The Ethylene Responsive Factor Required for Nodulation 1 (ERN1) Transcription Factor Is Required for Infection-Thread Formation in *Lotus japonicus*

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Several hundred genes are transcriptionally regulated during infection-thread formation and development of nitrogen-fixing root nodules. We have characterized a set of *Lotus japonicus* mutants impaired in root-nodule formation and found that the causative gene, *Ern1*, encodes a protein with a characteristic APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription-factor domain. Phenotypic characterization of four *ern1* alleles shows that infection pockets are formed but root-hair infection threads are absent. Formation of root-nodule primordia is delayed and no normal transcellular infection threads are found in the infected nodules. Corroborating the role of ERN1 (ERF Required for Nodulation1) in nodule organogenesis, spontaneous nodulation induced by an autoactive CCaMK and cytokinin-induced nodule primordia were not observed in *ern1* mutants. Expression of *Ern1* is induced in the susceptible zone by Nod factor treatment or rhizobial inoculation. At the cellular level, the *pErn1::GUS* reporter is highly expressed in root epidermal cells of the susceptible zone and in the cortical cells that form nodule primordia. The genetic regulation of this cellular expression pattern was further investigated in symbiotic mutants. Nod factor induction of *Ern1* in epidermal cells was found to depend on *Nfr1*, *Cyclops*, and *Nsp2* but was independent of *Nin* and *Nf-ya1*. These results suggest that ERN1 functions as a transcriptional regulator involved in the formation of infection threads and development of nodule primordia and may coordinate these two processes.

Development of legume root nodules hosting symbiotic nitrogen-fixing rhizobia results from two highly synchronized processes, namely, rhizobial infection and nodule organogenesis. In *Lotus japonicus* and *Medicago truncatula*, organogenesis is initiated by local reactivation of cell division in root cortical cells. This gives rise to nodule primordia that are targeted by infection threads initiated by invagination of root-hair plasma membranes. Both processes are triggered by rhizobial Nod factors

perceived by a Nod factor receptor complex that, in *L. japonicus*, consists of NFR1 and NFR5 as well as an interaction between SYMRK with NFR5 (Antolín-Llovera et al. 2014; Broghammer et al. 2012; Madsen et al. 2003; Radutoiu et al. 2003; Madsen et al. 2011). This initial recognition of Nod factor leads to expression of the EPR3 receptor, which subsequently perceives rhizobial exopolysaccharides and regulates rhizobial infection at the epidermis and the enclosure of bacteria by plant membranes during the subsequent progression of infection threads into nodule primordia (Kawaharada et al. 2015, 2017).

Transcriptome studies show that nodule development involves major changes in cellular, metabolic, and transcriptional activities (Colebatch et al. 2004; Høglund et al. 2009; Trujillo et al. 2014). Compared with uninfected root tissue, several hundred genes are transcriptionally up- or down-regulated in different stages of the developmental process forming these new organs (Verdier et al. 2013). This resetting of the transcriptional landscape is triggered by signal perception and the downstream signal transduction. One of the first detectable physiological hallmarks of this process is calcium oscillations (spiking) in the perinuclear space of root hairs, leading to calcium-mediated conformational changes that activate the CCaMK protein (Miller et al. 2013; Miwa et al. 2006; Shimoda et al. 2012). Downstream of this signaling activation of transcription factors, including members of the RWP-RK, GRAS, AP2/ERF (APETALA2/Ethylene Responsive Factor), C2H2, ARID, NF-Y, bHLH, coiled-coil, and MYB classes, has been shown to play a role in the transcriptional regulation of genes involved in different stages of root-nodule development (Andrianakaja et al. 2007; Cerri et al. 2012; Godiard et al. 2011; Middleton et al. 2007; Oldroyd 2013; Schauser et al. 1999, 2005; Sinharoy et al. 2013; Soyano and Hayashi 2014). The transcription factors were mainly identified by loss-of-function genetic approaches, and the mechanistic connection between the signaling and the spatio-temporal activities, interactions, and functional hierarchies of these transcription factors has only recently begun to emerge from molecular studies (Andrianakaja et al. 2007; Middleton et al. 2007). CYCLOPS, a coiled-coil transcription factor that is directly phosphorylated by activated CCaMK, induces *Nin* gene expression by phosphorylation-dependent binding at specific regulatory elements of the *Nin* gene promoter (Singh et al. 2014). The NIN transcriptional regulator then targets the promoters of *Nf-ya1* and *Nf-yb1* that encode subunits of the heteromeric CCAAT-box binding NF-Y nuclear factor complex (Soyano et al. 2013). These observations suggested a central position of CYCLOPS in the transcription-factor hierarchy. Supporting this notion, expression of a phosphomimic, CYCLOPS-DD, was sufficient to induce spontaneous nodules similar to those induced in

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the *snf1* mutant, an autoactive CCaMK (Singh et al. 2014; Tirichine et al. 2006). Overexpression of *Nin* also induces spontaneous nodule-like structures (Soyano et al. 2013), suggesting that CYCLOPS-DD-induced spontaneous nodules form through NIN and activation of the NF-Y complex. Taking the loss-of-function phenotypes of *nin* and *cyclops* mutants into consideration, these observations suggest that CYCLOPS and NIN are key regulators of both infection-thread formation and organogenesis (Fournier et al. 2015; Schauser et al. 1999; Yano et al. 2008). Another transcription-factor pair, NSP1/NSP2, operates downstream of CCaMK. In *M. truncatula*, NSP1 binds promoter regions of *Nin*, *Ern1*, and *ENOD11* and forms heteropolymers with NSP2 (Hirsch et al. 2009). It is still unclear whether this occurs in parallel with or downstream of CYCLOPS regulation. Detailed studies of transcription factors and promoter elements involved in *ENOD11* expression illustrate the complexity of transcription-factor interactions. In *M. truncatula*, ERN1 (ERF Required for Nodulation1) and the NSP1/NSP2 complex both activate *ENOD11* expression but different binding sites are involved (Cerri et al. 2012). In the early preinfection stage ERN1 activates Nod factor-elicited *ENOD11* expression via the so-called NF box, while NSP1/NSP2 regulates *ENOD11* expression during rhizobial infection. Additionally, ERN2, which overlaps the function of ERN1, can positively regulate *ENOD11* expression and *ern1 ern2* double mutants have a severe symbiotic phenotype compared with both *ern1* and *ern2* single mutants (Cerri et al. 2016). ERN3 appears to function as a repressor (Andriankaja et al. 2007). Further studies of *ENOD11* activation suggest that activation of *Ern1* expression differs from NIN-NF-YA1 and the NSP1/NSP2 complex (Laloum et al. 2014); however, it is not clear how ERN2 acts together with ERN1. Although several other transcription factors have been implicated in nodule development, their functions or positions in the hierarchy are less well-defined. A bHLH factor is involved in nodule vasculature patterning (Godiard et al. 2011), and a C2H2 factor controls *VAMP721a* expression and bacteroid development in late nodulation (Sinha et al. 2013). A MYB factor possibly interacts with NSP2 (Kang et al. 2014) or regulates nodule numbers (Libault et al. 2009), as observed with a basic leucine zipper and the miRNA172-controlled AP2s (Holt et al. 2015; Nova-Franco et al. 2015; Wang et al. 2014; Yan et al. 2013). Finally, at least two ERF-type transcription factors are positive regulators of nodulation in *L. japonicus* and *M. truncatula* (Asamizu et al. 2008; Vernié et al. 2008).

Many features of the molecular mechanisms governing the root-nodule developmental processes are similar in *L. japonicus* and *M. truncatula*. However, the formation of the new symbiotic organs follows distinct developmental paths. *M. truncatula* develops indeterminate nodules originating from cell division in the pericycle, endodermis, and inner cortex. These cell divisions lead to nodule primordia with a persistent meristem localized in the tip of the nodules, and these apical meristems produce new cells that are infected by continuously growing infection threads. *L. japonicus* nodules follow a determinate developmental path in which nodule primordia are initiated by cells in the outer cortex of *L. japonicus* roots and the nodules formed lack an apical meristem (Ferguson et al. 2010; Handberg and Stougaard 1992).

Here, we investigate the role of the *L. japonicus* ERF-type factor ERN1 in the nodule symbiosis signal pathway of determinate nodules connecting organogenesis with infection-thread formation in response to rhizobial signaling.

RESULTS

The ERN transcription-factor family.

The function of three ERN transcription-factor genes, *Ern1*, *Ern2*, and *Ern3*, has been investigated in *M. truncatula*, which

forms indeterminate nodules (Andriankaja et al. 2007; Cerri et al. 2016; Middleton et al. 2007). In *L. japonicus*, which develops determinate nodules, only two *Ern* genes were found by analyzing the v3.0 genomic sequence (Lotus Base). The *L. japonicus* ERN1 (Lj1g3v3975310.1) protein is 76% identical to *M. truncatula* ERN1, and *L. japonicus* ERN3 (Lj4g3v2604280.1) is 62% identical to *M. truncatula* ERN3. A *L. japonicus* homolog of the *M. truncatula* *Ern2* was not found. To examine whether this is a genuine difference between legumes forming indeterminate and determinate nodules, the phylogeny of the ERN proteins was assembled. *Cicer arietinum* (chickpea) and *Trifolium pratense* (red clover), which form indeterminate nodules (Afonso-Grunz et al. 2014; Webb et al. 2013), have ERN1, ERN2, and ERN3. *Glycine max* (soybean), which forms determinate nodules, has duplicated genes encoding ERN1-1 and ERN1-2 and ERN3-1 and ERN3-2 but no ERN2, suggesting ERN2 is not essential for determinate nodulation. However, *Phaseolus vulgaris* (common bean), which forms determinate nodules (Martínez-Romero 2003), has three *Ern* genes, two of which encode proteins closer to *M. truncatula* ERN1 than ERN2. In the phylogenetic analysis, ERN1 and ERN2 are widespread among land plants. Members of genera *Arabidopsis*, *Vitis*, *Ricinus*, *Oryza*, and *Sorghum* have one and members of genera *Brassica*, *Populus*, and *Solanum* have three ERN1 homologs (Fig. 1A). In contrast, only plants belonging to the Leguminosae (Fabaceae) family are represented in the ERN3 group. This analysis suggests that a common AP2/ERF transcription-factor gene has undergone gene duplications in several land plants and that the *Ern3* gene may have undergone neo-functionalization in legumes.

Characterization of *L. japonicus ern1* mutant phenotypes.

The *L. japonicus* *Ern1* gene, composed of one exon, is located on chromosome 1 and encodes a conceptual protein of 269 amino acids, corresponding to approximately 30.3 kDa. To characterize the symbiotic phenotype of *ern1* mutants, four monogenic recessive mutant alleles, *ern1-1*, *ern1-2*, *ern1-3*, and *ern1-4*, were obtained from the LORE1 mutant resource (Fukai et al. 2012; Małolepszy et al. 2016; Urbanski et al. 2012) (Fig. 2A; Supplementary Table S1). Gifu wild-type plants start forming nodule primordia 7 days postinoculation (dpi) with *Mesorhizobium loti* R7A. At 10 dpi, we observed three to five pink nodules per plant (Fig. 2C). In comparison, none of the four *ern1* mutants had developed any nodule primordia at 14 dpi. However, *ern1* mutants developed nodule primordia by 21 dpi. At 28 dpi, *ern1* mutants had one to three pink nodules, approaching the developmental stage that was reached by Gifu at 14 dpi (Fig. 2B and C; Supplementary Fig. S1). To further investigate the role of ERN1 in nodule organogenesis, we assayed the effect of expressing the *snf1* autoactive CCaMK (T265D) in transgenic roots. Of 17 *ern1-2* transgenic root systems transformed with a *pUbi:CCaMK* (T265D) construct, none developed spontaneous nodules (Supplementary Fig. S2A through D). In contrast, 11 of 12 transgenic Gifu roots transformed with *pUbi:CCaMK* (T265D) formed approximately 10 spontaneous nodules per root. To support this result, we tested the response of exogenous cytokinin in *ern1* mutants. No cytokinin-inducible nodule formation was observed in *ern1-1* and *ern1-2* mutants and Gifu plants treated with 10^{-8} M benzylaminopurine developed nodule primordia as previously observed (Heckmann et al. 2011). These results suggest that ERN1 promotes nodule organogenesis and that this function is required for the spontaneous nodule development driven by the autoactive CCaMK and cytokinin signaling. The absence of spontaneous nodules on 23 *nfr1-1* plants transformed with *pUbi:Ern1* suggests that ERN1 promotion of organogenesis requires a combination of transcription factors.

Ethylene influences the number of nodules in *L. japonicus* (Nukui et al. 2000) and several ERF-type transcriptional factors are controlled by ethylene signaling (Huang et al. 2016). The effect of an ethylene inhibitor (aminoethoxyvinylglycine [AVG]) and an ethylene precursor (1-aminocyclopropane-1-carboxylic acid [ACC]) on the nodulation of *ern1* mutants was investigated. A comparable effect was detected in inoculated *ern1* mutants and Gifu plants. AVG enhances nodulation whereas ACC reduces nodulation in both Gifu and *ern1* mutants (Fig. 2C). This indicates unchanged ethylene sensitivity in the *ern1* mutants.

ERN1 is required for development of root-hair infection threads.

When examined 7 dpi, curled root hairs, infection pockets, and infection threads containing rhizobia were observed in Gifu plants, whereas root-hair curling and infection pockets were rarely found in *ern1* mutants (Fig. 2E and F). Approximately 50 infection threads per root, including short and long infection threads, were observed in Gifu plants at 7 dpi, whereas no infection threads were observed in *ern1-1* and *ern1-3* mutants. One short infection thread, which reached halfway down the length of the root hair, was observed in one of 13 *ern1-2* mutant plants (Fig. 2D). The root-hair responses of *ern1* mutants are closer to those of *nsp1* and *nsp2* mutants (Heckmann et al. 2006) than to those of *nin* mutants, which show excessive root-hair responses in *L. japonicus* (Schauser et al. 1999) (Fig. 2F). The *L. japonicus ern1* infection-thread phenotype is also more comparable to that of the *M. truncatula ern1 ern2* double mutant than the *M. truncatula ern1* single mutant (Cerri et al. 2016).

Irregular peg-type infection of *ern1* mutants.

Microscopy of the pink nodules that developed in *ern1* mutants at 28 dpi revealed that bacteria were endocytosed into the nodule cells and had differentiated into bacteroids (Fig. 3C, E, and I). Closer inspection of 10 different nodule sections revealed an absence of normal transcellular cortical infection threads in *ern1* mutant nodules. Only irregular peg-type infections were found in these nodules (Madsen et al. 2010) and, most likely, they release rhizobia into the infected cells of *ern1* mutants (Fig. 3E and F). Rhizobia were also found between the plant cells in *ern1* nodules (Fig. 3H and I). Supporting these observations, crack entry sites were found adjacent to curled root hairs (Fig. 3G). These results suggest that ERN1 is required for and involved in infection-thread formation in the root epidermis and in cortical root nodules.

Complementation of *ern1* mutant with *L. japonicus* Ern1 and *Arabidopsis* Rap2.11.

The phylogenetic tree suggested that *Arabidopsis* RAP2.11 is a homolog of *L. japonicus* ERN1 and, previously, RAP2.11 was suggested as the ortholog of *M. truncatula* ERN1 (Kim et al. 2012). The AP2/ERF domain, which directly binds to promoter elements, is also well-conserved between *Arabidopsis* RAP2.11, *L. japonicus* ERN1, and *M. truncatula* ERN1 (Fig. 1B). In order to compare the function of *Arabidopsis* Rap2.11 and *L. japonicus* Ern1, comparable gene constructs were transformed into Gifu and *ern1-2* mutants. The *pUbi:Ern1* construct complemented *ern1-2* and infection threads were formed and effective nodules appeared 2 and 3 weeks postinoculation (wpi) with *M. loti* MAFF3030399 DsRed (Supplementary Fig. S3A

Fig. 1. Phylogeny of ERN (ethylene-responsive factor [ERF] required for nodulation) transcription factors in plants. **A**, Phylogenetic tree of ERN proteins from monocot and dicot plants. *Lotus japonicus* ERF1 (Asamizu et al. 2008) is included as an outgroup. The ERN1 group including *Medicago truncatula* ERN2 (top) and the ERN3 group (bottom). **B**, The alignment of AP2/ERF domains from *Arabidopsis thaliana* RAP2.11, *Lotus japonicus* ERN1, and *M. truncatula* ERN1.

and B). Interestingly, Gifu and *ern1-2* transformed with *pUbi:Ern1* express *Ern1* at levels 30-fold higher than that in roots transformed with the empty vector, and a reduction of nodule numbers in Gifu, reflecting a partial complementation of *ern1-2*, was observed. The *pUbi:Rap2.11* enhanced nodulation at the 2-wpi timepoint but any later effect was insignificant. These observations support the *ern1* mutant characterization.

Ern1 is not required for arbuscular mycorrhiza (AM) symbiosis.

Previous studies have shown that several genes serve a function in symbiosis with AM fungi and in root-nodule symbiosis (Kouchi et al. 2010). The AM symbiotic phenotype of *ern1* mutants was, therefore, tested with *Rhizophagus intraradices*. No difference was observed between Gifu and *ern1-2* mutants, although the *ccamk-13* mutants used as a negative control showed the expected absence of AM symbiosis. Corroborating this result, AM symbiosis did not induce *Ern1* expression, measured using *pErn1:GUS* activity in transgenic roots (Supplementary Fig. S4). These data suggest that *L. japonicus*

ERN1, like *M. truncatula* ERN1 and ERN2 (Cerri et al. 2016), has a specific function in root-nodule symbiosis and is unlikely to play a major role in AM symbiosis.

Expression of Ern1.

The symbiotic phenotype of *ern1* mutants showed the involvement of ERN1 in both infection-thread and nodule development. To investigate this at the cellular level, the spatiotemporal expression pattern was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and a *pErn1:GUS* reporter gene. Increased transcript levels of *Ern1* were detected in Gifu plants at 8 and 24 h postinoculation (hpi) with *M. loti* R7A (Fig. 4M), whereas *Ern1* upregulation was undetectable in Gifu plants inoculated with a *M. loti nodC* mutant unable to synthesize Nod factor (Fig. 4N). Similarly, *Ern1* induction was not detected in *M. loti* R7A-inoculated *nfr1-1* and *nfr5-2* mutants lacking the Nod factor receptor (Fig. 4N). In contrast, treatment of Gifu with purified Nod factor at 10^{-8} M induces rapid *Ern1* expression (Fig. 5). The cellular localization of expression was determined using a reporter

Fig. 2. Symbiotic phenotypes of *ern1* mutants inoculated with *Mesorhizobium loti*. **A**, Schematic gene model of *Ern1* and the position of LORE1 retrotransposon insertions in *ern1* mutants. **B**, Symbiotic phenotype of *ern1* mutants 28 days postinoculation (dpi) with *M. loti* R7A. From left, two plants of Gifu, *ern1-4*, *ern1-1*, *ern1-3*, and *ern1-2*. Panels at the bottom show images of nodule and nodule primordia in Gifu and *ern1-2*. Red triangles indicate pink nodules. **C**, Nodulation kinetics of Gifu and *ern1-2* mutants inoculated with *M. loti* R7A with and without 3.5 μ M aminoethoxyvinylglycine (AVG) or 1.0 μ M 1-aminocyclopropane-1-carboxylic acid (ACC). **D**, Number of infection threads formed 7 dpi with *M. loti* R7A DsRed in Gifu, *ern1-1*, *ern1-2*, and *ern1-3*. **E**, Infection threads formed in Gifu and defective infection threads and excessive root-hair curling in the *ern1-2* mutant. **F**, Infection threads and root-hair deformation 7 dpi with *M. loti* R7A DsRed in Gifu, *ern1-2*, and *nin-2* mutants. Red arrows indicate infection thread, and white arrows show root-hair curling in Gifu and excessive root-hair curling in the *nin* mutant. Two asterisks (**) ($P < 0.01$) indicate significant differences comparable to total number of nodules or infection threads in Gifu plant. Bars are 1 cm and 0.5 mm in B, 20 μ m in E, and 100 μ m in F.

gene, *pErn1::GUS*, with a 2.2-kb *Ern1* promoter fused to a β -glucuronidase (GUS) reporter. Expression in uninoculated transgenic roots was barely detectable, whereas a high level of GUS activity was found in epidermal cells and root hairs of the susceptible zone at 3 dpi (Fig. 4B, F, and J). Between 5 and 10 dpi, GUS activity was detected in cortical cells initiating nodule primordia formation and in young nodules (Fig. 4C, D, G, H, K, and L). At this stage of nodule development, *pErn1::GUS* expression was decreasing in epidermal cells (Fig. 4D).

Genetic position of *Ern1*.

The arrested infection-thread formation phenotypes in *ern1* mutants are reminiscent of phenotypes observed in *cyclops*, *nsp1*, *nsp2*, and *nin* mutants (Heckmann et al. 2006; Schauser et al. 1999; Yano et al. 2008). In order to position *Ern1* in the genetic hierarchy of these transcription factors, we tested expression in the corresponding loss-of-function mutants 12 h after treatment with 10^{-8} M Nod factor. Induction of *Ern1* was *Nfr1* dependent, and normal *Ern1* transcript levels were found in *nin-2* and *nf-ya1* mutants, whereas an intermediate induction was detected in *cyclops-2*, *nsp1-1* and *nsp2-3* mutants (Fig. 5). Corroborating this result, *pErn1::GUS* expression following *M. loti* inoculation was indistinguishable in epidermal cells of Gifu, *nin-2*, and *nin-7* mutants. In contrast, *pErn1::GUS* expression was undetectable in *nfr1-1*, *nsp2-3*, and *cyclops-2* mutants, although expression was found in epidermal cells of *nsp1-1* mutant at 5 dpi with *M. loti* (Supplementary Fig. S5). At later timepoints, when *cyclops-2* and *nsp1-1* mutants had formed

nodule primordia, *pErn1::GUS* expression was detected in the cortical nodule primordia. This result suggests that *Cyclops* is necessary for *Ern1* induction in the epidermis but not in the cortex. *pErn1::GUS* expression was also found in *snf2*-induced spontaneous nodules.

Downstream components.

Given the phenotype of *ern1* mutants and the role of AP2/ERF proteins as transcription factors, we examined the *L. japonicus* transcriptome for genes acting downstream of ERN1. Candidates were found among genes from a transcriptome study (Høgslund et al. 2009), and genes induced 1 to 7 dpi with *M. loti* but not by the *M. loti nodC* were selected (Supplementary Fig. S6; Supplementary Table S2). The following set was investigated further: *Chi4*, encoding a legume-specific chalcone isomerase involved in flavonoid synthesis (Shimada et al. 2003); *Pub1a* and *Pub1b*, homologs of *M. truncatula Pub1* encoding a U box-dependent E3 ubiquitin ligase (Mbengue et al. 2010); and *Puch/ERF086*, a homolog of *Arabidopsis Puchi* encoding an AP2 transcription factor (Hirota et al. 2007; Kazusa DNA Research Institute et al. 2000). RT-qPCR showed that *Chi4*, *Pub1a*, and *Pub1b* were induced 4 to 8 hpi with *M. loti* R7A, and *Puchi* was induced 12 to 24 hpi (Supplementary Fig. S7A). Induction of all these genes depends on *Nfr1*, *Nfr5*, and Nod factor. Distinct expression patterns were detected among this gene set. *Chi4*, *Pub1a*, and *Pub1b* were expressed early, whereas *Puchi* was expressed at later timepoints. The gene dependencies of these two patterns were, therefore, investigated in *L. japonicus* loss-of-

Fig. 3. Transcellular infection threads in the nodule. **A** to **C**, Gifu nodules at 14 days postinoculation (dpi) with *Mesorhizobium loti* R7A. **D** to **I**, *ern1-2* (**D**, **G**, **E**, **H**) and *ern1-4* (**F**, **I**) mutant nodules at 28 dpi with *M. loti* R7A. **A** and **D**, show whole nodule sections of Gifu and *ern1* mutant stained by toluidine blue. Dark blue indicates infected cells containing bacteroids. **B** and **C**, Transcellular infection in Gifu wild-type nodules. **E** and **F**, Irregular peg-type infection in *ern1* nodules. **G**, Crack entry site adjacent to curled root hairs in *ern1* mutant. **H** and **I**, Rhizobia between nodule cells. Red arrows and stars indicate transcellular infection threads. Yellow arrows and stars show a peg-type of infection. White stars show colonization of rhizobia between nodule cells. A small black arrow shows bacteria colonization in the root-hair curling and a large black arrow shows the crack entry site. Bars show 200 μ m in **A** and **D**, 10 μ m in **B**, **E**, **G**, and **H**, 1 μ m in **C** and **I**, and 2 μ m in **F**.

function mutants at 12 h posttreatment with 10^{-8} M Nod factor (Fig. 5). Induction of *Chi4*, *Pub1a*, and *Pub1b* depends on *Nfr1*, *Nsp2* and *Ern1*, but was independent of *Nin* and *Nf-ya1*. Interestingly, *Chi4* is independent of *Cyclops* and *Pub1b* is independent of *Nsp1* but *Pub1a* depends on both *Cyclops* and *Nsp1*. These RT-qPCR results suggest that *Chi4*, *Pub1a*, and *Pub1b* expression is downstream of ERN1. In contrast, *Puchi* expression clearly depends on *Nsp1*, *Ern1*, and *Nin*.

DISCUSSION

Several transcription factors, including CYCLOPS, NSP1, NSP2, and NIN, have been characterized in both *M. truncatula* and *L. japonicus*. These transcription factors are essential for root-nodule symbiosis and CYCLOPS, NSP1, and NSP2 also play a role in AM symbiosis (Heckmann et al. 2006; Takeda et al. 2013; Yano et al. 2008). Here, we present the *L. japonicus* ERN transcription factors and characterize ERN1, using four LORE1 retrotransposon mutants. All *ern1* alleles showed the same delayed nodule formation phenotype after inoculation with *M. loti*, and further detailed analyses of *ern1* mutants revealed that root-hair infection threads and normal transcellular infection threads were absent. The *ern1-2* allele was complemented for both root-hair infection threads and nodule formation by the *Ern1* gene. This phenotype distinguishes *ern1* mutants from other *L. japonicus* mutants with a defect in infection-thread development, such as *nap*, *pir*, and *cerberus* (Yano et al. 2009; Yokota et al. 2009). These mutants develop excessive numbers of small, uninfected nodule primordia, whereas *ern1* mutants form a reduced number of nodules infected by crack entry after a lag period. This phenotype suggests that ERN1 is required for infection-thread formation. Previous results showed that ERN1 is required for expression of the EPR3 exopolysaccharide receptor that controls infection-

thread formation and that lack of *Epr3* gene expression in the *ern1* mutants is likely to contribute to the infection-thread phenotype (Kawaharada et al. 2017). In the nodule developmental process, ERN1 has a function in promoting organogenesis, with *ern1* mutants developing primordia with a delay. The suggested function of ERN1 in nodule organogenesis is also supported by the lack of spontaneous nodules in *ern1* roots transformed with the *snf1* autoactive CCaMK (T265D) and the absence of cytokinin-induced primordia. These data suggest that ERN1 promotes organogenesis rather than being strictly required in the process.

As shown in Figure 4B and D, *M. loti* inoculation rapidly induces *Ern1* expression in epidermal cells. Later on, this epidermal expression fades out when nodule primordia develop from cell divisions in the cortex. It may be important that *Ern1* expression follows this developmental pattern and is decreased with proper timing. Supporting this suggestion, constitutive overexpression of *Ern1* from the *pUbi:Ern1* construct results in a reduced nodule number in Gifu control plants and, in *ern1-2*, the normal number of nodules was not obtained. These data suggest that *Ern1* may function both positively and negatively, similar to NIN (Cerri et al. 2016; Vernié et al. 2015; Yoro et al. 2014).

In *M. truncatula*, both ERN1 and ERN2 contribute to rhizobial infection and nodule organogenesis (Cerri et al. 2016) and ERN1 is up-regulated in root-nodule symbiosis and ERN2 is up-regulated in AM symbiosis (Hogekamp and Kuster 2013; Hogekamp et al. 2011). From complement analysis, it has been suggested that, during evolution, *M. truncatula* ERN1 was duplicated from ERN2 to serve for rhizobial interaction (Vanneste et al. 2014; Young et al. 2011). We did not find an *Ern2* homolog in *L. japonicus*, although involvement of an unidentified AP2/ERF transcriptional factor that overlaps the function of *M. truncatula* ERN2 cannot be excluded (Handa et al. 2015). Another difference is that *L. japonicus* *Ern1* is only

Fig. 4. *Ern1* expression during symbiosis. A to L, Gifu plants transformed with *pErn1:GUS* at 0 to 10 days postinoculation (dpi) with *Mesorhizobium loti* R7A. A, E, and I, Uninoculated transgenic roots, and roots at B, F, and J, 3 dpi, C, G, and K, 5 dpi, and D, H, and L, 10 dpi. M, The relative expression of *Ern1* 8 or 24 hpi with *M. loti* R7A on Gifu (M), and N, 24 hpi with *M. loti* R7A or *nodC* mutant on Gifu, *nfr1-1* or *nfr5-2*. These graphs show *Ern1* expression levels relative to H₂O treatment in each genotype. The relative expression ratio for the three biological and technical replicates and error bars represent the 95% confidence interval. Scale bars are 0.5 mm in A to H and 50 μ m in I to L.

induced by rhizobia and not by AM symbiosis (Handa et al. 2015). In addition, the delayed nodule primordia of *L. japonicus ern1* mutants are infected by rhizobia via an alternative crack entry mechanism. Previous research found that *M. truncatula* ERN1 and ERN2 bind directly to the *Enod11* promoter region at the NF binding box (Andrianakaja et al. 2007; Cerri et al. 2012). Recent results have shown that the *pEpr3::GUS* expression in the

epidermal cells depends on *Ern1* and an ERN1 binding site (GCCGTC) in the *Epr3* promoter region, similar to the original AP2/ERF (GCCGCC) and RAP2.11 (GCCGTC) binding sites, was delimited (Kawaharada et al. 2017; Kim et al. 2012). These AP2 binding sites are also present in the promoter regions of the downstream *Pub1a*, and *Pub1b* genes (Supplementary Table S3). However, no NF binding box or AP2 binding sites

Fig. 5. Gene expression in symbiotic mutants. The relative expression of *Ern1*, *Nf-ya1*, *Chi4*, *Pub1a*, and *Pub1b* 12 h after treatment with 10⁻⁸ M Nod factor in Gifu, *nfr1-1*, *nsp1-1*, *nsp2-3*, *ern1-2*, *nin-2*, or *nf-ya1* mutants. These graphs show gene expression levels relative to H₂O treatment. The relative expression ratio for the three biological and technical replicates and error bars represent the 95% confidence interval. N. D. is not detected gene expression.

Fig. 6. Model of the genetic pathway required for *Ern1* induction. In the epidermis, Nod factor induces the symbiotic signal pathway through direct interaction with NFR1 and NFR5 receptors. Signal transduction through the common symbiotic pathway activates CCaMK-CYCLOPS and NSP2 (NSP1). CCaMK-NSP2 (NSP1) and CCaMK-CYCLOPS subsequently induce *Ern1*.

were identified in the 2.1-kb *Chi4* putative promoter regions. The different behavior of ERN transcription factors between *M. truncatula* and *L. japonicus* is also reflected in the dependencies for gene expression. Laloum et al. (2014) suggested *M. truncatula* has two induction pathways regulating *Ern1*. One is via the NF-YA1 transcription factor that is downstream of NIN and directly controls *Ern1* expression. The other pathway involves NSP1/NSP2 transcription factors that also induce *Ern1* expression (Cerri et al. 2012; Laloum et al. 2014). Supporting this pathway, *M. truncatula* NSP1 was observed to bind directly to the *Ern1* promoter (Hirsch et al. 2009). In *L. japonicus*, our data suggest similar *Ern1* expression in *nin* and *nf-ya1* mutants and Gifu wild type after Nod factor treatment (Fig. 5). Likewise, *pErn1::GUS* induction in the epidermis is independent of *Nin*. These expression data and phenotypic observations suggest that ERN1 may be positioned differently in the transcription-factor hierarchy of epidermal cells of *M. truncatula* and *L. japonicus*.

Based on our RT-qPCR and promoter GUS data, we propose a model of the transcription-factor hierarchy acting downstream of CcAMK in the epidermis (Fig. 6). Calcium spiking activates CcAMK and, subsequently, NSP2 and maybe also NSP1 are activated in one branch and CYCLOPS in another (Yano et al. 2008). Both of these pathways are needed for ERN1 and NIN to support *Nf-ya1* induction (Fig. 5). Supporting this dual regulation of *Ern1*, putative CYCLOPS-binding sites (underlined in the following sequences: TGGCAAGCATGGCT and TGGCAGTCGTTCA TGCCT) were found in the *Ern1* promoter region. Future experiments are needed to investigate the interactions of ERN1 and NIN in the epidermis and cortex as well as the regulation of the downstream components identified in this study.

MATERIALS AND METHODS

Plant material and growth conditions.

Lotus japonicus ecotype Gifu (Handberg and Stougaard 1992) was used as the wild-type plant. The *ern1* and *nf-ya1* mutants were LORE 1 retrotransposon insertions (Fukai et al. 2012; Urbanski et al. 2012) (Fig. 1). *nfr1-1*, *nfr5-2*, *nsp1-1*, *nsp2-3*, *cyclops-2*, *nin-2*, and *nin-7* mutants were previously described (Heckmann et al. 2006; Madsen et al. 2003, 2005; Radutoiu et al. 2003; Schauser et al. 1999; Yano et al. 2008). Seed sterilization and plant-growth setups were carried out as previously described (Kawaharada et al. 2015). Plants were grown at 21°C with day and night cycles of 16 and 8 h, respectively. Plants were inoculated with bacterial suspensions at an optical density at 600 nm = 0.02, if necessary.

Bacterial strains and growth conditions.

Wild-type strains *Mesorhizobium loti* R7A (Kelly et al. 2014; Sullivan et al. 2002) and MAFF303099 (Kaneko et al. 2000) along with the *nodC* mutant were grown in tryptone yeast and yeast mannitol broth medium at 28°C. The construction of DsRed-tagged strains used in microscopy experiments has previously been described (Kawaharada et al. 2015; Kelly et al. 2013; Maekawa et al. 2009). *Agrobacterium rhizogenes* AR1193 strains (Hansen et al. 1989; Stougaard et al. 1987) were used for all hairy root transformation experiments, according to previously described methods (Petit et al. 1987). The following concentrations of antibiotics were used for *M. loti* and *Agrobacterium* strains: ampicillin, 100 µg ml⁻¹; tetracycline, 2 to 10 µg ml⁻¹; gentamicin, 50 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; and spectinomycin, 100 µg ml⁻¹.

AM symbiosis.

Seedlings were grown in the dark for 5 days and, then, after germination, in day and night cycles of 16 and 8 h, respectively, for 8 days. They were sandwiched within nitrocellulose

membranes (GSWP, 0.22 µm; Merck Millipore), together with 50 spores of *Rhizophagus irregularis* (Symbion Ltd.) in Magenta boxes containing sand (Muregrus 0 to 4 mm; Solid Products, Inc.) (Giovannetti et al. 1993). These were supplied with 50 ml of Long Ashton solution (0.75 mM MgSO₄, 1 mM NaNO₃, 1 mM K₂SO₄, 2 mM CaCl₂, 3.2 µM Na₂HPO₄, 25 µM FeNa-EDTA, 5 µM MnSO₄, 0.25 µM CuSO₄, 0.5 µM ZnSO₄, 25 µM H₃BO₃, 0.1 µM Na₂MoO₄). Plants were grown at 21°C with day and night cycles of 16 and 8 h, respectively, for 3 weeks, after which roots were scored for AM colonization by ink staining as previously described (Małolepszy et al. 2015).

Light and transmission electron microscopy (TEM).

Nodule sections for light and TEM were prepared using previously described methods (Madsen et al. 2010). Semithin sections (1 µm) of nodule for light microscopy and ultra-thin (70 nm) sections for TEM were cut using a Leica UCT ultramicrotome. Light microscopy was performed using a Zeiss Axioskop and TEM using a JEOL JEM 1400.

GUS staining.

L. japonicus roots were stained with 0.5 mg 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid per milliliter, 100 mM potassium phosphate buffer (pH 7.0), 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 0.1% Triton X-100. *L. japonicus* roots were incubated at 21°C overnight.

Promoter and expression constructs.

A putative *Ern1* promoter region of 2,178 bp and a full-length *Ern1* in *L. japonicus* and a *Rap2.11* in *Arabidopsis* genes were amplified from *L. japonicus* Gifu or *A. thaliana* Col-0 genomic DNA by PCR, using primers containing *attB* sites and recombined into *pDONR207* (Invitrogen), using the Gateway BP reaction (Invitrogen) to create the entry clones *pDONR207::pErn1*, *pDONR207::Ern1*, and *pDONR207::Rap2.11*. Entry clones were recombined using Gateway LR reactions (Invitrogen) with destination vector *pIV10::GW::GUS* and *pCAMBIA1300::Ljubiquitin promoter::GW* (Maekawa et al. 2008) to create the *pIV10::pErn1::GUS*, *pCAMBIA1300::Ljubiquitin promoter::Ern1*, and *pCAMBIA1300::Ljubiquitin promoter::Rap2.11* constructs.

RT-qPCR gene expression analysis.

For gene expression analysis, 10 plants were grown on 1/4 Broughton and Dilworth plates and were either inoculated with *M. loti* R7A or water, or were treated with 10⁻⁸ M Nod factor or water within 24 h, or three transgenic hairy root plants were inoculated at 3 wpi with *M. loti* MAFF303099 before the roots were harvested. Three biological replicates were performed for each combination of mutant and timepoint. mRNA extraction, quality control checks, and gene expression analysis using a Lightcycler 480 instrument (Roche) was performed as previously described (Kawaharada et al. 2015). Primer sets and conditions are shown in Supplementary Table S4.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Lotus Base database: <https://lotus.au.dk>