Changes in the Common Bean Transcriptome in Response to Secreted and Surface Signal Molecules of *Rhizobium etli*[1][OPEN]

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Establishment of nitrogen-fixing symbiosis requires the recognition of rhizobial molecules to initiate the development of nodules. Using transcriptional profiling of roots inoculated with mutant strains defective in the synthesis of Nod Factor (NF), exopolysaccharide (EPS), or lipopolysaccharide (LPS), we identified 2,606 genes from common bean (*Phaseolus vulgaris*) that are differentially regulated at early stages of its interaction with *Rhizobium etli*. Many transcription factors from different families are modulated by NF, EPS, and LPS in different combinations, suggesting that the plant response depends on the integration of multiple signals. Some receptors identified as differentially expressed constitute excellent candidates to participate in signal perception of molecules derived from the bacteria. Several components of the ethylene signal response, a hormone that plays a negative role during early stages of the process, were down-regulated by NF and LPS. In addition, genes encoding proteins involved in small RNA-mediated gene regulation were regulated by these signal molecules, such as Argonaute7, a specific component of the trans-acting short interfering RNA3 pathway, an RNA-dependent RNA polymerase, and an XH/XP domain-containing protein, which is part of the RNA-directed DNA methylation. Interestingly, a number of genes encoding components of the circadian central oscillator were down-regulated by NF and LPS, suggesting that a root circadian clock is adjusted at early stages of symbiosis. Our results reveal a complex interaction of the responses triggered by NF, LPS, and EPS that integrates information of the signals present in the surface or secreted by rhizobia.

Plants have the capacity to sense complex signals from the environment and adjust expression of their genetic programs accordingly. In particular, the ability of plant roots to recognize microorganisms in the rhizosphere is critical to distinguish beneficial from detrimental organisms. Nitrogen-fixing bacteria produce signaling molecules that are secreted or exposed on the cell surface, allowing recognition by receptors present in the plant as part of the so-called molecular dialogue. Upon recognition of these signals, two independent, but highly coordinated genetic programs are triggered in the root, one associated with the entrance of bacteria through the epidermis to reach cortical cells (infection) and the other resulting in the organogenesis of the nodule, where nitrogen fixation will take place (Oldroyd and Downie, 2008). During evolution, legumes have acquired a complex recognition system that allows them to identify one or a few species of bacteria with which they can establish a mutualistic symbiosis (Oldroyd et al., 2011); even in particular cases, they are able to distinguish between different strains from the same species, selecting the most efficient partners for the interaction (Aguilar et al., 2004; Den Herder and Parniske, 2009). Failure of the initial recognition steps will lead to misidentifying beneficial bacteria as pathogens, triggering defense responses by the plant, or allowing infection by detrimental organisms, with serious consequences for the plant.

Under low nitrogen conditions, legume roots exudate flavonoids and isoflavonoids, which are sensed by rhizobia and trigger the biosynthesis of Nod Factors (NFs). NFs are lipo-chito-oligosaccharides secreted by rhizobial species as a mix of molecules with different substitutions in a common chitin backbone (D'Haeze and Holsters, 2002). Specific NF receptors present in legume roots trigger molecular responses, followed by physiological and morphological changes (Gage, 2004). NF perception is required for initial steps of infection, such as root hair curling and formation of a tubular structure called the infection thread, but also to sustain the progress of this infection structure toward the

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actively dividing cortical cells, where bacteria will be released to form nitrogen-fixing symbiosomes. In addition to the well-known role of NF in symbiosis, other molecules associated with the cell wall, like the lipopolysaccharide (LPS), or secreted by bacteria, such as exopolysaccharides (EPSs), cyclic glucans, and K antigens, have been proposed to be part of the molecular dialogue with the plant (Gibson et al., 2008; Downie, 2010). Evidence of the role of EPS and LPS as suppressors of defense responses during symbiosis is originated in the phenotypic analysis of plant roots after exogenous application of the purified molecules or inoculation with strains defective in the synthesis of these polysaccharides. For example, simultaneous addition of purified LPS of *Sinorhizobium meliloti* suppressed the alkalinization and oxidative burst reaction induced by yeast (*Saccharomyces cerevisiae*) elicitors in alfalfa (*Medicago sativa*) cell cultures (Albus et al., 2001). Similarly, the lipid A of the *S. meliloti* LPS is sufficient to suppress oxidative burst in *Medicago truncatula* roots (Scheidle et al., 2005). On the other hand, two *Bradyrhizobium japonicum* mutants defective in EPS production formed effective nodules but exhibited delayed nodulation on the host plants *Glycine max* and *Glycine soja*, which responded to these strains with phytoalexin accumulation in the rhizodermis and the induction of a chitinase in the infected zone of the nodules (Parniske et al., 1994). An *S. meliloti* strain that fails to synthesize the acidic EPS I was unable to induce infection of root nodules on alfalfa. This strain elicited the formation of uninfected pseudonodules with high callose deposition and phenolic compounds on their cell walls (Niehaus et al., 1993). More recently, a transcriptome analysis of *M. truncatula* roots revealed that a *S. meliloti* mutant deficient in the production of EPS I induced hundreds of defense- or pathogenesis-related genes (Jones et al., 2008). Although these studies provide support to the hypothesis that both LPS and EPS molecules are involved in the suppression of plant defense responses, the precise role played during the interactions has not been determined in detail (Downie, 2010). Recently, a receptor-like kinase that binds EPS directly and controls rhizobial infection was identified in *Lotus japonicus*, revealing a second mechanism of bacterial recognition after NF perception (Kawaharada et al., 2015).

In this work, we aimed to transcriptionally profile plant genes modulated by signaling molecules (i.e. NF, EPS, and LPS) in the common bean (*Phaseolus vulgaris*)-*Rhizobium etli* interaction. Common bean is a grain legume of worldwide economic importance that accounts for more than one-half of the grains consumed by humans (Broughton et al., 2003). Additionally, this species offers an interesting model for plant recognition associated with partner selection, because Mesoamerican variants are able to establish a preferential interaction with sympatric bacterial strains, which are more efficient in terms of nodule formation compared with strains that are predominant in Andean soils (Aguilar et al., 2004; Zanetti et al., 2010; Mazziotta et al., 2013). We took advantage of the availability of *R. etli* mutant strains deficient in the synthesis of NF, EPS, or LPS (Noel et al., 1984; Vázquez et al., 1991) to characterize the transcriptome of the susceptible zone of common bean roots in response to wild-type or mutant strains of rhizobia. Direct RNA sequencing (RNAseq) revealed a set of genes differentially regulated specifically by one signal molecule, as well as genes that showed a transcriptional response that is common to either two or the three signals, suggesting that the plant can distinguish and integrate information from different determinants of rhizobia identity. Our results highlight unique aspects of plant responses to trigger genetic programs associated with symbiosis, such as circadian rhythms and gene regulation mediated by transcription factors and small RNAs.

RESULTS AND DISCUSSION

Transcriptome Changes in Response to Rhizobium Signaling Molecules

To analyze how the common bean root transcriptome is modulated by the NF, LPS, and EPS from rhizobia, we took advantage of available strains of *R. etli* that are deficient in the production of these signal molecules (Fig. 1A; Table I). UBP102 is a CE3 derivative strain mutated in the *nodA* gene (hereafter, NodA$^-$; Vázquez et al., 1991). The product of this gene introduces an essential fatty acid modification at the nonreducing end of NF, thus mutations in this gene abolish nodulation. On the other hand, strain CE109, an *R. etli* mutant unable to produce the saccharide I of the LPS, promotes nodule organogenesis, but the mutation affects infection and release of bacteria (Noel et al., 1986). Strain CE338 is affected in the synthesis of the acidic EPS, forming nonmucoid colonies; however, nodulation of common bean roots was not affected (Diebold and Noel, 1989). All of these strains are derived from CE3, which was used as the reference for normal infection, whereas plants mock inoculated with culture media (yeast-extracted mannitol [YEM]) were used as controls. Mature nodules formed by CE3 and CE338 developed lenticels and were pink, indicating the presence of leghemoglobin. On the contrary, roots inoculated with NodA$^-$, CE109, or the double mutant CE343 (mutated in the LPS and EPS production) did not form nodules, except for two empty nodules formed in one of 51 roots observed upon inoculation with CE343 (Fig. 1B). The observed nodulation phenotypes are coincident with those previously described (Table I), except for CE109, which formed empty nodules in the Negro Xamapa accession but did not form nodules or bumps in NAG12 under our experimental conditions (Noel et al., 1984; Diebold and Noel, 1989; Vázquez et al., 1991; Eisenschenk et al., 1994).

Samples for RNAseq were obtained from roots at 24 h postinoculation (hpi) with YEM or each of the rhizobial strains, a time when root hairs are curled and infection threads are not yet visible. At this time, molecular responses associated with infection and nodule organogenesis

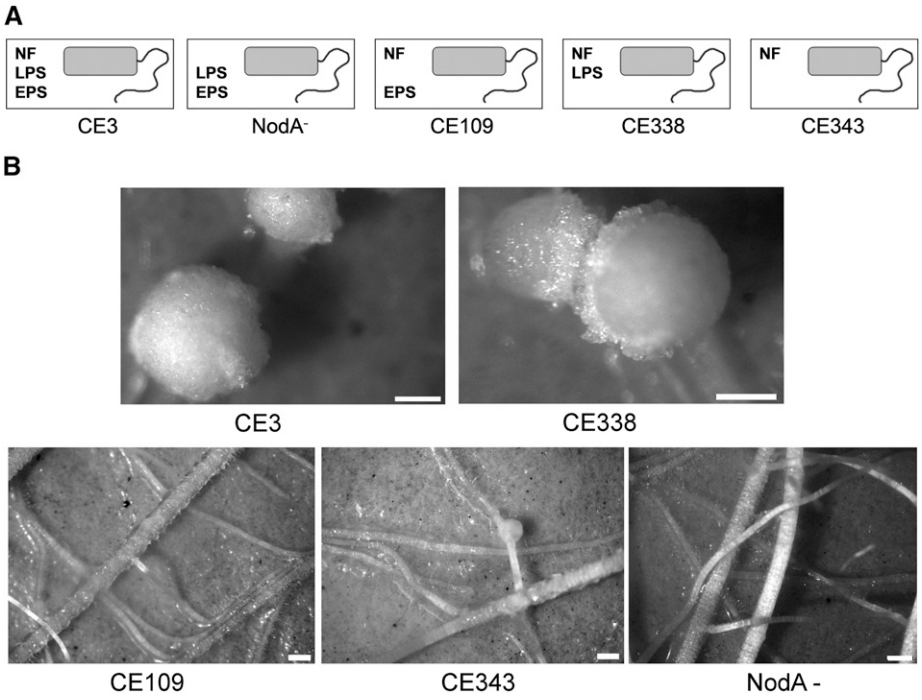

Figure 1. Nodulation phenotype in response to rhizobial strains. A, Schematic representation of the strains used in this study. B, Images of nodules developed in common bean roots 21 d postinfection with the indicated *R. etli* strains. After inoculation with the CE343 mutant, only two empty bumps were observed in a total of 51 roots from 15 plants. Bars = 1 mm.

are already induced (Meschini et al., 2008). Transcriptional profiling by direct RNAseq was obtained using Illumina technology. This approach enables quantification of all expressed genes, as well as their alternative transcripts. A minimum of 91 million single-end reads of 50 bp were obtained for each sample from two independent biological replicates, with an average of 87% of reads mapped to the common bean genome (Supplemental Table S1). Expression analysis showed an even distribution among samples (Supplemental Fig. S1; Supplemental Table S2). We considered pairwise comparison of all possible combination using Cuffdiff and selected genes that meet the following criteria: a fold change of at least 2 between samples, $P < 0.05$, and a fragment per kilobase per million (FPKM) reads value greater than 1 in at least one of the samples (Fig. 2A; Supplemental Table S3). This analysis identified a total of 2,606 differentially expressed genes whose expression significantly changed between at least two of the samples analyzed, i.e. roots inoculated with *R. etli* strains or mock treated with YEM (Supplemental Table S4).

Genes Regulated by NF, LPS, and EPS

Genes positively modulated in response to NF, LPS, or EPS were defined as those that are reduced in the corresponding mutant compared with CE3, whereas genes with higher levels in plants inoculated with mutants strains than CE3 were categorized as negatively regulated by these molecules. A total of 233 and 339 genes responded positively and negatively to NF, respectively. LPS and EPS increased expression of 47 and 110 genes, whereas 52 and 109 genes were negatively regulated in response to these signals, respectively. Several genes were modulated by more than one signal, including 32 that differentially responded to the three molecules (Fig. 2B; Supplemental Table S5). Surprisingly, many genes that were differentially regulated in response to single mutants were not differential in response to the LPS/EPS double mutant (Fig. 2C; Supplemental Table S6), whereas more than one-half of the genes differentially regulated by the double mutant were not significantly different in single mutants. Many of these genes showed higher expression in plants inoculated with the double mutant than in single mutants, particularly in certain categories, such as redox and stress responses, photosynthesis, and metabolism, whereas genes involved in transport or translation showed a different pattern of expression. This suggests that the absence of both molecules, LPS and EPS, produces synergistic effects on the plant transcriptional response.

Differentially expressed genes were functionally classified based on Gene Ontology (GO) annotation. The most represented categories correspond to metabolism,

Table I. *R. etli strains and their phenotypes*

Strain	Characteristics[a]	Symbiotic Phenotype[b]
CE3	Wild type	Ndv^+ Fix^+
UBP102 ($NodA^-$)	$NodA^-$ (NF)	Ndv^- Fix^-
CE109	Ops^- (LPS)	Ndv^- Fix^-
CE338	Exo^- (EPS)	Ndv^+ Fix^+
CE343	Ops^- Exo^- (LPS and EPS)	Ndv^- Fix^-

[a]$NodA^-$, Ops^-, and Exo^- are deficient in the production of NF, LPS, and EPS, respectively. [b]Ndv refers to normal nodule formation (Ndv^+) or absence of nodules (Ndv^-), whereas Fix corresponds to pink nitrogen-fixing (Fix^+) or nonfixing white nodules (Fix^-).

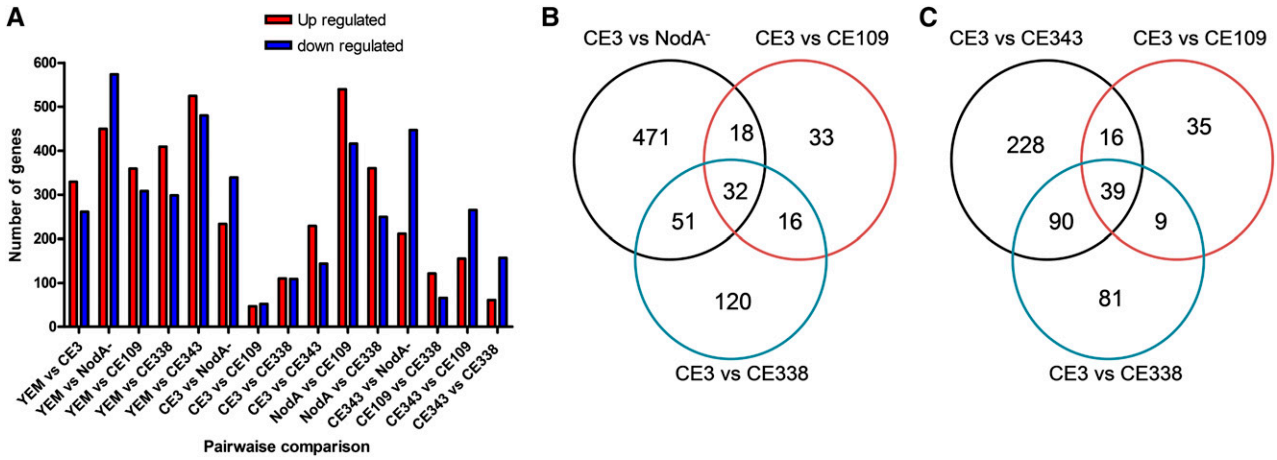

Figure 2. Genes up- and down-regulated in response to rhizobial strains. A, Number of up- and down-regulated genes in each pairwise comparison. B, Venn diagram representing the number of genes differentially regulated by NF (CE3 versus NodA⁻), LPS (CE3 versus CE109), and EPS (CE3 versus CE338). C, Venn diagram representing the number of genes differentially regulated in response to the wild type and the LPS and EPS single mutants (CE3 versus CE109 and CE3 versus CE338) or the LPS/EPS double mutant (CE3 versus CE3434). Expression of common bean genes was estimated by RNAseq, and those significantly regulated in response to signal molecules were identified using Cuffdiff.

transcriptional regulation, and perception and signaling (Fig. 3). Genes with unknown functions were particularly abundant in genes either up- or down-regulated by NF, LPS, or EPS, whereas genes in the transcriptional regulation category were overrepresented in genes up-regulated by these signal molecules. Among genes up-regulated by each of these molecules, defense response and cell wall remodeling categories were overrepresented, suggesting that, at this early time point, defense responses were still active in the root. Stress response genes were also overrepresented among genes that are expressed at higher levels in response to CE3 than in response to the CE109 mutant. The percentage of genes belonging to the metabolism category was also high among genes either up- or down-regulated by NF.

Validation of Expression Data

Expression of genes previously described in the literature as up-regulated in response to rhizobia was analyzed by reverse transcription (RT)-quantitative PCR (qPCR) and compared with data obtained by RNAseq. *Early nodulin40* (*ENOD40*), *nodule inception* (*NIN*), and *ethylene responsive factor required for nodulation1* (*ERN1*) showed similar results in both analyses, validating the RNAseq approach and confirming molecular responses of plants at 24 hpi, when morphological changes are hardly visible (Fig. 4). The three genes were significantly induced by the wild-type strain CE3, but this induction was completely abolished or reduced in roots inoculated with the NodA⁻ strain. Inoculation with CE109, CE338, and CE343 also induced expression of these early nodulation markers. These results are consistent with that observed in our previous RT-qPCR experiments in common bean, as well as with those described in other legume species (Campalans et al., 2004; Marsh et al., 2007; Middleton et al., 2007; Zanetti et al., 2010; Battaglia et al., 2014).

We also selected a set of differentially expressed genes from several functional categories to validate expression data obtained by RNAseq, including genes involved in transcriptional regulation, signal transduction, cell wall remodeling, transport, and defense responses. RT-qPCR analysis of these genes confirmed expression data obtained by RNAseq for nine out of 11 genes (Supplemental Fig. S2). The two exceptions were a receptor-like kinase (Phvul.007G049400) and the pleiotropic drug resistance12 (Phvul.001G054500). For both genes, induction by CE3 strain was detected by either RNAseq or RT-qPCR, but the effect of inoculation with the NodA⁻ strain was different depending on the technique used to measure transcript abundance.

Transcription Factors Differentially Expressed

To understand how LPS and EPS act as signal molecules in combination with the NF, we focused our analysis on genes belonging to categories related to early responses to rhizobial infection, such as transcriptional regulation, signal perception and transduction, stress and defense responses, and cell wall modifications. A total of 248 transcription factors were identified as differentially expressed in all the pairwise comparisons (Fig. 5; Supplemental Table S7), which were classified according to the Plant Transcription Factor database v3.0 (Jin et al., 2014). Most of the genes were up-regulated by infection and strongly affected by the presence of NF (Supplemental Fig. S3). The list includes the known symbiotic transcriptional regulators NIN and ERN1 (Fig. 4). NIN is a family characterized by the presence of

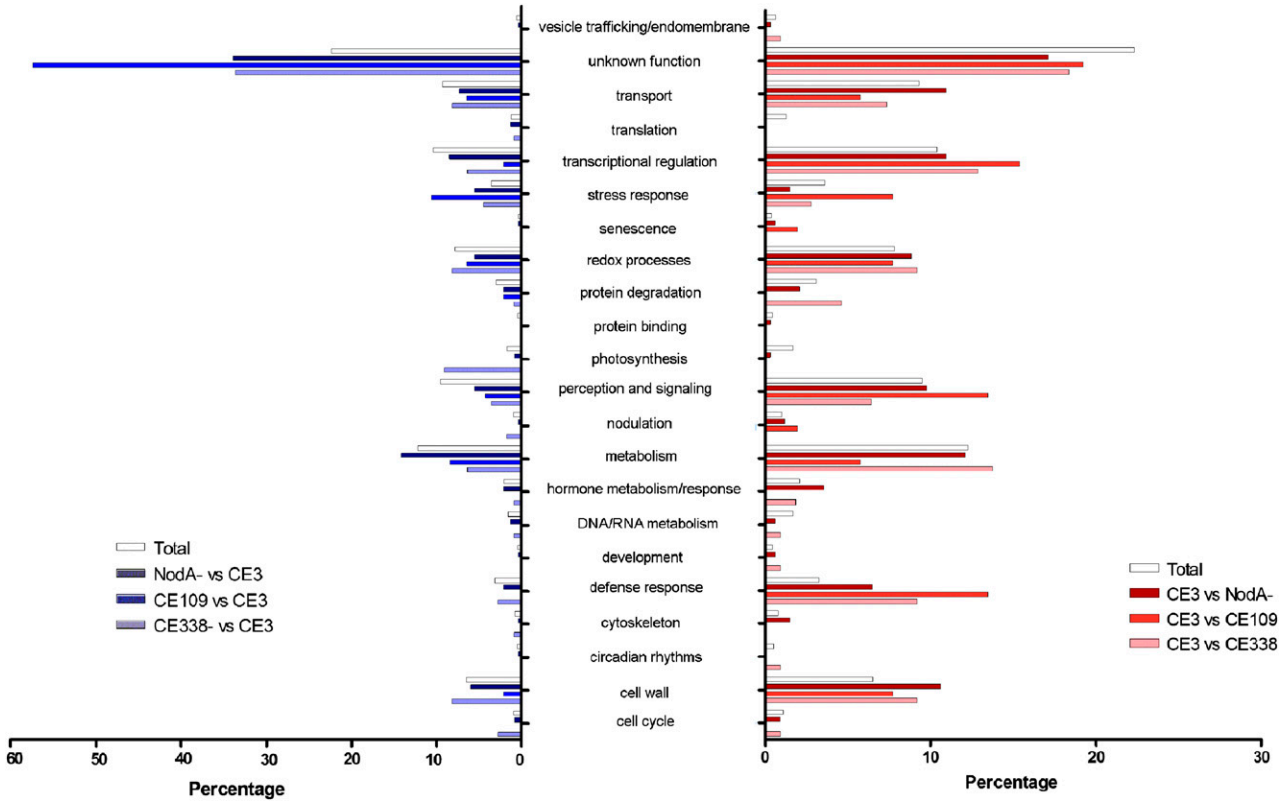

Figure 3. Functional categories of genes regulated by NF, LPS, and EPS. Differential expressed genes were defined as up-regulated when their expression values were lower in response to the mutant compared with the wild-type strain (right) or down-regulated by these rhizobial signal molecules when values were higher in the mutant than in the wild type (left). Genes were categorized based on the GO annotation and presented as percentages of the total. Total accounts for genes identified as differentially expressed in this analysis (2,606 genes).

RWP-RK domains, named after the first member described in root legume symbiosis (Schauser et al., 1998; Marsh et al., 2007). Phylogenetic and synteny analyses showed that one of the two *NIN* genes induced in response to NF, Phvul.008G291800, is the putative ortholog of *NIN* from *M. truncatula* (Supplemental Fig. S4A).

Several members of the family APETALA2/Ethylene Responsive Factor (AP2/ERF) have been associated with developmental processes and responses to biotic and abiotic stresses (Solano et al., 1998; Berrocal-Lobo and Molina, 2004; Zhu et al., 2014). We detected 41 members of this family with differential expression, including Phvul.001G111800, the ortholog of *ERN1* from *M. truncatula* (Supplemental Fig. S4B). Interestingly, levels of some *AP2* genes from common bean were down-regulated in nodules, concomitantly with an increase of the microRNA 172 (Nova-Franco et al., 2015). This microRNA has complementary sites in six AP2 genes; however, none of these genes were detected as differentially expressed in our analysis at an early stage of the interaction. The other AP2/ERF genes identified in our screening were up-regulated by infection with the wild-type strain CE3 and either up- or down-regulated in response to NF and the combination of EPS/LPS.

The GRAS family of transcription factors has been largely associated with legume-rhizobia symbiosis, because several members were shown to be required for the interaction (Catoira, 2000; Kaló et al., 2005; Smit et al., 2005; Battaglia et al., 2014). In this study, we identified seven GRAS genes differentially regulated, including Phvul.009G122700, the closest homolog of Nodulation Signaling Pathway2 (NSP2) according to the phylogenetic analysis of common bean GRAS transcription factors and *M. truncatula* NSP1 and NSP2 (Battaglia et al., 2014). NSP2 is essential for nodulation and mycorrhization (Kaló et al., 2005; Liu et al., 2011). Whereas Phvul.009G122700 was induced by NF, two closely related homologs (Phvul.008G165200 and Phvul.002G269800) belonging to the HAIRY MERISTEM (HAM) subfamily were repressed by infection (Supplemental Table S7). Interestingly, two members of the Phytochrome A Signal Transduction1 (PAT1) subfamily of GRAS, Phvul.005G089900 and Phvul.008G275500, were induced by the wild-type strain CE3. This induction was lower in response to the CE338 mutant strain, indicating that these genes could be modulated by EPS. Scarecrow-Like13 (SCL13) Involved in Nodulation1, another member of the PAT1 branch, was associated with the symbiotic response in common bean, but levels of this gene were

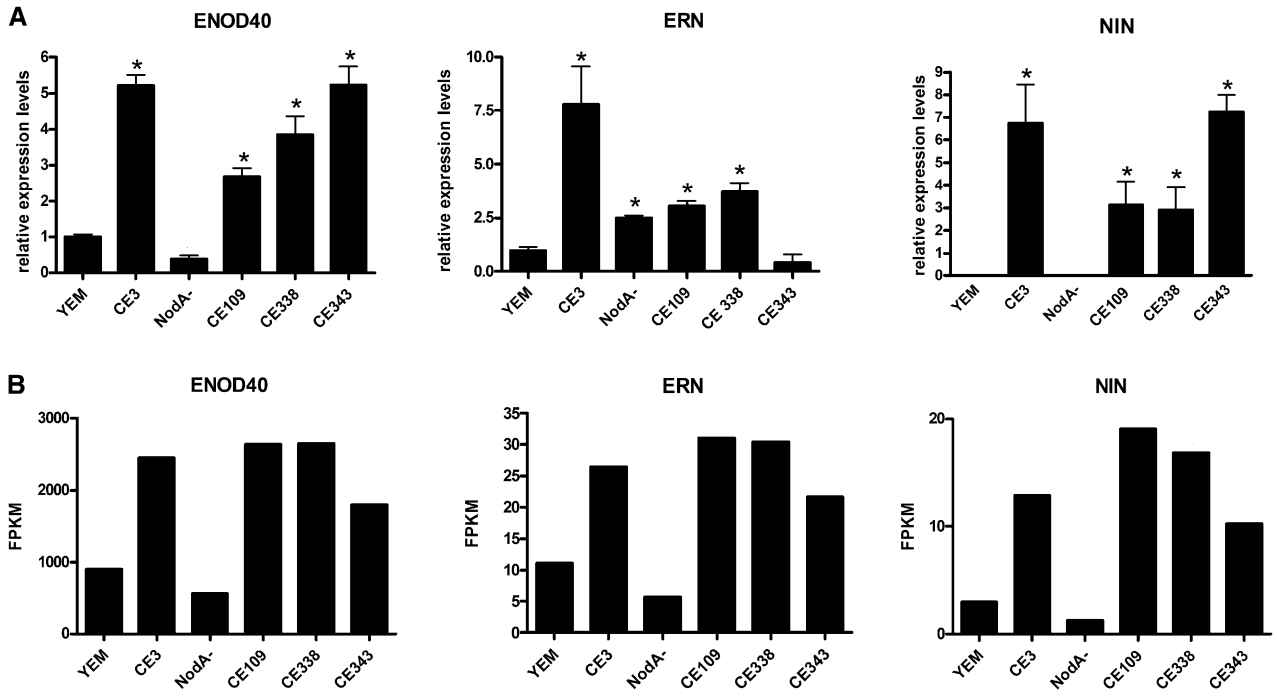

Figure 4. Expression of early nodulation markers quantified by RNAseq and RT-qPCR. Plants were inoculated with the indicated *R. etli* strains or mock inoculated with YEM media, and root tissue from the susceptible zone was collected 24 hpi. A, Abundance of *ENOD40*, *ERN*, and *NIN* transcripts was measured by RT-qPCR using gene-specific primers, normalized to EF-1α, and presented relative to the values of YEM. Bars represent media and SD. Asterisks indicate significant differences in an unpaired two-tailed Student's *t* test with the YEM value. B, FPKM for *ENOD40*, *ERN*, and *NIN* transcripts obtained by RNAseq.

regulated at later stages of the interaction (Battaglia et al., 2014). The two other GRAS transcription factors were Phvul.011G063100 and Phvul.005G145200, which are part of the *Lilium longiflorum* SCR-like (LISCL) subfamily. This subfamily does not contain any of the genes previously involved in nitrogen-fixing symbiosis but includes SCL14, which has been shown to play a role in the activation of defense- and stress-inducible promoters (Fode et al., 2008).

Sixteen genes encoding transcription factors of the NAC family (named after the founding members No apical meristem [NAM], ATAF1 and Cup-shaped cotyledon2 [CUC2]) showed differential responses to signal molecules. One of them, Phvul.004G077400, requires LPS and EPS for induction and has increased levels in absence of NF. This gene is phylogenetically close to NAC969 from *M. truncatula* (Supplemental Fig. S4C), a gene that participates in symbiotic nodule senescence and stress responses in the root (de Zélicourt et al., 2012).

The list of transcription factors also includes 20 members of the CO-like family, which contains pseudoresponse regulators (PRRs) and have been involved in the control of the circadian clock. Most of these genes were induced by CE3 and also by NF and EPS deficient mutants. Two CONSTANS genes showed opposite expression patterns: whereas Phvul.003G149000 levels increased in response to CE3 and all mutant strains, accumulation of Phvul.006G005200 transcripts was repressed in all tested conditions compared with the control sample inoculated with YEM (Supplemental Table S7). GATA transcription factors are regulated also by circadian rhythms and are involved in regulation of the nitrogen metabolism (Reyes et al., 2004; Bi et al., 2005). Two differentially expressed genes that are part of the Group I of TIFY proteins described in Arabidopsis (*Arabidopsis thaliana*) were induced in response to the three signal molecules. TIFY transcription factors are characterized by the presence of the conserved motif TIFY. Interestingly, Phvul.002G002000 encodes a protein containing TIFY and CCT domains (named after CONSTANS, CO-like and TOC1), which are typically bound to DNA in response to circadian oscillations or light (Teakle et al., 2002). It has been shown that CCT domains can interact with Nuclear Factor Y (NF-Y) heterotrimeric transcription factors, integrating vernalization and photoperiod signals (Li et al., 2011). Work from several groups has shown the involvement of several subunits of NF-Y complexes in nodulation in the model legumes *L. japonicus* and *M. truncatula*, but also in common bean (Combier et al., 2006; Zanetti et al., 2010; Mazziotta et al., 2013; Soyano et al., 2013). We detected one NF-YA and five NF-YB genes differentially regulated in our samples. Phvul.007G267100 was classified as NF-YA9 and shown to be induced at 24 hpi with rhizobia in common bean (Rípodas et al., 2014). Considering these results, it will be interesting to further explore whether NF-Y proteins are involved in the connection between circadian clock and nodulation through their interaction with CCT domain-containing proteins.

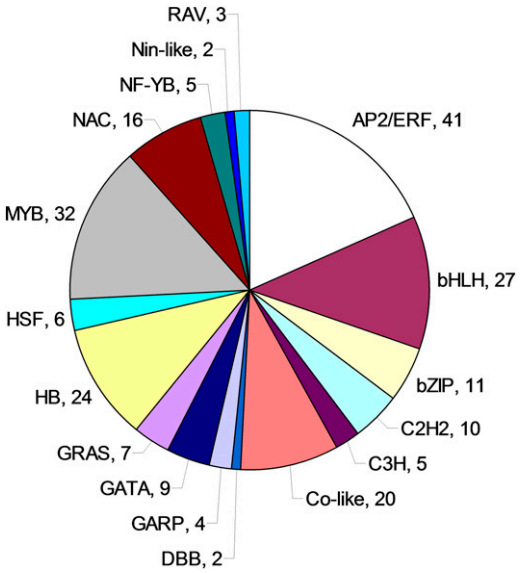

Figure 5. Differentially expressed transcription factors classified in gene families. Transcription factors identified as differentially expressed in all the pairwise comparisons were classified according to the families defined in the Plant Transcription Factor Database 3.0. Families with only one member were not included in the graph for clarity (Dof, GeBP, GRF, LSD, MADS, NF-YA, SBP, and TCP).

Other transcription factors represented in our list include MYB transcription factors, which participate in metabolism, response to stress, and synthesis of the cell wall (Dubos et al., 2010; Ko et al., 2014), as well as other families implicated in several aspects of plant development and stress responses. Similar results were reported in nodules of *M. truncatula*, where MYB, Homeobox (HB), RWP-RK, basic Leucine Zipper (bZIP), basic Helix-Loop-Helix (bHLH), Cys2-His2 (C2H2), NAC, Zinc finger, Zinc-finger protein expressed in Inflorescence Meristem (ZIM) domain, ERF, and GRAS transcription factors were differentially expressed in nodules or in infected root hairs (Moreau et al., 2011; Breakspear et al., 2014).

Our results highlight the vast array of transcription factors that are modulated at the transcriptional level by signal molecules from the bacterium. Expression patterns show that these molecules, i.e. NF, LPS, and EPS, can exert opposite effects on members of the same family (e.g. AP2/ERF genes), suggesting that root cells are able to mount a unified response that integrates different signals from the environment through complex regulatory networks. It will be of great interest to further elucidate the role of these genes at early stages of symbiosis.

Genes Involved in Transcriptional and Posttranscriptional Silencing

Several genes associated with the regulation of gene expression mediated by small RNAs were also detected as regulated by rhizobial signals. One of them (Phvul.003G046700) is up-regulated when NF, EPS, or LPS is not produced by the bacterium and encodes a protein highly similar to Argonaute7 of Arabidopsis and *M. truncatula* (Fig. 6). This particular Argonaute is involved in the production of trans-acting small interfering RNA auxin response factors (ARFs) derived from the noncoding transcript *trans-acting short interfering RNA3*, which regulates auxin responses mediated by ARF transcription factors. Previous studies have shown that this pathway is regulated at early stages of the symbiotic interaction between legumes and rhizobia (Reynoso et al., 2013; Li et al., 2014). Another differentially expressed gene encodes a protein containing an XH/XS domain (Phvul.003G281400), homologous to double strand RNA binding proteins that direct DNA methylation (Butt et al., 2014). In this case, transcript levels decrease upon inoculation with CE3, NodA$^-$, CE109, or CE343 compared with uninoculated roots, whereas the inoculation with the EPS mutant has a mild effect. Other genes associated with transcriptional silencing were also identified as differentially regulated, such as a methyl-CpG-binding domain7 (Phvul.004G006200), an RNA-dependent RNA polymerase family protein (Phvul.004G176400), and a nucleolar histone methyltransferase-related protein (Phvul.007G184700). These genes constitute a very interesting link between early molecular responses to rhizobia and gene regulation mediated by small RNAs at both transcriptional and posttranscriptional levels.

Differential Expression of Circadian Clock Genes

Circadian clocks are biological timing mechanisms used by pluricellular organisms to predicatively adjust physiological and molecular processes to the anticipated environmental changes that occur as a consequence of the day-night cycle. In our transcriptomic analysis, we found six genes encoding the pseudoresponse regulators PRR3, PRR5, and PRR7 that were down-regulated by NF and EPS (Fig. 7), suggesting that the circadian rhythms can be modulated at early stages of symbiosis. These three elements are part of the sequential circadian waves described in the Arabidopsis circadian clock (Fig. 7A), showing peaks at mRNA and protein levels along the day (Matsushika et al., 2000). Interestingly, other genes involved in the molecular clock were also detected as differentially regulated: two genes encoding proteins with high sequence identity with GIGANTEA (GI; Phvul.007G083500 and Phvul.004G088300), a membrane protein that controls the clock and flowering time in Arabidopsis (Fowler et al., 1999; Martin-Tryon et al., 2007), and five *SUPPRESSOR OF PHYA105* (*SPA1*; Phvul.002G296100, Phvul.005G071800, Phvul.010G111100, Phvul.010G111200, and Phvul.011G158700) genes encoding SPA1-related proteins. SPA1 was identified as a negative regulator of phytochrome A-mediated responses (Hoecker et al., 1998) and shown to be involved in the regulation of circadian rhythms and flowering time in Arabidopsis (Ishikawa et al., 2006). Most of these genes showed the same transcriptional pattern as the five PRR genes, a moderate induction by the wild-type strain CE3, but a higher and significant increase in mRNA

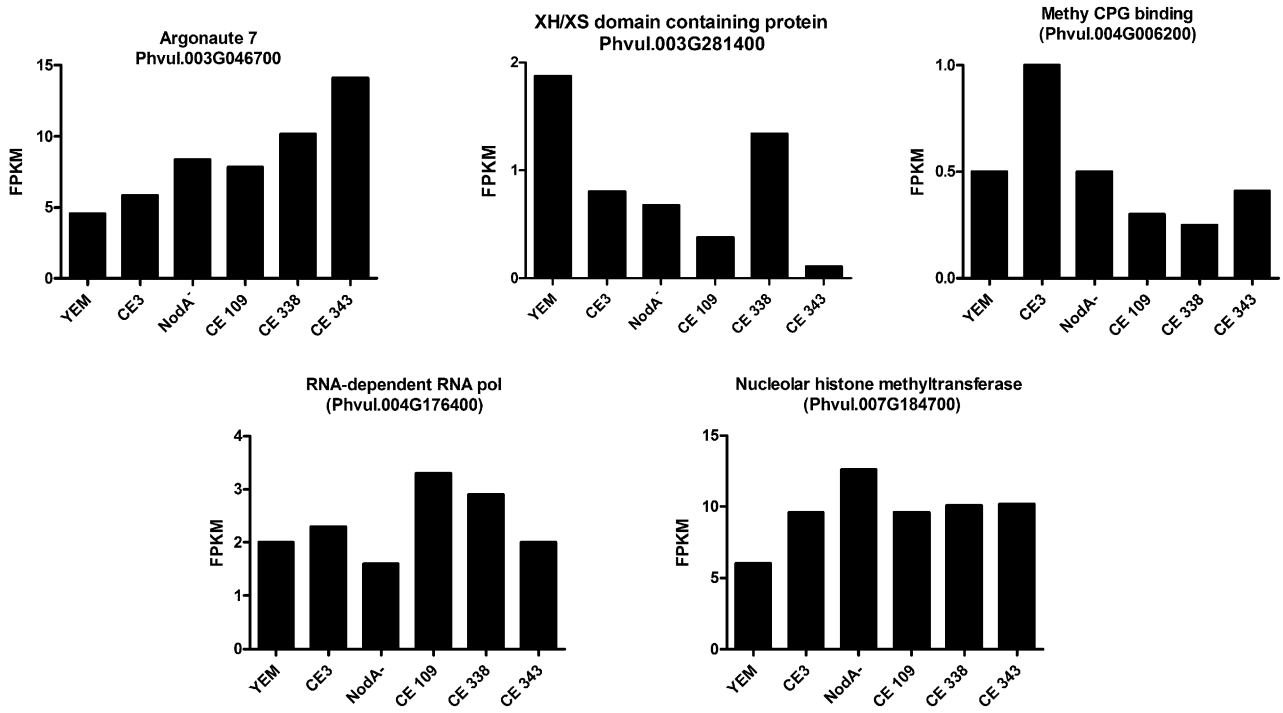

Figure 6. Genes involved in transcriptional and posttranscriptional silencing identified as differentially expressed. Expression values estimated by RNAseq are expressed in FPKM.

levels upon inoculation with strains that do not produce either NF or EPS, whereas the absence of LPS had no effect (Fig. 7B).

Two of the main components of the clock are CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTIL (LHY). These two proteins act as negative regulators of PRR genes and GI. Both genes were included in our list of differentially expressed genes and showed the same pattern of expression described above for the other genes related to circadian rhythms (Fig. 7B), revealing a connection between the clock and early stages of nodulation. Interestingly, GI, PRR5, and PRR7 were also found as differentially regulated in mature nodules formed by wild-type plants or roots silenced in a bHLH transcription factor (Chiasson et al., 2014). The authors suggested the existence of a nodule circadian clock that links availability of photosynthetic C and the demand of the nitrogen fixation process throughout the light/darkness cycle. Our results suggest that these changes occur very early in the interaction, long before the nodule is formed and nitrogen fixation takes place. It is known that circadian rhythms can sense external signals to anticipate changes in the environment through modification of developmental programs and stress responses. It is unclear how the clock integrates signals from aboveground with physiological processes in the root. It has been suggested that a root clock can operate independently of the main one operating in the shoot (James et al., 2008). In Arabidopsis, only the morning loop seems to operate in the root, without the requirement of the evening loop. Consistently, TOC1, the last PRR in the circadian wave, did not show changes in expression after rhizobia inoculation (Fig. 7B). A BLASTX search against the common bean genome using CONSTANS (another protein of the central oscillator) from Arabidopsis as a query did not produce a significant hit. Consistently with this result, a recent report showed that the function of this protein is not conserved in legumes (Wong et al., 2014). Taken together, our results showed that the circadian rhythms are adjusted in the root after perception of signal molecules of rhizobia, possibly to anticipate metabolic adjustments for nitrogen fixation in nodules.

Receptors and Signal Transduction Genes

A total of 247 genes involved in signal perception and transduction were found as differentially expressed in our analysis. According to the PlantsP classification (http://plantsp.genomics.purdue.edu/), 76 protein kinases belong to Class I, which correspond to transmembrane receptor kinases, including proteins with Lysin Motifs (LysM; four genes), leucine-rich repeats (LRR; 32 genes), and lectin (four genes) extracellular domains (Supplemental Table S8). These ectodomains are involved in the recognition of polysaccharides (LysM and lectin) or proteins (LRR) and have been largely associated with bacteria recognition in symbiotic or pathogenic interactions (Endre et al., 2002; Stracke et al., 2002; Limpens et al., 2003; Arrighi et al., 2006; Kawaharada et al., 2015). Of particular interest are the four genes predicted to

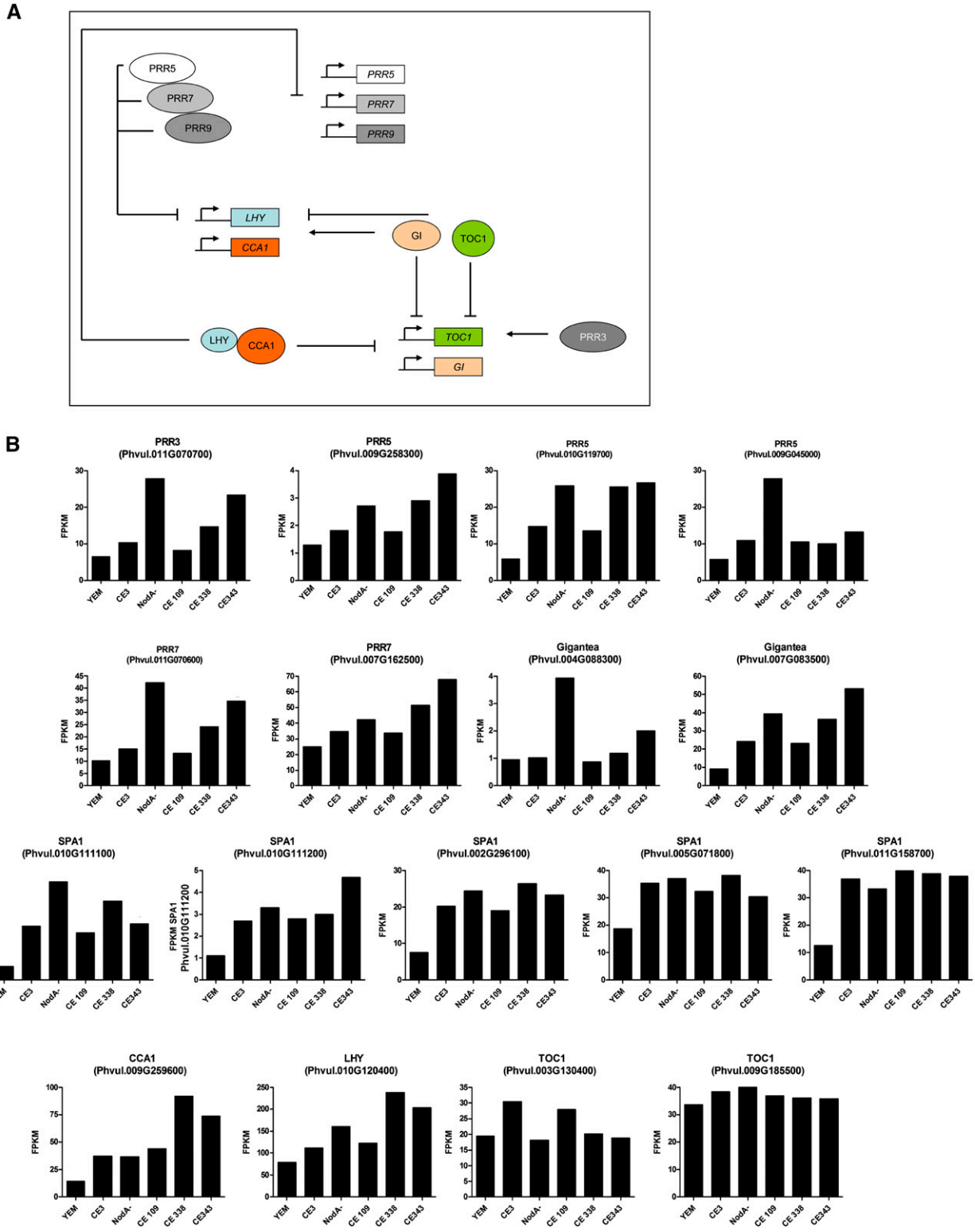

Figure 7. Regulation of circadian rhythm genes in response to rhizobial strains. A, Schematic representation of key circadian clock components identified in Arabidopsis and their transcriptional interactions. PRR5, PRR7, and PRR9 repress transcription of *LHY* and *CCA1*. Reciprocally, LHY and CCA1 proteins repressed transcription of these pseudoregulator genes. Another loop involves *TOC1* and *GI*, which are positively controlled by PRR3 and repressed by LHY, CCA1, and their own proteins. The central oscillator is composed by other loops and involves other levels of regulation. For more information, see McWatters and Devlin

encode proteins with LysM domains, which constitute peptidoglycan-binding modules. Remarkably, Phvul.002G05900 showed the highest percentage of sequence identity with the *L. japonicus* EPS receptor, EXOPOLYSACCHARIDE RECEPTOR3, recently described by Kawaharada et al. (2015), in a BLASTP search against the common bean protein database. Phvul.002G05900 showed higher levels of transcripts in roots inoculated with the CE3 strain with respect to YEM and a strong dependence of the presence of NF, consistent with what was reported in *L. japonicus* (Kawaharada et al., 2015). Another gene of this subfamily, Phvul.002G025500, showed a similar expression pattern. The rest of the Class I kinases belong to families with other extracellular domains or a group of receptors without close relatives. Phvul.007G129500 and Phvul.007G271700 encode proteins highly similar to the ethylene receptor ETR2, which has Ser/Thr kinase activity. These receptors belong to a gene family whose members are regulated by abiotic stresses and hormones (Watanabe et al., 2004; Yau et al., 2004).

Differentially expressed genes also include several receptors associated with pathogen recognition, such as LRR, Toll/Interleukin-1 receptor (TIR)-Nucleotide Binding Site (NBS)-LRR, and NBS-Apoptosis Repressor with Caspase recruiting (NBS-ARC). The expression of these receptors does not follow a common pattern, with members that are strongly induced or repressed by infection or any of the signal molecules (Supplemental Table S8).

The symbiotic process involves communication between different tissues to coordinate and integrate physiological processes taking place in the epidermis, cortical cells, and even aerial tissue. Small peptides are good candidates to participate in this cell-to-cell signaling. We detected four putative ligands homologous to Clavata3 (Phvul.002G095900, Phvul.002G095900, Phvul.011G160700, and Phvul.002G081400), one small signal peptide from the DEVIL (Phvul.007G098500), and one from the ralf-like (Phvul.002G018200) families (Supplemental Table S8).

Receptors differentially modulated in response to infection, NF, LPS, and EPS are excellent candidates to participate in the specific recognition of bacteria, particularly lectin receptors, which can recognize glycan-containing molecules, such as the saccharides present in the surface of rhizobia. On the other hand, small peptides can function as secondary signals coordinating tissue-specific genetic programs.

Differentially Expressed Genes Involved in Hormone Responses and Biosynthesis

Our transcriptome analysis revealed several genes involved in synthesis, signaling, or response to hormones that are modulated by NF, LPS, or EPS (Supplemental Table S9). Ethylene is a gaseous phytohormone that activates and regulates plant immunity (Khatabi and Schäfer, 2012). Several components of the ethylene signaling and response pathway are negatively regulated by LPS and EPS, as it was previously mentioned for the two putative receptors ETR2 and the ethylene-regulated transcription factors of the AP2/ERF family. In addition, three members of the *ETHYLENE-INSENSITIVE3* (*EIN3*)-*binding F box protein* (*EBF*) showed higher transcript accumulation in response to mutants in LPS and EPS molecules compared with CE3 or the NF-deficient strain. EBF1 and EBF2 negatively regulate the ethylene response by ubiquitination of EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003), a transcriptional factor that positively regulates ERF transcription. It has been described that ethylene has a negative effect on nodulation and prevents NF signal transduction (Oldroyd et al., 2001; Penmetsa et al., 2003). Mutation of the *EIN2* gene results in a higher number and sustained growth of infection threads, leading to hypernodulation (Prayitno et al., 2006). Further support of the negative effect of ethylene during nodulation came from silencing of two genes encoding 1-amino-cyclopropane 1-carboxylate oxidases, an enzyme that is required for ethylene biosynthesis (Miyata et al., 2013). Expression of one of these genes is induced by NF, suggesting that NF triggers a negative feedback on nodulation through ethylene signaling.

Additionally, genes encoding proteins involved in the biosynthesis of phytohormones were differentially expressed. Zeaxanthin epoxidase (Phvul.003G243800) and abscisic aldehyde oxidase3 (Phvul.008G210000), enzymes that are part of the biosynthetic pathway of abscisic acid, were down-regulated by NF/EPS and NF, respectively. Another differentially expressed gene, induced by NF, encodes a GA 20-oxidase, a protein that is part of the biosynthetic pathway of GAs (Phvul.009G131500). This is consistent with previous results showing genes of the GA synthesis metabolism induced in *M. truncatula* after infection with *S. meliloti* (Hayashi et al., 2012; Breakspear et al., 2014).

Genes involved in cytokinin biosynthesis and auxin responses were also represented in the list of differentially expressed genes. Several members of the Small Auxin-Up RNA-like auxin-responsive protein family were differentially modulated by infection, with members that were up- or down-regulated in response to rhizobial inoculation. Levels of these genes change rapidly in response to auxin levels. Auxin signaling plays a major role during the organogenesis of the nodule (Mathesius, 2008) but also at early stages of rhizobial infection, as recently described by Breakspear et al. (2014).

Figure 7. (*Continued.*)
(2011), Nagel and Kay (2012), and Farré and Liu (2013). B, Expression of genes involved in circadian rhythms in common bean roots 24 hpi with *R. etli* strains. Expression values estimated by RNAseq are expressed in FPKM.

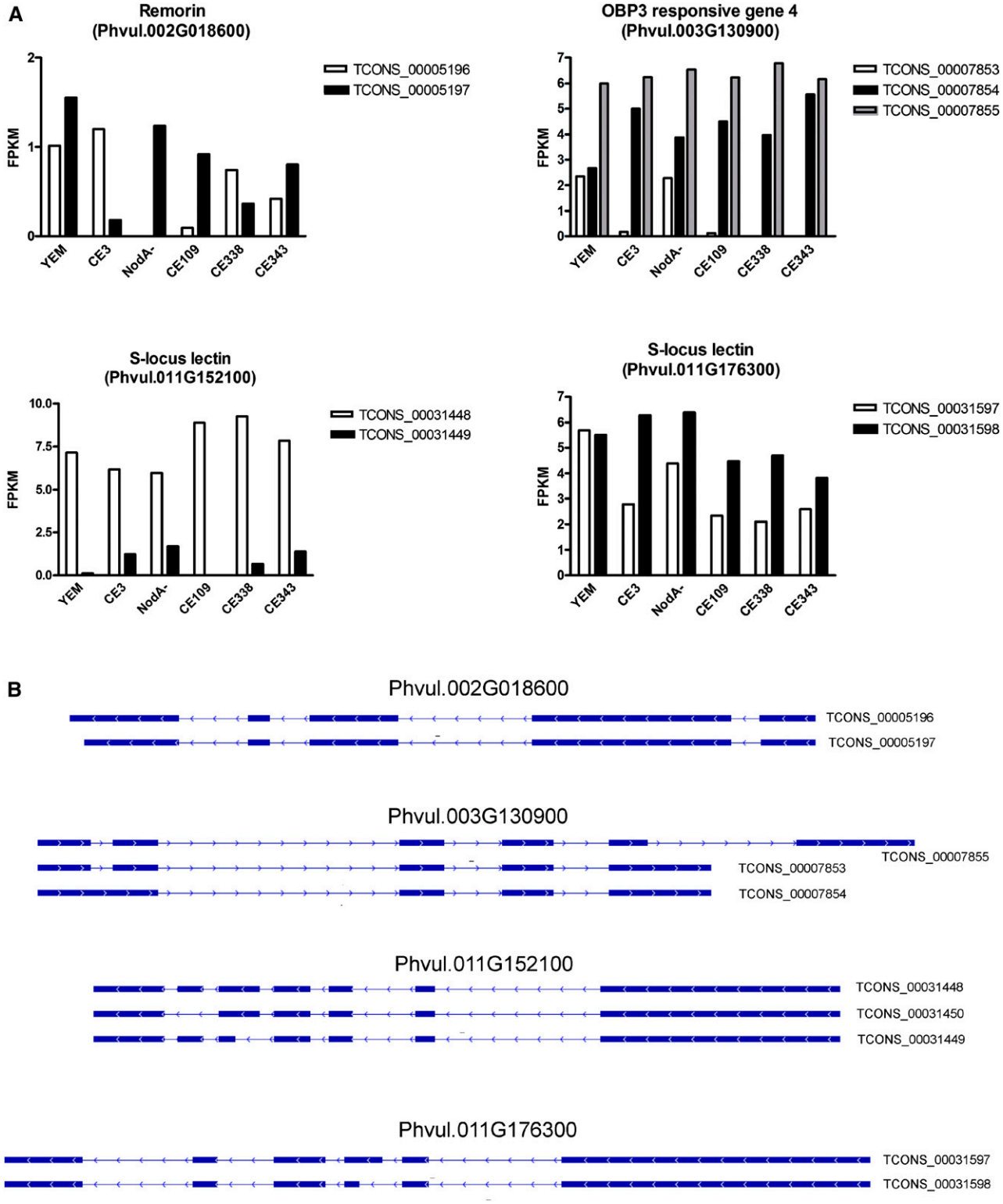

Figure 8. Examples of genes with alternative transcript variants differentially regulated by *R. etli* strains. Expression values at 24 hpi with *R. etli* strains (A) and gene models (B) for selected genes are shown for each alternative transcript variant.

Altogether, our results are in accordance with the key role played by hormones during different stages of root nodule symbiosis. Of particular interest is the case of ethylene, a hormone that plays a key role modulating defense responses, infection thread formation, and regulation of nodule number.

Other Genes of Interest

Several genes participating in defense, oxidative responses, and cytoskeleton rearrangements, as well as cell wall remodeling, were detected as differentially regulated (Supplemental Table S4). At 24 hpi, the infection process is already taking place, involving reorientation of the root hair directional growth, loosening of the cell wall at the tip, and the formation of the infection thread. These events require the reprogramming of genes that act in the cell wall synthesis, vesicle trafficking, and cytoskeleton. The list of differentially expressed genes includes many genes in these categories, providing the basis to start functional analysis by reverse genetics to better understand these early physiological events. Concomitantly with the early stages of infection, the cell division is reactivated in cortical cells to form the nodule. We detected 28 genes related to the cell cycle regulation that can be related to this process.

Differentially Expressed Genes with Alternative Transcript Variants

Computational analyses identified 172 differential expressed genes that are subjected to alternative transcript processing and regulated in response to rhizobial strains (Supplemental Table S10). Comparison with the total list of differentially expressed genes showed that DNA/RNA metabolism, metabolism, transcriptional responses, and vesicle trafficking were the categories overrepresented among genes with alternative splicing or transcript processing (Supplemental Fig. S5).

An interesting example of gene expression regulated at the level of alternative termination is the Phvul.002G018600 gene, which has a form that is induced by CE3 and one whose repression seems to be mediated by EPS (Fig. 8). This gene encodes a remorin, a plant-specific lipid raft protein involved in signaling in the context of nodulation. In *M. truncatula*, it was shown that a remorin can interact with the core receptors of symbiosis (Lefebvre et al., 2010).

Phvul.003G130900 is a gene whose expression is regulated by a plant-specific DNA-binding with one finger (Dof) transcription factor inducible by salicylic acid (Kang et al., 2003). Whereas one of the splicing variants is induced by infection independently of NF, EPS, or LPS (TCONS_00007854), another variant is repressed in a NF-dependent manner (TCONS_00007853) and the third one is not significantly altered (Fig. 8).

Two proteins of the S-locus lectin protein kinase family showed two alternative spliced forms that respond differentially to rhizobia infection. Members of this family contain an extracellular Man-binding lectin domain, making them excellent candidates to participate in the specific recognition of extracellular molecules containing Man. The LPS from the CE3 strain of *R. etli* contains residues of Man in the O-antigen portion of the molecule (Forsberg et al., 2000) and the species-specific EPS molecules typically contain Man among other common monosaccharides in their repetitive units (Janczarek, 2011). One of the receptors, Phvul.011G152100, showed a transcript variant that is slightly repressed by *R. etli* infection, whereas an alternative isoform is induced, but only when NF or EPS was present in the bacteria (Fig. 8).

These genes are good examples of differentially expressed transcripts that were not detected as differential when the sum of all transcript variants was considered. They also illustrate how mRNAs can be modulated at the splicing or processing level, changing the relative abundance of alternative transcript variants during the symbiotic process, and in response to the different signal molecules produced by rhizobia. In agreement with this idea, it has been previously shown that *NF-YA1* was subjected to alternative splicing in response to rhizobia (Combier et al., 2008).

CONCLUSION

Data generated by RNAseq are an important source of information to elucidate the molecular mechanisms involved in biological processes, such as the establishment of the symbiosis between legumes and rhizobia. Our analysis revealed that signal molecules present in the surface or secreted by rhizobia induce changes in the expression of plant genes that participate in a plethora of biological processes, such as transcriptional regulation, circadian rhythms, hormone biosynthesis, or signaling and stress responses. In addition, our results illustrate how alternative transcript processing can be modulated by bacterial-derived signal molecules in the context of root nodule symbiosis. Several receptors identified as differentially expressed are excellent candidates to participate in the recognition of rhizobial molecules, particularly signal molecules of glycosidic nature. Interestingly, many of the genes detected as differentially expressed respond to more than one of such signals, suggesting a complex regulatory system that integrates perception of different molecules from bacteria to distinguish pathogenic and symbiotic organisms, and even particular strains from the same species. This is of particular interest to understand and manipulate ecological interactions in agronomical systems, where strains of rhizobia optimized for nitrogen fixation are outcompeted by other rhizobia present in the soil. Our results highlight, to our knowledge, new biological and molecular aspects that can help to understand recognition and downstream responses in the context of nitrogen-fixing symbiosis.

MATERIALS AND METHODS

Biological Material and Plant Inoculation

Growth and inoculation of common bean (*Phaseolus vulgaris*) 'NAG12' were performed essentially as previously described (Blanco et al., 2009). *Rhizobium etli* strains used in this study were previously described (Noel et al., 1984; Diebold and Noel, 1989; Vázquez et al., 1991; Eisenschenk et al., 1994) and are listed in Table I. A minimum of five plants was inoculated with the indicated strains or YEM medium, and roots were frozen in liquid nitrogen 24 hpi for RNA extraction. Harvested root tissue corresponds to the susceptible zone, which presents actively growing root hairs and is susceptible to rhizobia infection. Two biological replicates were prepared independently.

Library Construction

Total RNA was extracted from root tissue using TRIZOL (Invitrogen) and digested with RNase-Free DNase (Promega). RNA quality was evaluated by capillary gel electrophoresis in an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano kit. Libraries were prepared using 3.5 μg of RNA and the Illumina TruSeq RNA Sample Preparation Kit (v2) following provider's instructions. SuperScript II (Invitrogen) reverse transcriptase was used for complementary DNA synthesis. PCR fragments were purified using AMPure XP beads (Beckman Coulter Genomics). Size of the synthesized fragments was verified in an Agilent 2100 Bioanalyzer using the DNA-1000 kit (Agilent). Libraries were multiplexed following Illumina recommendations for barcode compatibility and sequenced in an Illumina HiSeq2500 at the Genomics Core of the University of California, Riverside's Genomic Institute. The number and quality of the 50-base single-end reads obtained for each condition is listed in Supplemental Table S1. Raw data were deposited at National Center for Biotechnology Information BioProject database under the accession number PRJNA280590 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA280590).

Data Analysis

Illumina reads were aligned to the common bean genome v1.0 using Tophat 2 (Kim et al., 2013). To optimize parameters for plant genomes, minimum and maximum intron lengths were set as 60 and 6,000 according to what was described for splicing in Arabidopsis (*Arabidopsis thaliana*; Márquez et al., 2012); own junction usage and indel search options were activated. Transcript assembling was performed using Cufflinks and differentially expressed genes were identified with Cuffdiff (Trapnell et al., 2010, 2012) using quartile normalization, BIAS correction, and multiread correct options. To quantify the expression of genes and transcripts, the result of a Cuffmerge with the v1.0 of the common bean genome and assemblies from our libraries (Cufflinks) were used (Trapnell et al., 2010). Reproducibility of biological samples was analyzed with CummeRbund (Trapnell et al., 2012). Clustering was performed applying the k-means algorithm using the GENESIS software (Sturn et al., 2002). Functional classification of genes was based on GO annotations using Blast2GO (http://www.blast2go.com/) but was further manually redefined using BLAST searches and database annotations. Transcription factors were classified according to the Plant Transcription Factor database v3.0 (Jin et al., 2014), and protein kinases were classified according to the PlantsP database (Gribskov et al., 2001).

Analysis of Alternative Transcript Variants

FPKM values from differentially expressed transcripts obtained after Cuffdiff analysis were selected. Only alternative transcripts with at least a 2-fold change between samples, $P < 0.05$, and FPKM value greater than 1 in at least one sample were considered. The different transcript variants along with reads aligned to the genome were visualized using the Integrative Genomic Viewer (Thorvaldsdóttir et al., 2013).

RT and qPCR

Expression analysis by RT-qPCR was performed essentially as previously described (Meschini et al., 2008; Blanco et al., 2009). For each primer pair, the presence of a unique PCR product of the expected size was verified in ethidium bromide-stained agarose gels. Absence of contaminant genomic DNA was confirmed in reactions with DNase-treated RNA as template. Amplification of common bean *Elongation Factor 1 Alpha* was used to normalize the amount of template complementary DNA. At least three biological replicates were performed per condition. Primers used are listed in Supplemental Table S11.

Supplemental Material

The following supplemental materials are available.

Supplemental Figure S1. Distribution of FPKM values among samples.

Supplemental Figure S2. RNAseq compared with RT-qPCR data of selected genes.

Supplemental Figure S3. Cluster analysis of the expression profiles of differentially expressed transcription factors.

Supplemental Figure S4. Genetic relationships between NIN, ERN1, and NAC969 from *M. truncatula* and the corresponding common bean proteins.

Supplemental Figure S5. Percentage of differentially expressed genes with alternative transcript variants.

Supplemental Table S1. Summary of total read number, percentage of mapped reads, and quality of common bean root libraries.

Supplemental Table S2. Normalized expression values for all genes.

Supplemental Table S3. Genes considered as differentially expressed in systematic pairwise comparisons.

Supplemental Table S4. Total genes identified as differentially expressed.

Supplemental Table S5. Differentially expressed genes in comparisons between CE3, NodA$^-$, CE109, and CE338.

Supplemental Table S6. Differentially expressed genes in comparisons between CE3, CE109, CE338, and CE343.

Supplemental Table S7. Classification of differentially expressed transcription factors.

Supplemental Table S8. Classification of differentially expressed genes encoding receptors.

Supplemental Table S9. Classification of differentially expressed genes involved in hormone synthesis, signaling, and response.

Supplemental Table S10. Transcripts differentially expressed.

Supplemental Table S11. Sequence of primers used for qPCR.

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