and was not detected in the root hair cell wall, suggesting that the timing of NPL expression, as directed by NIN, determines where it is secreted, as has been shown for the transmembrane protein MtPT4 (Zhang et al., 2015). The conservation of NPL function and its regulation by NIN in *M. truncatula*, along with the existence of NPL orthologs in lupin (*Lupinus albus*) and *Arachis ipaensis* (Supplemental Fig. S2), suggest that this key regulatory module may be conserved across the legume family. It is unclear whether NPL's recruitment to NIN's regulon occurred after NIN's recruitment to nodulation or before, as part of the broader role of NLPs in growth-related processes. Also dependent on NIN were a subset of secreted peroxidases with mostly symbiotic-specific expression (Breakspear et al., 2014; Chen et al., 2015). Peroxidases are generally considered to be involved in cell wall loosening and hardening, thus may play a role in cell wall cross-linking for formation of the cell wall and matrix of the growing infection thread (Wisniewski et al., 2000; Santos et al., 2001; Passardi et al., 2004; Chen et al., 2015).

Development of infection threads also needs extensive expansion of the plasma membrane, echoing our finding that NIN is required for induction of many membrane protein-encoding genes that are ostensibly involved in rhizobial infection, including many early nodulins. *NIN* also regulates the gene expression of two putative annexins, including calcium-binding Annexin 1, which localizes to the nuclear periphery in *S. meliloti* activated cells (de Carvalho-Niebel et al., 2002). Annexins have multiple membrane-associated functions, including the formation of calcium ion channels (Gerke et al., 2005; Mortimer et al., 2008). In addition, the infection-induced gene *ABIL1* was dependent on *NIN*. ABIL1 has been proposed to mediate NAP1 and SCAR/WAVE binding (Basu et al., 2005) and so may act in concert with NAP1, PIR1, ARPC1, and SCARN to mediate actin nucleation, which is required for the development of infection threads (Yokota et al., 2009; Miyahara et al., 2010; Hossain et al., 2012; Qiu et al., 2015). Actin cytoskeletal dynamics play a central role in cell wall formation, particularly during polar growth (Pratap Sahi et al., 2018), further supporting NIN's role as a growth module.

In addition to uncovering NIN's role in growth processes, comparison of the *nin* transcriptome with that of *ern1* and *nf-ya1* allowed us to gain insights into their regulatory relationships. Generally, the impact on gene expression was much stronger in *ern1* than in *nf-ya1*, reflecting the strength of the infection phenotypes of these mutants: in *ern1*, no wild-type infection threads are observed, resulting in a fix− phenotype, while in *nf-ya1*, many normal-looking infection threads can be found. Based on the absence of transcriptional interdependence and nonoverlap of the most strongly affected genes in the *ern1* and *nin* mutants, we can conclude that the *nin* phenotype is not due to a failure to activate *ERN1* expression, and vice versa. This finding appears not to be due to the redundant action of ERN2, as *NIN* and *RPG* are induced normally in response to Nod factors in the *ern1 ern2* double mutant (Cerri et al., 2016). Further support for the independence of these regulons comes from *L. japonicus*, where *ERN1* induction by rhizobia was found to be dependent on CYCLOPS and NSP2 and independent of NIN (Yano et al., 2017). Also, in accordance with results in *L. japonicus*,

we found that the up-regulation of *NF-YA1* in response to rhizobia is dependent on *NIN*. However, we found no evidence for positive regulation of *ERN1* expression by NF-YA1 in root hairs, contrasting with results in *M. truncatula* showing that NF-YA1 can transcriptionally activate *ERN1* (Laloum et al., 2014). However, the functional redundancy of *NF-YA2* in the *nf-ya1* mutant (Laloum et al., 2014) and the rhizobial induction of NIN-independent CCAAT-box subunits in the *nin* mutant (this study) make it impossible to conclude whether ERN1 is regulated by the CCAAT-box complex. The fact that CCAAT-box signaling is likely at least partially active in *nin* may have allowed us to more clearly extract information on the remaining NIN regulon. A detailed analysis of higher order CCAAT-box mutants is needed to clarify the role of this complex in nodulation. Interestingly, it was recently shown that Arabidopsis *nlp7* has changed expression of several genes encoding NF-YA subunits (Zhao et al., 2018). This suggests that, as with the *CLEs*, these genes were part of NIN's regulon prior to its neofunctionalization into nodulation.

We also find evidence for the partial dependence of the induction of *IPD3L* and other transcription factors on NIN, suggesting further interconnectedness of the gene regulatory network directing rhizobial infection (Fig. 6).

The *ern1* mutant had widespread changes in the expression of ROS-related genes as well as genes encoding receptor-like kinases, a class of regulators that has many members with central roles in ROS regulation (Duan et al., 2010; Osakabe et al., 2010; Liu et al., 2013; Idänheimo et al., 2014). Interestingly, a study on the Arabidopsis ortholog of ERN1 implicated it in ROS homeostasis, suggesting potential functional conservation for this transcription factor across plants (Kim et al., 2012). The regulation of REDOX homeostasis is crucial for infection, and therefore these changes in ROS-related gene expression may contribute to the *ern1* infection phenotype (Chang et al., 2009). Further genetic and biochemical studies are needed to determine the role of ERN1 and its role in symbiotic infection.

The improved sensitivity and specificity of single cell-type gene expression profiling has revealed NIN's role in controlling genes involved in nutrient uptake, assimilation, and signaling, a role founded in the ancestral function of the NLP family. It is not yet clear whether other key infection genes like *NPL* and *RPG* were part of NIN's original regulon or were adopted subsequent to its symbiotic recruitment. Nonetheless, the further regulation of growth-related processes, including genes for GA biosynthesis, cell cycle activation, and cell wall remodeling, points to a broader role for NIN as a growth module that serves to support rhizobial infection.

MATERIALS AND METHODS

Isolation of Root Hairs from *Sinorhizobium meliloti*-Inoculated Roots

Medicago truncatula root hairs were isolated as described by Breakspear et al. (2014). Essentially, seedlings were grown vertically on plates between layers of Whatman Envelope Strip filter paper (VWR), where they were inoculated with *S. meliloti* 1021. At 5 dpi, the seedlings were removed and immediately immersed in a liquid nitrogen bath, where the root hairs were removed using a small paint brush. Approximately 100 seedlings were used in each biological replicate, and three biological replicates were used for each mutant. RNA was then isolated, labeled, and hybridized to the Affymetrix Medicago GeneChip as previously described (Breakspear et al. (2014).

Normalization and statistical analysis were carried out using Genespring 12.0 GX. Background correction, normalization, and probe summarization were performed using Robust Multichip Averaging. Unpaired Student's *t* tests were used to detect genes with significant changes in expression for each comparison. Probe sets were retained that had a value of greater than 50 for all three biological replicates in at least one of the two conditions being compared. Benjamini-Hochberg false discovery rate was used to derive asymptotically and multiple test-corrected *P* values. All data are available through the Medicago Gene Expression Atlas server (http://mtgea.noble.org/roothair/). GO term enrichment analysis was carried out using Phytomine.

RT-qPCR Analysis of Complemented *M. truncatula* Composite Roots and Seedlings

For comparison of gene expression in response to Nod factors, wild-type and *ern1-1* mutant *M. truncatula* A17 lines carrying a stably integrated p*ENOD11*:*GUS* gene fusion (Journet et al., 2001; Middleton et al., 2007) were transformed via *Agrobacterium rhizogenes* with respective p*ERN1*:*YFP-ERN1* or p*35S*:*YFP* binary vectors and transferred to Fahraeus-agar/paper support, as described by Boisson-Dernier et al. (2005). Root systems of composite plants were treated for 6 h with a solution of 10^{-9} M purified Nod factors (kindly provided by F. Maillet and J. Denarié) 3 to 5 d later. Harvested samples were kept at −80°C before RNA extraction using the Macherey-Nagel total RNA isolation kit. The DNA-free RNA samples were quantified and RNA integrity was checked by Agilent RNA Nano Chip (Agilent Technologies). First-strand complementary DNA synthesis was performed using 1 μg of total RNA with an anchored oligo(dT; 17T+V) and SuperScript II (Invitrogen) or Roche reverse transcriptases following their manufacturers' protocols. RT-qPCR was performed on 384-well plates with the Light Cycler 480 system and the SYBR Green I Master mix (Roche) according to Cerri et al. (2012). The following primer pairs were used to amplify the respective target genes: *CYM1*, Medtr3g081140 (forward, 5′-ACCTCCTCCTCCTGTTGGTTAT-3′; reverse, 5′-TCCCTTCCAGAA TCCATCAC-3′); *SPI1*, Medtr5g045470 (forward, 5′-CTCGAGTGCCAAAAA TTGGT-3′; reverse, 5′-GAATGCCATGACAAGCAACA-3′); and *Ubiquitin* (forward, 5′-TTGTGTGTTGAATCCTAAGCAGG-3′; reverse, 5′-CAAGAC CCATGCAACAAGTTCT-3′). The data shown represent transcript levels of respective genes normalized to those of endogenous *Ubiquitin* and are mean values obtained from two independent experiments with three technical repeats.

For experiments using seedlings, seeds of *M. truncatula* wild type (Jemalong A17 or R108) or mutants (*nin-1*, *ern1-1*, *nin-2*, and *ern1-3*) were sterilized, transferred onto water agar plates, and left in 4°C for 2 d. Then the seeds were transferred to 22°C for 16 h. The germinated seedlings then were transferred to plates containing 1.5% (w/v) agar and 100 nM aminoethoxyvinylglycine and inoculated as described before (Breakspear et al., 2014) with either distilled deionized water or *S. meliloti* (Rm1021; OD_{600} = 0.02). At 3 dpi, root tips of the treated seedlings were removed, and only the root zone containing root hairs was collected for extraction of RNA. This early time point prior to nodule emergence was chosen to avoid the secondary effects of gene expression caused by delayed nodulation in the mutants, which would result in reduced expression of most nodulation genes. Eight seedlings were used per replicate, and three biological replicates were used for RT-qPCR. *TIP41* was used as an internal control for normalization, and the relative expression was calculated using the delta-delta Ct method.

GeneChip Experiment and Analysis

M. truncatula mutants *nin-1* (Marsh et al., 2007), which has an 11-bp deletion that affects the RWP-RK DNA-binding domain, *ern1-1* (Middleton et al., 2007), where the *ERN1* gene is completely deleted, and *nf-ya1-1*, which has a nonsynonymous mutation leading to a premature stop codon at amino acid position 137 (Laporte et al., 2014), were used in this study. The methods of isolation of root hairs, RNA extraction from root hairs, and GeneChip analysis were as previously described (Breakspear et al., 2014). Briefly, analyses were performed using robust multichip averaging in Genespring 12.0 GX. Means comparisons

were carried out using unpaired Student's *t* tests. Multiple test corrections were made using the Benjamini-Hochberg procedure with a false discovery rate of 0.05. Normalized data were further analyzed in Microsoft Excel. For RT-qPCR confirmation of genes in the NIN regulon, the *nin-2* mutant and wild-type (R108) control were sterilized and germinated at 4°C on water-agar plates for 9 d, then transferred to vermiculite:perlite substrate (1:1) for 7 d, at which point the seedlings were inoculated with Sm2011. After 1 d, the seedlings were fertilized with 0.5 mM KNO_3 and then harvested for RNA isolation from the entire roots at 6 dpi. Three biological replicates were used for each genotype, and each replicate constituted three to four seedlings. *Elongation Factor*α (*EF*α) was used as a reference gene. The following primers were used: *EF*α, 5′-CTTTGCTTG GTGCTGTTTAGATGG-3′ and 5′-ATTCCAAAGGCGGCTGCATA-3′; *CPS1*, 5′-TTCTGCTTTGCTGGTCAATG-3′ and 5′-GTCCAATGCATAAGCCACCT-3′; *RPG*, 5′-GGCATGGAGAGTCCAAAGAG-3′ and 5′-CCTCCGTCAATT CCTTCAAA-3′; *NSP1*, 5′-ACGAAATGCCGAAGATGAAC-3′ and 5′-ACC GGTTATGGCTACAGCAC-3′; and *Nod26*, 5′-CTGGAGTTGCCACCGATA CT-3′ and 5′-GGTTCATCGATGCTCCTGTT-3′.

Promoter-GUS Analysis

The promoter GUS fusion constructs of *SPI1* (Medtr5g045470), *CYM1* (Medtr3g081140), and *SIR1* (Medtr4g016610) were made by using Gateway cloning. An upstream fragment from each gene was PCR amplified from *M. truncatula* A17 genomic DNA using Phusion High-Fidelity DNA Polymerase (NEB) by using primer pairs as follows: proSPI1 forward and proSPI1 reverse, proCYM1 forward and proCYM1 reverse, proSIR1 forward and proSIR1 reverse (see sequences below). The fragment was cloned into pDONR207 using Gateway BP Clonase II enzyme mix (Invitrogen). Then the fragment was introduced into a destination vector pKGWFS7 by an LR reaction to make the final promoter-GUS constructs. The *ProMtNPL:GUS* construct was made by Golden Gate cloning (Patron et al., 2015). A 2.5-kb *MtNPL* promoter, GUS gene (EC75111), and 35S terminator (EC41414) were synthesized by Life Technologies and used as level 0 modules, then cloned into level 1 vector EC47802, which was further cloned into level 2 vector EC50505 (https://www.ensa.ac.uk/). Composite plants were transferred to sand-Terra Green 4 weeks after *A. rhizogenes*-mediated transformation. The plants were then inoculated with Rm1021-LacZ. The roots were harvested 14 d later for GUS staining and stained for at least 4 h at 37°C in 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution with 100 mM potassium phosphate buffer (pH 7), 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 10 mM EDTA. Primer sequences used were as follows: proSPI1 forward, 5′-ggggacaagtttgtacaaaaaagc aggcttcgagaagtctagtggagttatttgg-3′; proSPI1 reverse, 5′-ggggaccactttgtacaagaa agctgggttccggctagccaaggtattct-3′; proCYM1 forward, 5′-ggggacaagtttgtacaa aaaagcaggcttcttctgctgattctgcaaacata-3′; proCYM1 reverse, 5′-ggggaccactttgta caagaaagctgggtttttggaaagcaaaggaaacaa-3′; proSIR1 forward, 5′-ggggacaagttt gtacaaaaaagcaggcttccagttcatagtttgttctcacattgttt-3′; and proSIR1 reverse, 5′-ggg gaccactttgtacaagaaagctgggttacgtccctacaaccttcatcatc-3′.

Mutant Screening, Genotyping, and Phenotyping

Plant roots transformed with promoter-GUS constructs were generated in *M. truncatula* A17 or *nin-1* background by hairy root transformation mediated by *A. rhizogenes* Arqua1 or AR1193 (Boisson-Dernier et al., 2001). The composite plants were transferred to a mixture of sand and Terra Green (1:1) 4 weeks after transformation and inoculated with *S. meliloti* (Rm1021) harboring pXLGD4 (*hemA*:*lacZ*). The roots were harvested 3 weeks after inoculation for GUS staining.

Subcellular Localization

The *ProMtNPL:MtNPL-GFP* and *ProLjUBI1:MtNPL-GFP* constructs were made by using Golden Gate cloning. A 2.5-kb *MtNPL* promoter and *MtNPL*-coding DNA were synthesized by Life Technologies and assembled as described (Patron et al., 2015). Plant roots transformed with the constructs were generated by hairy root transformation mediated by *A. rhizogenes* AR1193. The subcellular localization experiment was done by using the imaging method described by Fournier et al. (2008). Transgenic roots were inoculated with *S. meliloti* (Rm1021; OD_{600} = 0.001) tagged with CFP, and at 7 dpi root hairs at different infection stages were imaged with a Leica SP5 confocal laser scanning microscope. CFP and GFP were excited at wavelengths 457 and 488 nm, respectively. Cell wall autofluorescence was excited at wavelength 561 nm. The emission wavelengths used were 465 to 485 nm for CFP, 500 to 530 nm for GFP, and 620 to 720 nm for cell wall autofluorescence. The confocal images were processed using Leica AF Lite software, and the bright-field images of root hairs were adjusted using Adobe Photoshop Elements 8.0.

Gene Network Analysis

Gene network analysis was carried out using NetProphet v2.0 (Haynes et al., 2013; Kang et al., 2018). Before the gene network analysis, the data from this work were normalized with previously published data (Breakspear et al., 2014) using LIMMA. LIMMA uses preprocessing steps prior to statistical analysis that preserve information (Ritchie et al., 2015) and provides a superior analysis to what was originally reported by Breakspear et al. (2014), so we provide these results in Supplemental Table S2. For NetProphet analysis, we included probe sets that were significantly up-regulated greater than 2-fold relative to their respective controls (SL44; *S. meliloti* Δ*nodD1ABC*) at 1, 3, or 5 dpi by *S. meliloti* inoculation. For differential expression, data for each mutant inoculated with Rm1021 (*nin-1*, *ern1-1*, and *nf-ya1-1*) were compared with the Rm1021-inoculated control (5 dpi). For coexpression analysis, the following samples were used (*nin-1*, *ern1-1*, *nf-ya1-1*, wild type 1, 3, and 5 dpi with Rm1021, and wild type 1 d post treatment with Nod factors). Although *nin-1* and *nf-ya1-1* are null alleles (Marsh et al., 2007; Laporte et al., 2014), they still produce stable transcripts that are induced upon infection. It was therefore necessary to set the expression values for *NIN* and *NF-YA1* probe sets in the mutants to baseline expression levels for the analysis. NetProphet uses a weighted scoring of coexpression and differential expression values in a gene perturbation analysis to rank transcription factor potential targets. All data used in this analysis are available through http://mtgea.noble.org/roothair/ and the Gene Expression Omnibus.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers provided in Tables 1 and 2 and Supplemental Data S2. The gene models corresponding to the mutants analyzed in this article were *NIN* (Medtr5g099060), *NF-YA1* (Medtr1g056530), *ERN1* (Medtr7g085810), and *NPL* (Medtr3g086320).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Relative expression of selected genes identified using GeneChip analysis for the wild type (R108) versus *nin-2* using qPCR.

Supplemental Figure S2. Phylogenetic tree of pectate lyases.

Supplemental Figure S3. Promoter-GUS analysis of the *MtNPL* gene and rhizobial infection phenotype of *Mtnpl*.

Supplemental Figure S4. Complementation of *Mtnpl* mutant.

Supplemental Figure S5. Genes involved in production of antioxidant compounds are more highly expressed in *ern1-1* following rhizobial inoculation.

Supplemental Figure S6. The expression of *SPI1*, *CYM1*, and *SIR1* is symbiosis specific and is dependent on *ERN1*.

Supplemental Figure S7. Infection phenotype of the *ern1-3* mutant.

Supplemental Table S1. The number of genes present in the *M. truncatula* NIN regulon for which homologs were identified in *L. japonicus* NIN using ChIP-seq (Soyano et al., 2014).

Supplemental Table S2. Expression of *ERN1*, *ERN2*, *NF-YA1*, *NIN*, and genes downstream of *ERN1* across nodule zones.

Supplemental Data S1. Gene expression levels in root hairs of *nin*, *ern1*, and *nf-ya1* versus the wild type after inoculation with *S. meliloti*.

Supplemental Data S2. Comparison of relative gene expression levels in root hairs of *nin*, *ern1*, and *nf-ya1* versus the wild type after inoculation with *S. meliloti*.

Supplemental Data S3. Genes that are down-regulated in *M. truncatula nin* that have close homologs that were identified in an independent ChIP-seq analysis for LjNIN by Soyano et al. (2014).

Supplemental Data S4. GO term enrichment analysis of genes down-regulated in *nin* mutant root hairs 5 dpi with rhizobia.

Supplemental Data S5. Most GA biosynthesis and signaling genes are down-regulated in *nin*.

Supplemental Data S6. Expression of JA-related genes is increased in *nin*.

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