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LIN, novel type U-box protein in symbiosis

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LIN, a novel type of U-box/WD40 protein, controls early infection by rhizobia in legumes

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ABSTRACT

The formation of a nitrogen fixing nodule requires the coordinated development of rhizobial colonization and nodule organogenesis. Based on its mutant phenotype \( \text{LIN} \) functions at an early stage of the rhizobial symbiotic process, required for both infection thread growth in root hair cells and the further development of nodule primordia. We show that spontaneous nodulation activated by the calcium- and calmodulin-dependent protein kinase (CCaMK) is independent of \( \text{LIN} \), thus \( \text{LIN} \) is not necessary for nodule organogenesis. From this we infer that \( \text{LIN} \) predominantly functions during rhizobial colonization and the abortion of this process in \( \text{lin} \) mutants leads to a suppression of nodule development. Here, we identify the \( \text{LIN} \) gene in \textit{Medicago truncatula} and \textit{Lotus japonicus}, showing it codes for a predicted E3 ubiquitin ligase containing a highly conserved U-box and WD40 repeat domains. Ubiquitin-mediated protein degradation is a universal mechanism to regulate many biological processes by eliminating rate-limiting enzymes and key components such as transcription factors. We propose that LIN is a regulator of the component(s) of the Nod factor signal transduction pathway and its function is required for correct temporal and spatial activity of the target protein(s).
INTRODUCTION
The soil bacteria rhizobia are able to establish nitrogen-fixing symbioses with leguminous plants by inducing the formation of a new organ, the nodule, on the roots of the host plant. Symbiotic infection is initiated and maintained by an exchange of signaling molecules between the host plant and the microsymbionts. In most legumes, flavonoid compounds produced by leguminous plants activate bacterial regulators to induce nodulation (nod) genes required for the synthesis of lipochitooligosaccharide Nod factors. Nod factors initiate multiple early responses on plant hosts including a burst of intracellular calcium levels in root hairs, calcium oscillations (calcium spiking) and the induction of nodulation specific genes whose products are referred to as ‘nodulins’. In addition, they induce a rearrangement of the root hair cytoskeleton leading to root hair deformation and curling, which traps surface-attached rhizobia, establishing a site that acts as an infection focus. Bacteria penetrate into the curled root hairs towards the root cortex via host-derived tubular structures, called infection threads. Simultaneously, Nod factors stimulate the reinitiation of mitosis in cortical cells, leading to the formation of nodule primordia, which give rise to the cells that receive the invading bacteria (Oldroyd and Downie 2008). Exopolysaccharides (EPS), capsular polysaccharides (K antigens or KPS), and lipopolysaccharides (LPS) serve as further bacterial signals required for successful infection of nodules in many legumes (Jones et al., 2007). The infecting rhizobia are released into intracellular membrane compartments of plant origin called symbiosomes where they differentiate into bacteroids capable of reducing atmospheric nitrogen to ammonium, which is provided to the plants in exchange for carbon and amino acid compounds (Brewin, 2004).

Analysis of nodulation-defective *Medicago truncatula*, *Lotus japonicus* as well as *Pisum sativum* mutants has led to insights into the mechanisms by which Nod factors are perceived and trigger subsequent signal transduction cascades (Geurts et al., 2005; Stacey et al., 2006; Oldroyd and Downie, 2008). In *M. truncatula* and *L. japonicus*, the Nod factor signal is probably sensed by LysM-type receptor kinases such as NFP/NFR5 (Amor, et al., 2003; Radutoiu, et al., 2003) and LYK3/NFR1 (Smit et al., 2007; Radutoiu, et al., 2003). Another receptor-like kinase known as NORK/DMI2/SYMRK (Endre et al., 2002a; Stracke et al., 2002) is also involved in the transmission of the Nod factor signal. Downstream in the pathway, DMI1/POLLUX and CASTOR (Ane et al., 2004; Imaizumi-Anraku et al., 2005), putative ligand-gated ion channels (Charpentier et al., 2008), nucleoporins NUP133 and NUP85 (Saito et al., 2007), a Ca$^{2+}$-calmodulin dependent protein kinase (CCaMK) (Levy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006a) and a protein of unknown function, Cyclops, (Yano et al., 2008) are necessary for the activation of transcriptional regulators
such as NSP1 (Smit et al., 2005; Heckmann et al., 2006), NSP2 (Kalo et al., 2005; Heckmann et al., 2006) and ERN1 (Middleton et al., 2007) responsible for the transcriptional changes that are required for the initiation of nodule morphogenesis. In addition to the signaling pathway outlined above, legumes have many genes required to enable rhizobia to infect the roots. Only a few of these genes have been identified; the RPG gene required for infection was identified but the function of its product has yet to be defined (Arrighi et al., 2008). Some genes such as NIN and ERN encode predicted regulators that may affect both the Nod-factor signaling and infection pathways (Schauer et al., 1999, Marsh et al., 2007, Middleton et al., 2007), and the hcl mutation (in MtLYK3 gene) affecting a predicted Nod-factor receptor also causes an infection defect (Smit et al., 2007). The NAP and PIR genes are required for normal infection and their loss affects polar growth of some cell types and probably are required for the polar growth of the infection thread (Yokota et al., 2009). The NAP and PIR proteins have been identified as components of the SCAR/WAVE complex, which is involved in polymerization of actin-related proteins (Li et al., 2004).

The lin mutant (C88) was identified as a M. truncatula mutant, in which infection was arrested and reduced to a quarter of the frequency seen in wild-type plants (Penmetsa and Cook, 2000; Kuppusamy et al., 2004). In those cases when infections were initiated, bacteria were arrested after very limited progression within root hairs. Although nodule primordia were formed in the root cortex, differentiation was arrested at an early stage. Interestingly, infection and initiation of cortical cell division recurred continually in the presence of rhizobia, suggesting that lin is required for appropriate regulation of nodule initiation, and for control of nodule number (Kuppusamy et al., 2004). The early nodulins (RIP1, ENOD20 and ENOD40) were expressed in lin at comparable levels to wild-type, while late nodulins such as MtN6, ENOD2 and ENOD8 failed to be induced in lin. This suggests that LIN acts downstream of the early Nod factor signal transduction pathway, but is required for infection initiation and persistence and nodule differentiation (Kuppusamy et al., 2004). Based on the phenotype of the mutant the LIN function is probably needed either in the process of nodule development/maturation – with indirect effect on the block of infection thread growth or in the invasion process resulting in the interruption of nodule development. It is also possible that LIN acts at both levels as a synchronizing regulator between the parallel processes in the root epidermis and the cortex.

In this work we show the identification of the LIN gene revealing that LIN encodes a large protein containing multiple domains including a U-box (modified RING) domain, indicating a function as an E3 ubiquitin ligase. Spatiotemporal analysis of the promoter
activity of LIN demonstrated that the expression of the gene correlated with the early nodule primordia formation and bacterial invasion during the symbiosis. We used a gain-of-function mutation in CCaMK that induces spontaneous nodulation in the absence of rhizobia to show that LIN is not required for nodule organogenesis. This indicates that LIN functions exclusively during rhizobial colonization and the defect in nodule development in lin is a response to aborted infection.

RESULTS

LIN is not required for nodule organogenesis

EMS and fast neutron mutagenised M. truncatula populations were screened to identify new loci required for nodulation; two of the mutants identified had phenotypes similar to lin, namely impaired nodulation and infection with the infection threads arrested in the root hair cells. Though nodule primordia emerged three to four days after inoculating the roots with wild type rhizobia, nodule development was always blocked before the stage of nodule differentiation. No non-symbiotic phenotype was detected in these mutants. Genetic crosses revealed that the two mutants (EMS6:T7 and 14P) carried mutations allelic to lin-1 (Table I), but not allelic to the other nodulation mutations tested (data not shown). EMS6:T7 was derived from EMS mutagenesis and will be defined as lin-2, while 14P was derived from fast neutron mutagenesis and will be defined as lin-3.

To determine if LIN is required for nodule organogenesis we tested whether an autoactive form of CCaMK could induce spontaneous nodulation in lin mutants. In M. truncatula, transgenic expression of an autoactive CCaMK (DMI31–311), comprised of the kinase domain alone, is capable of inducing spontaneous nodules and has been useful in determining the order of gene product function within the early NF signaling pathway relative to CCaMK (Gleason et al., 2006). When we transformed lin-1, lin-2 and lin-3 mutants with this autoactive CCaMK construct, we could clearly detect nodules on the lin-1 (3/21 plants), lin-2 (9/19 plants) and lin-3 (40/103 plants) mutants. The observation that 52 nodules were induced on 143 lin mutant plants transformed with autoactive CCaMK suggests that LIN is not essential for nodule organogenesis and therefore the defects in nodule development observed in the lin mutants during rhizobial invasion is likely to be a result of the abortion of bacterial infection.
Positional cloning of LIN in *M. truncatula*

Preliminary mapping data previously positioned *lin-1* (C88) between molecular markers DSI and SCP on the lower arm of linkage group 1 (LG1) within a relatively large distance of several cM (Kuppusamy et al., 2004). Using newly generated markers and a new segregating population of 290 F2 individuals and 508 F3 individuals of selected F2 plants, we mapped *LIN* within the region on LG1 between markers 4E6R and e53J20F (Fig. 1. A). This region is spanned by the sequenced BACs: mth2-69D21, mte1-13O17 and mte1-53J20 (Fig 1. A, B). Within this region there were numerous good candidates for *LIN* among the predicted genes including genes encoding AP2 transcription factors, a bZIP transcription factor, a protein kinase, a putative RING zinc finger protein and genes with unknown function, but represented with nodule expressed sequence tags (ESTs) in the *Medicago* database. No mutation was detected in *lin-1* for any of these candidate genes.

Further sequencing of this region in *lin-1* and comparisons with the sequence from wild-type revealed a single nucleotide difference in *lin-1* in the predicted coding region MTCON310-47 (indicated by the star in Fig. 1B). Since no EST had been reported for this gene, we validated that this region was actively transcribed in *M. truncatula*. PCR amplification products could easily be obtained from plant cDNA isolated from roots four days after *S. meliloti* inoculation. The cDNA of MTCON 310-47 could be assembled from the sequences of overlapping fragments. The intron/exon boundaries were similar to what had previously been predicted for the genomic sequence, with the exception that exon 12 was found to be 24 bp longer (indicated by an open triangle in Fig. 1C) resulting in a predicted total protein of 1488 amino acids. The mutation we identified in *lin-1* was in the last nucleotide position of intron 4 (indicated by an arrow in Fig. 1C). RT-PCR amplification of the affected region using primers Lin-3F (in exon 3) and Lin-3R (in exon 5) on *lin-1* RNA samples of inoculated roots revealed that indeed there was an error in the RNA splicing resulting in a longer transcript (Fig. 2A). Sequencing of this longer fragment showed that due to the mutation intron 4 was not spliced out during mRNA processing (while correct splicing of intron 3 was detected in the same fragment), causing a premature stop codon to be introduced. Amplification of all other parts of the *lin-1* cDNA resulted in the expected fragment sizes (e.g. the fragment amplified by Lin-8R and Lin-8R primers in Fig. 2A).

Sequencing of the genomic DNA amplified from the *lin-2* mutant revealed a point mutation in the first exon that appears at nucleotide position 662 in the cDNA sequence, introducing a premature stop codon into MTCON 310-47 very early in this gene (indicated by an arrow in Fig. 1C). In addition, Southern analysis of *lin-3* revealed an apparent large
deletion or rearrangement in this same gene (Fig. 2B). RT-PCR experiments on lin-3 mutant demonstrated the presence of a short mRNA (1568 nucleotide) transcribed from this allele (indicated by an arrow in Fig. 1C). Another mutant line, C105 originating from the same population (Penmetsa and Cook, 2000) and having a similar phenotype to C88 (lin-1) is allelic with C88 (R. Dickstein, personal communication); sequencing of MTCON 310-47 in C105 revealed the same mutation, suggesting that C88 and C105 are siblings. The identification of mutation events in three different alleles of lin provides strong evidence that MTCON 310-47 is indeed LIN.

**Complementation of the Medicago lin mutants on transgenic hairy roots**

To confirm that MTCON 310-47 corresponds to LIN, we tested complementation of the lin alleles with a construct carrying MTCON 310-47 expressed off the 35S promoter and also harboring a constitutively expressed GFP reporter gene to facilitate the identification of the transgenic roots. The lin mutants were transformed using Agrobacterium rhizogenes mediated hairy root transformation and transgenic hairy roots were detected by the fluorescent GFP signal (Fig. 3A). For screening the symbiotic phenotype of the roots, wild type symbiotic bacteria were applied on the well-grown root systems following one week of nitrogen starvation. Complementation of the symbiotic phenotype of all three lin mutant alleles was observed, indicated by the formation of mature nodules induced by S. meliloti strain 1021 carrying a hemA promoter: lacZ fusion (Fig. 3B). The lin mutant alleles do form bumps following S. meliloti inoculation, however, in these complementation experiments, we could clearly discriminate between the mature complemented nodules which were infected (Fig. 3B) and the nodule primordia bumps where infections always arrested in the root hairs on the non-transgenic roots of lin mutants or on transgenic roots induced by A. rhizogenes carrying the empty vector (Fig. 3C). Using β-galactosidase staining of the symbiotic bacteria we could clearly see the presence of rhizobia within the nodule and in infection threads ramifying throughout the nodules on complemented roots. The identification of mutations in MTCON310-47 in lin-1, lin2 and lin-3, coupled with the complementation of these mutants by this gene, proves that MTCON310-47 encodes LIN.

**Identification and trans-complementation of a L. japonicus lin mutant**

Mutants of L. japonicus with a phenotype similar to the M. truncatula lin mutant were described previously and two of the mutant loci, sym7 and itd3, map to a 10 cM region on the upper arm of chromosome 5 (Lombardo et al., 2006) in a region predicted to show synteny with the map location of LIN in M. truncatula (Choi et al., 2004). To identify a LIN-
like gene from *L. japonicus*, the TIGR database of expressed sequence tags was checked for sequences similar to *MtLIN*. The most similar *L. japonicus* EST sequence was BP053520 (E-value 8.7 e\(^{-47}\)) and two reverse primers designed from this sequence were used in combination with various *MtLIN* forward primers to amplify cDNA from *L. japonicus*. This resulted in the amplification of about 3.5 Kb of cDNA. Where the primer combinations produced multiple fragments, nested PCR was used to produce individual fragments for DNA sequencing. The 5’ end of the gene was obtained by using a *L. japonicus* GenomeWalker\textsuperscript{TM} library (Heckmann et al., 2006). The assembled *L. japonicus* cDNA sequence of 4470 bp (GenBank accession number EU926664) is 86% identical to the coding region of the *M. truncatula* LIN gene.

The *MtLIN*-like gene was amplified and sequenced from mutants SL1450-5 (carrying an allele of *sym7*) and SL1947-2 (carrying *itd3*), revealing one change, a G-A substitution at position 3799 in SL1450-5 and no change in SL1947-2. This mutation in SL1450-5 resulted in the alteration of aspartic acid 1267 (GAC) to asparagine (AAC). Roots of SL1450-5 were transformed with the same *MtLIN* construct that was used for complementation of the *M. truncatula* lin mutants. *MtLIN* complemented both nodulation (Table II and Fig. 4A) and infection (Fig. 4B) of SL1450-5 in transformed hairy roots. Normal looking pink nodules could be observed in the complemented plants indicating that they were functional; nodulation, scored only using those transgenic roots showing GFP fluorescence, was significantly different from the controls lacking *MtLIN* (Table II). This shows that (a) the *L. japonicus* mutant phenotype can be rescued by the *M. truncatula* wild type LIN gene, (b) *MtLIN* is the orthologue of *LjSYM7* and (c) the identified mutation caused the nodulation-defective phenotype of SL1450-5. A few nodules were observed on hairy roots in the negative controls (Table 2,) but microscopy revealed only a few infected cells in these nodules as had been seen previously with the SL1450-5 mutant (Lombardo et al., 2006). No nodules were observed on hairy roots of SL1947-2 (*itd3*) transformed with *MtLIN* confirming previous observations (Lombardo et al., 2006) that SL1947-2 and SL1450-5 do not carry allelic mutations.

**LIN belongs to a unique family of proteins containing domains with homology to E3 ubiquitin ligases**

Analysis of LIN revealed regions with high similarity to protein domains of known function. A U-box domain is present between residues 516 and 580 (Fig. 5). U-box domains are modified RING finger domains, without the full complement of Zn\(^{2+}\)-binding ligands and known for their E3 ubiquitin ligase activity. At the C-terminal region of the protein the
NCBI Conserved domain-search program identified a large region belonging to the WD40 superfamily (Fig. 5), while the predictions by InterProScan suggested three clear WD40 repeats with scores above the threshold from several programs (SMART, Pfam, SPRINT). According to the PANTHER Classification system (www.pantherdb.org/panther/) this part shows homology to a more general family of F-box and WD40 domain proteins (PTHR22844) trained by 71 sequences from several organisms. Between these two main identified domains a large Armadillo repeat-type region was predicted (Fig. 5) by only one protein analysis program (residues 662-1001 – InterPro-Gene3D: IPR011989 Armadillo-like helical domain). No further significant similarity to known domains was found. By comparing the predicted domain structure of LIN and the identified cDNA sequences of the three lin alleles in M. truncatula it is clear that none of the possible truncated proteins would carry the U-box, Armadillo-like and WD40 domains. The mutation identified in L. japonicus SL1450-5 mutant altered aspartic acid 1267 (GAC) to asparagine (AAC) in the second predicted WD40 repeat indicating that the WD40 motif is essential for function.

When the Conserved Domain Architecture Retrieval Tool (CDART) was used to find proteins with similar structural features, the closest group was proteins with the combination of RING finger and WD40 repeat domains (RING finger and WD40 repeat domain 3 Homo sapiens). Another set of proteins with a similar structural arrangement involves pre-mRNA processing factor 19 homologs (Bos taurus), which contain U-box and WD40 repeats, but always together with a PRP19/PSO4 domain. An additional large family includes proteins with the combination of F-box/WD40 repeat domains (like Pop1 from Schizosaccharomyces pombe). However, LIN is unusual when comparing to any of these families, as it contains a U-box instead of an F-box or RING domain and being exceptionally large with significant regions of unknown function. The structure of LIN, a large and novel N-terminal region, followed by a U-box, a probable Armadillo repeat and WD40 repeats at the C-terminal domain, appears to be unique for plants, since genes with similar structure could not be found in other organisms. Genes homologous to LIN are clearly present in poplar and grapevine, identified as being duplicated in the Populus genome (Pt LGII, accession number is NC008468, and Pt LGV, accession number is NC008471). The hypothetical protein product of the Vitis homolog is predicted under the GenBank accession number CAN74785. The amino acids of the predicted proteins are closely aligned, even the N-terminal region between residues 1-450 shows strong similarity with remarkably high conservation at positions 1-300 (Fig. 5. and Supplemental Figure 1. online). The next most similar protein to LIN was identified in rice (Os01g0229700; Fig. 5 and Supplemental Figure 2 online) and contains similar domain structure, but the level of
similarity clearly drops at the N-terminal region indicating that domain to be diverse. In the Arabidopsis genome no single homologous sequence could be identified, and no gene encoding a protein with similar domain structure was identified. Instead, two proteins show homologous regions with parts of the LIN protein, At3G06880 codes for a longer protein with WD40 repeats, while At1G23030 codes for a shorter U-box containing protein (Fig. 5. and Supplemental Figures 3. and 4. online).

**LIN promoter activity is associated with infection and primordium formation**

In order to reveal the promoter activity of the *M. truncatula* LIN gene throughout the nodulation process, a 1.2 kb segment upstream the coding region of LIN was fused to the GUS reporter gene. This pLIN-GUS construct was subsequently introduced into *M. truncatula* A17 plants by *A. rhizogenes* mediated transformation. Transformed hairy roots were monitored for GUS activity on uninfected roots, and at different time points after inoculation with *S. meliloti*.

There was no detectable signal of GUS activity throughout the most parts of the uninoculated roots, except a very faint signal just above the detection limit at the apical region (Fig. 6A). Three days after inoculation with rhizobia, GUS activity seemed to be associated with dividing cortical cells leading to the formation of nodule primordia (Fig. 6B). Six days after inoculation strong overall GUS staining was detected in the young, emerging nodules, where infection of plant cells by rhizobia takes place (Fig. 6C). In elongated mature nodules (21 days after inoculation), strong GUS activity was detected but was mainly restricted to a relatively broad area of nodule apices including the infection zone. Much lower expression was detected in the nitrogen-fixing zone (Fig. 6D).

These data indicated that the cloned promoter segment enabled a gene expression associated with the bacterial infection as well as with the cortical cell division leading to the formation of symbiotic nodules. Although this showed an activity that strongly correlated to the position where the expression of the LIN gene would be needed to recondition the mutant phenotype (i.e. early nodule primordia), to further support its activity complementation experiment was carried out using the wild type LIN cDNA driven by the same promoter segment. Fully developed nodules appeared on the transgenic roots of lin-1 mutant plants and bacterial invasion was detected in the apical zone of the nodules (Supplemental Figure 5 online). This confirmed that promoter activity of this segment ensures a LIN expression necessary for nodule development and restoration of the infection process during early nodule invasion, but not sufficient for the complete occupation of the nodule by bacteria. This suggests that the function of LIN might also be needed at later steps of nodule invasion.
DISCUSSION
The formation of a nodule requires the coordinated development of rhizobial colonization and nodule organogenesis. These two processes are coordinated both spatially and temporally to ensure rhizobial infection of the developing nodule. However rhizobial infection and nodule organogenesis can be separated genetically (Gleason et al., 2006; Murray et al., 2006; Tirichine et al., 2006a; Tirichine et al., 2006b) indicating that these two processes constitute different developmental pathways. Based on its mutant phenotype LIN functions at an early stage of the rhizobial symbiotic process, required for both infection thread growth in root hair cells and the further development of nodule primordia. The lin mutation in *L. japonicus* SL1450-5 did not affect Nod-factor-induced calcium spiking (Lombardo et al 2006). No apparent nonsymbiotic phenotypes were observed in either of the three *M. truncatula* lin mutants and colonization of the lin-1 mutant by mycorrhizal fungi is similar to that of wild type *M. truncatula* (M. Harrison personal communication). Gain-of-function mutations in CCaMK (autoactive CCaMK) allowed nodule development in the absence of rhizobial infection and the use of this mutant revealed that LIN is not necessary for nodule organogenesis. From this we infer that LIN predominantly functions during rhizobial colonization and the abortion of this process in lin leads to a suppression of nodule development.

Detailed genetic studies in *M. truncatula*, *L. japonicus* and pea have revealed an ordered array of loci functioning at different stages of nodulation (Tsyganov et al., 2002; Jones et al., 2007; Oldroyd and Downie, 2008). From studies in pea it has been proposed that three potential checkpoints for rhizobial infection exist, while only a single checkpoint exists for cortical cell division and this occurs as a result of a malfunctioning infection process. In the mutants where infection thread growth in the root hair is abolished (pea mutants *sym2*, *sym36*, *sym37*, *sym38*), nodule tissue development is aborted after nodule primordia development and before nodule meristem formation. The lin mutants show an equivalent phenotype and our work with autoactive CCaMK validates this hypothesis, indicating that the level of nodule organogenesis is dictated by the extent of infection thread invasion. Further supporting this assumption promoter-reporter gene fusion experiments revealed that expression of LIN is associated with the dividing cortical cells leading to the
formation of nodule primordia and in nodule tissues where infection of plant cells by rhizobia takes place.

The LIN protein contains many domains of which the presence of a U-box places it among E3 ubiquitin ligases, while other regions might be responsible for different interactions with target/substrate proteins or regulation of LIN by post-translational modifications. E3 ubiquitin ligases play roles in the ubiquitination of the target protein achieved by enzymatic reactions that act in concert. The substrate proteins are labelled with ubiquitin that serves as a degradation tag, in three consecutive steps catalyzed by a set of enzymes E1, E2, and E3 leading to proteolysis of target proteins by the 26S proteasome complex (Glickman and Ciechanover, 2002). This system plays a key role in the control of cellular functions as diverse as cell cycle progression, endocytosis, protein sorting, embryogenesis, hormone responses, defense against pathogens, and senescence through degradation of a wide range of proteins in the nucleus and cytoplasm (Pickart, 2001; Frugis and Chua, 2002; Vierstra, 2003; Smalle and Vierstra, 2004). In higher plants, a large gene family composed of diverse isoforms encodes the E3 ubiquitin ligases. In the Arabidopsis genome there are twelve hundred genes encoding the E3 components, whilst forty-one genes encode E2 components and only two genes encode the E1 components (Vierstra, 2003; Kraft et al., 2005). Therefore it is thought that the E3 ubiquitin ligases must play a central role in selecting the appropriate candidate proteins during the ubiquitination process (Zeng et al., 2006). On the basis of subunit composition, E3 ligases can be divided into four groups: HECT, SCF, APC and RING/U-box. However, regardless of their subunit types, E3 ubiquitin ligases are responsible for identifying proteins that should be ubiquitinated, thereby determining target specificity (Callis and Vierstra, 2000; Vierstra, 2003; Smalle and Vierstra, 2004; Kraft et al., 2005). For this function another domain is responsible that usually guides protein-protein interactions, e.g. WD40 repeats, Armadillo repeats, leucine rich repeats. Besides its U-box domain responsible for linking and presenting the ubiquitin tag onto the target protein, LIN has several additional domains, including WD40 repeats, that are likely to define specificity of target substrates.

The complete structure of LIN appears to be unique to plants, since genes coding for proteins with similar structures could not be found in other organisms. Even in the plant kingdom it is sometimes absent, e.g. in the Arabidopsis genome no single homologous sequence could be identified. Instead, two proteins show homologous regions with parts of the LIN protein, At3G06880 codes for a longer protein with WD40 repeats, while At1G23030 codes for a shorter U-box containing protein. On the other hand, the genome of the phylogenetically more distant rice does carry a gene coding for a protein with a domain
structure (Os01g0229700) similar to LIN, although the level of similarity is poor at the N-terminus. *L. japonicus*, *P. trichocarpa* and *V. vinifera* show genes with a high degree of similarity to LIN. We show that the homologous protein in *L. japonicus* has an equivalent function to LIN, although the presence of homologs in non-legumes suggests functions unrelated to nodulation in these species.

The emerging picture for ubiquitin regulation in plant-microbe interactions is supported so far mostly of examples in plant defenses suggesting multiple levels of regulation: from the resistance proteins to downstream signaling components and regulators (Zeng et al., 2006). Several U-box-type E3 ligases are identified as components that regulate plant defenses. It is likely that an equivalent level of regulation exists in nodulation signaling as well. Based on the symbiotic phenotype of the lin mutants it can be hypothesized that inappropriate regulation of plant defenses during rhizobial invasion in the root hairs might be causing the block in infection. A gene expression study on a small scale microarray (representing ~ 1000 genes) identified several genes misregulated in the *M. truncatula* lin-1 mutant, including genes predicted to be involved in defense responses; cell cycle regulation, signaling, membrane composition and transport (Endre et al., 2008). Therefore a possible function of LIN may be the fine-tuning of the pathogen response upon rhizobial infection.

There are already indications for an involvement of E3 ligases in the legume/rhizobial symbiosis with two E3 ligases showing a role in nodule development. Recently, nsRING, a novel RING finger protein required for rhizobial invasion and nodule formation was identified in *L. japonicus* (Shimomura et al., 2006). The possible involvement of nsRING in phytohormone-related signaling was suggested. In *M. truncatula*, it has been shown that the SINA family of E3 ligases is important for infection thread growth and symbiosome differentiation (Den Herder et al., 2008). Overexpressing SINAT5DN from *Arabidopsis* caused a significant reduction in nodule number, indicating a negative regulatory role for this protein. Based on the mutant phenotype we can assume that LIN negatively regulates certain transcription factors and/or signal transduction proteins required for bacterial invasion, and which need fine tuned regulation for their proper action. Good candidates were identified in a suppression subtractive hybridization approach that revealed candidate transcription factors: a bHLH, a WRKY and a C2H2 zinc-finger protein, that were upregulated, following *S. meliloti* inoculation in lin (Godiard et al., 2007). Understanding of LIN targets should provide insights into the maintenance of infection thread growth and may also provide insights into the mechanisms by which infection thread growth and nodule organogenesis are coordinated.
METHODS

Plant growth and bacterial strains

*Medicago truncatula* cv Jemalong genotype A17 was used as the wild-type control for phenotypic and genotypic analysis. The plants were grown as previously described by Cook et al. (1995). Plants were infected with *S. meliloti* 1021 carrying pXLGD4 plasmid, expressing the *lacZ* reporter gene under a *hemA* promoter. In the cloning procedures, *E. coli* DH5α (Gibco BRL) and One Shot Omnimax 2T1 (Invitrogen) strains were used. *S. meliloti* and *E. coli* strains were grown at 30 and 37 °C respectively, in Luria-Bertani (LB) medium supplemented with antibiotics when required: In hairy root transformation experiments, *Agrobacterium rhizogenes* ARqua1 strains electroporated with the appropriate binary vector constructs were used.

Gene isolation

Mapping was done on an F2 segregating population originating from a cross between C88 and A17 as described in Kuppusamy et al. (2004), but on a different set of 290 individuals. For testing more individuals with close recombination events to the *lin-1* mutation, 508 F3 individuals of selected F2 plants were also genotyped. Genetic markers used in this study (LP = length polymorphism; SNP = single nucleotide polymorphism) with their respective primer pairs are as follows: 4E6R (LP): 4E6R_F, 5’-TGAGCGTCCAATCAAATTGAC-3’; 4E6R_R, 5’-TTCACAAATACAAACCCTCAC-3’. 16L4R (SNP): 16L4R_F, 5’-GGCATTTCAAGGTTTTTGTG-3’; 16L4R_R, 5’-TAACTCATTTGATGTCATAAGAG-3’. TC110346_2 (SNP): TC110346-2_F, 5’-TGATTTCAACAAATGGACAGG-3’; TC110346-2_R, 5’-CATGAATAATGAAAAACCT- TAATTG-3’. 18E17F (SNP): 18E17F_F, 5’-TAGCTCTCTACACCTCCG-3’; 18E17F_R, 5’-CTCCGTCTCTACACTTCGTC-3’. TC96353-1 (LP): TC96353-1_F, 5’-GAATAGTTGAGATAAACACGGGAG-3’; TC96353-1_R, 5’-TTGTGGACACAAACGAGT-3’. e13O17F (SNP): e13O17F_F, 5’-CCTAGGCGATTGCATCTGG-3’; e13O17F_R, 5’-CAAGCTGAACGAACATGG-3’. e53J20F (SNP): e53J20F_F, 5’-GAGGGAAATGAAACGATGAAAG-3’; e53J20F_R, 5’-AGAAGTCAACACACGAGG-3’. Zero recombination was found for all tested markers with *lin* between flanking markers 4E6R and e53J20F, therefore the region carrying the LIN gene could not be further delimited. The identified BAC clones were sent to and sequenced by the group at the University of Oklahoma (lead by B. Roe) responsible for sequencing *M. truncatula*. 

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chromosome 1. Gene predictions of the region is from the IMGAG annotation of the two sequenced BAC clones: www.tigr.org/tigr-scripts/medicago/CONTIGS/GBROWSE/gbrowse2?name=MTCON310).

The following primer pairs were used to demonstrate the imperfect splicing of the messenger RNA in lin-1 mutant: Lin-3F, 5’- GGATGAAGATGTTGAACCAA -3’ and Lin-3R, 5’- CCTGTGATTGGACAAACAA -3’; Lin-8F, 5’- GGACGCAAGGAAGAGAAT -3’ and Lin-8R, 5’- CCAGTGAAGAATGAAGTTGATG -3’. Primers Lin-3Fb: TTGTGTGTCCAATCACAGG and Lin-12Rc: ACAGTCCTGCAAGTTTTCTGTGT were used to amplify fragment for the Southern hybridization. All amplification reactions were carried out as described earlier (Endre et al., 2002b) using adequate annealing temperature for the respective primer pairs.

**Sequence Analysis**

LIN sequences and homologs were aligned using VectorNTI. For different domain, motif and structure predictions the following programs/websites were used:

NCBI Conserved domain search program on A Conserved Domain Database and Search Service, v2.16; CDART: Conserved Domain Architecture Retrieval Tool (searches the NCBI Entrez Protein Database for similarity based on domain architecture, defined as the sequential order of conserved domains in proteins; (Geer et al., 2002) http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi; PSORT II program (http://psort.ims.u-tokyo.ac.jp/form2.html);

**A. rhizogenes mediated complementation of different lin mutants**

For complementation experiments, LIN cDNA was recombined from the pCR8GW-TOPO entry clone into the pK7WG2D vector (Karimi et al., 2002), under the control of the CaMV 35S promoter. This construct also harbors a constitutively expressed GFP gene to facilitate the identification of the transgenic roots. For the pLIN::LIN cDNA construct, the 1.2 kb LIN promoter was amplified from genomic DNA using the following oligonucleotides: GGACTAGTCATCTATCAAGAAAAATCACAA;CCCAAGCTTATAATCTATGTCCG carrying the SpeI and HindIII restriction sites, respectively. LIN cDNA was also recombined from the pCR8GW-TOPO entry clone into the pK7WGF2 vector (Karimi et al., 2002) in which the 35S promoter was exchanged with the 1.2 kb LIN promoter segment using HindIII and SpeI. The resulted destination clones were electroporated into A. rhizogenes ARqua1 and used for hairy root transformation of M. truncatula plants as was described earlier (Boisson-Dernier et al., 2001), only without using antibiotic selection for
the transgenic roots. Plants harboring 4-5 cm long hairy roots (approx. two weeks after *A. rhizogenes* treatment) were transferred into pots and let grow and strengthen for another 3-4 weeks in Turface. One week before the date of inoculation by rhizobia nitrogen was omitted from the liquid media. Plants were inoculated afterwards with *S. meliloti* 1021 strain carrying a *hemA* promoter::*lacZ* fusion (Leong et al., 1985) allowing the detection of bacteria within plant tissues by histochemical staining for *lacZ* activity. Roots were screened and scored for the presence of nodules or nodule primordia 21 days after inoculation. Transgenic hairy roots expressing GFP constitutively from the inserted T-DNA were selected and monitored for nodulation and for nodule occupation by *S. meliloti*.

**Histochemical localization of the LIN promoter activity**

To generate pLIN-GUS fusion construct, a 1.2 kb segment upstream the coding region of *LIN* was amplified and cloned into the binary vector pMDC164 (Curtis and Grossniklaus, 2003). The resulting plasmid constructs was introduced into *M. truncatula* by *A. rhizogenes* mediated hairy-root transformation as described above. In order to detect the activity of the cloned promoter in the transgenic hairy-roots, root and nodulated root samples were collected 0, 3, 6 and 21 days after inoculation with *S. meliloti* 1021. Histochemical detection of β-glucuronidase (GUS) activity was carried out using a standard protocol and 5-Bromo-6-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt (Magenta-Gluc CHA salt, Duchefa Biochemie, Haarlem, The Netherlands) as substrate (Jefferson and Wilson, 1991). Uninoculated roots and six-day-old nodules were studied as intact organs. Developed nodules were embedded in 4 % agarose, and 100 µm longitudinal sections were made with a microcut H1200 (Bio-Rad, Munich, Germany). Microscopic analysis was carried out with a Leica DM LB2 light microscope (Leica Microsystems, Wetzlar, Germany) coupled with a MicroPublisher 3.3 RTV (Q imaging, Surrey, Canada) digital camera.

**Accession numbers**

Sequence data from this article are deposited in the Genbank/EMBL data libraries under the following accession numbers: MtLIN EU926660; MtLIN_CDS EU926661; Mtlin-1 EU926662; Mtlin-2 EU926663; Ljlin_CDS EU926664; Ljlin-1 EU926665

**Supplemental Data**

The following materials are available in the online version of this article.
Supplemental Figures S1. – S4. Multiple sequence alignments of the LIN proteins and respective homologs.

Supplemental Figures S5. Positive complementation of the nodulation phenotype on the transgenic hairy roots on the *lin-1* mutants using the pLIN::LIN construct.

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**FIGURE LEGENDS**

**Figure 1.** *M. truncatula* LIN identification by positional cloning. (A) Genomic region around the *lin* mutation, the overlapping BAC clones mth2-69D21, mte1-13O17 and mte1-53J20 represent the region where *lin* was delimited by the identified recombination events. Vertical bars correspond to the polymorphic markers generated in the region and used for identifying recombinations (numbers under the bars) from *lin* to set the borders of the region. (B) Predicted coding regions in MTCON310. The position of MTCON310-47 is indicated by the star. (C) Gene model of MTCON310-47; the exon-intron structure of the LIN coding region: numbered blocks are the coding exons; open triangle: position of the extra 24 nucleotides in the actual cDNA compared to the predicted cDNA; arrows: indicate the positions of the mutations in the three *M. truncatula lin* alleles.

**Figure 2.** Visualization of two mutations in the *M. truncatula* LIN gene. (A) RT-PCR products of *lin-1* mutant and wild type A17 plants on the RNA samples isolated from roots four days after *S. meliloti* inoculation. DNA fragments with primers Lin-3F and Lin-3R include intron 4 that is spliced out from the wild type allele but not spliced from the mutant. Amplification with primers Lin-8F and Lin-8R resulted in identical fragments representing exons 11-14 on the same cDNA templates. (B) Southern blot using EcoRV-digested genomic DNA of the wild type (wt) and *lin-3* mutant plants (as well as *nsp2-4* mutant included as another control) and a probe of 500 bp from exon 5 amplified with primers Lin-3Fb and Lin-12Rc revealed an apparent deletion or rearrangement affecting the large part of the gene.

**Figure 3.** Positive complementation of the nodulation phenotype on the transgenic hairy roots on the *lin-1* mutants. (A) Detection of the transgenic roots and nodules by GFP fluorescence. Hairy roots were induced without antibiotic selection allowing the emergence of both transgenic (bright green fluorescent roots) and non-transgenic (yellowish) roots. (B) Mature nodule on the transgenic root on the *lin-1* mutant occupied by *S. meliloti* strain 1021 carrying a hemA promoter: *lacZ* fusion, stained for β-galactosidase activity. Bacteria visualized in the nodule revealed that wild type nodule development and invasion process occurred in the complemented roots. (C) Nodule primordia on control non-transgenic root on the *lin-1* mutant inoculated with the same bacteria showed the mutant phenotype with the nodule primordia and arrested infections in the root hairs. Scale bars represent 200 μm (B) and 10 μm (C), respectively.
**Figure 4.** Complementation of the *Lotus japonicus sym7* mutant (SL1450-5) by *MtLIN*. (A) Transgenic roots on SL1450-5 mutant identified by GFP fluorescence. Pink nodules could be observed in the complemented plants indicating that they were functional. (B) Light microscopy of the SL1450-5 (*sym7*) nodules from *MtLIN* transgenic roots showing a regular infection pattern. Bar = 50 μm.

**Figure 5.** Schematic presentation of the domain structure of the *M. truncatula* LIN protein and similarity levels with closely related plant proteins. Predicted domains of the *M. truncatula* LIN protein are indicated in the schematic drawing at the top of the figure. Graphical representation of the multiple alignment of the *M. truncatula* LIN protein with the best plant homologs below the domain structure are plotted by the AlignX program of VectorNTI (Invitrogen) as follows: specific values (in a 0-1 range) are assigned to each residue at a given alignment position in each aligned sequence, depending on whether the residue is identical, similar or weakly similar to the corresponding residue of the consensus sequence.

**Figure 6.** Promoter activity of the cloned upstream region of *LIN* in roots and nodules. Samples were collected from transformed hairy-roots carrying the *LIN*-promoter-*GUS* fusion, from uninoculated roots (A), and 3 (B), 6 (C), 21 (D) days after inoculation with wild type *S. meliloti* 1021. Histochemical staining of samples were carried out using Magenta-gluc as substrate. Scale bars represent 200 μm.
**Table I.** Results of the *lin* allelism test in *M. truncatula*. All individuals identified as symbiotic mutants in the progeny of the respective crosses. Numbers in the cells: individuals tested; numbers in brackets: pod numbers from which seeds originated.

|       | *lin-1* | *lin-2* | *lin-3* |
|-------|---------|---------|---------|
|       | C88     | EMS6:T7 | 14P     |
| *lin-1* | na      | 3 (1)   | 17 (4)  |
| *lin-2* | 15 (4)  | na      | 11 (5)  |
| *lin-3* | 15 (5)  | 24 (7)  | na      |
Table II. Hairy root complementation tests of *L. japonicus* mutants for nodulation with *M. truncatula* LIN gene gave positive result for the sym7 mutation.

| *L. japonicus* mutant | Transforming binary plasmid | Transformed plants | No. of plants nodulated* |
|-----------------------|-----------------------------|--------------------|-------------------------|
| SL1450-5 (sym7)       | pK7WG2D                     | 77                 | 5<sup>a</sup>           |
| SL1450-5 (sym7)       | pK7WG2D-35S:LIN             | 90                 | 33<sup>b</sup>          |
| SL1947-2 (itd3)       | pK7WG2D                     | 35                 | 0<sup>a</sup>           |
| SL1947-2 (itd3)       | pK7WG2D-35S:LIN             | 80                 | 0<sup>a</sup>           |

*Different superscript letters signify a significant difference (*P*<0.001, $\chi^2$-test).
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**Figure 3.** Positive complementation of the nodulation phenotype on the transgenic hairy roots on the *lin-1* mutants. (A) Detection of the transgenic roots and nodules by GFP fluorescence. Hairy roots were induced without antibiotic selection allowing the emergence of both transgenic (bright green fluorescent roots) and non-transgenic (yellowish) roots. (B) Mature nodule on the transgenic root on the *lin-1* mutant occupied by *S. meliloti* strain 1021 carrying a hemA promoter:*,lacZ* fusion, stained for β-galactosidase activity. Bacteria visualized in the nodule revealed that wild type nodule development and invasion process occurred in the complemented roots. (C) Nodule primordia on control non-transgenic root on the *lin-1* mutant inoculated with the same bacteria showed the mutant phenotype with the nodule primordia and arrested infections in the root hairs. Scale bars represent 200 μm (B) and 10 μm (C), respectively.
Figure 4. Complementation of the *Lotus japonicus* *sym7* mutant (SL1450-5) by *MtLIN*. (A) Transgenic roots on SL1450-5 mutant identified by GFP fluorescence. Pink nodules could be observed in the complemented plants indicating that they were functional. (B) Light microscopy of the SL1450-5 (*sym7*) nodules from *MtLIN* transgenic roots showing a regular infection pattern. Bar = 50 μm.
Figure 5. Schematic presentation of the domain structure of the *M. truncatula* LIN protein and similarity levels with close plant homologs. Predicted domains of the *M. truncatula* LIN protein are indicated in the schematic drawing at the top of the figure. Graphical representation of the multiple alignment of the *M. truncatula* LIN protein with the best plant homologs below the domain structure are plotted by the AlignX program of VectorNTI (Invitrogen) as follows: specific values (in a 0-1 range) are assigned to each residue at a given alignment position in each aligned sequence, depending on whether the residue is identical, similar or weakly similar to the corresponding residue of the consensus sequence.
Figure 6. Promoter activity of the cloned upstream region of LIN in roots and nodules. Samples were collected from transformed hairy-roots carrying the pLIN-GUS fusion, from uninoculated roots (A), and 3 days (B), 6 days (C), and 21 days (D) after inoculation with wild type S. meliloti 1021. Histochemical staining of samples were carried out using Magenta-gluc as substrate. Scale bars represent 200 μm.