学位論文

Molecular genetic analyses and molecular cloning of *TOO MUCH LOVE*; the root regulatory gene in the long-distance regulation of the root-nodule symbiosis in *Lotus japonicus*.

（和訳：根粒着生数を根で制御する因子 *TOO MUCH LOVE* の遺伝子検索と分子遺伝学的解析）

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Abstract

The interaction of legumes with N_2-fixing bacteria collectively called rhizobia, results in the root nodule development. The number of nodules is tightly restricted through the negative feedback regulation by hosts. The fact that the *HAR1*-mediated control of nodule number needs the *HAR1* expression in the shoots exibits a long distance communication between the shoot and the root. However, the large part of the mechanism remains to be elucidated. Previously, we have shown that *too much love-1* (*tml-1*), a hypernodulating mutant in *Lotus japonicus*, has a defect in the negative feedback regulation and that *TML* functions in the roots downstream of *HAR1*. To better understand the mechanism by which legume plants control nodule number, I conducted molecular biological and genetic analyses using *tml* mutants. From the results of the double mutant analyses, I conclude that *TML* acts at the final stage of AON downstream of *CLE-RS1*/*RS2* and *HAR1* to negatively regulate the nodule number by inhibiting the organogenesis induced by the cytokinin signaling. Identification of the deleted regions in the large deletion alleles *tml-1*, *tml-2* and *tml-3* and the fine mapping of the EMS allele *tml-4* suggested that the gene corresponding to the EST sequence (GenBank accession number AK339024) was the strong candidate for the *TML* gene. Therefore, I confirmed that the knockdown of the candidate gene induced the same phenotype as the *tml* mutants. Hence, I finally concluded that the gene encoding a Kelch repeat-containing F-box protein with two NLSs is the *TML* gene.

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Chapter 1

General introduction

1.1 Legume-*rhizobium* symbiosis

In this section, I briefly mention the importance of the studies in this field.

Unlike animals, plants can not move around. Therefore, they must obtain essential nutrients from the soil in order to grow or to respond to the environmental change. In another word, their growth depends on the availability of the nutrients in the soil. Nitrogen is an indispensable component of living organisms. Even if the nitrogen molecule (N_2) is plentiful in the atmosphere on the earth, most plants can not directly use N_2 because they can use only the oxidized (NOx) and reduced form (NH_4^+) of nitrogen. Therefore, nitrogen acquisition is the important work for plants (Kraiser et al. 2011).

Legume-*Rhizobium* symbiosis is one of the well studied mutually-beneficial interactions involved in the nitrogen acquisition of the host legume plants. In this symbiosis, the host legume provides the nitrogen-fixing bacteria, collectively called rhizobia, with the photosynthetic products. In return, the rhizobia provide the host legume with ammonia as the fixed nitrogen. Through this symbiosis, legumes can survive in the nitrogen-limiting environment. Therefore, some researchers launch projects for the greening of deserts using the legume plants (Marui et al. 2012). In addition, Galloway et al and Canfield et al estimated that the nitrogen fixation rate was 2.4×10^{12} mol $year^{-1}$ in the mid-1990s and they quoted that the large part of the fixed nitrogen was from the rhizobia associated with seed legumes (e.g. *Pisum sativum* and *Glycine max*) and fodder legumes (e.g. *Astragalus sinicus* and *Trifolium repens*) (Galloway et al. 2004, Canfield et al. 2010). Replacing the fixed nitrogen from fodder legumes to the nitrogen fertilizer would cost 7 to 10 billion dollars annually (Graham et al. 2003). Furthermore, Legume-*Rhizobium* symbiosis is a good example of the endosymbiosis in which the symbiont lives within the cell of the host organism. *Rhizobium* released into the cell of the nodule becomes surrounded by a

plant-derived membrane and differentiates into a specialized cell type called a bacteroid. These processes result in the formation of the organelle-like structure called a symbiosome that is capable of fixing nitrogen (Maunoury et al. 2010). Therefore, Legume-*Rhizobium* symbiosis might be a good model providing the better understanding of endosymbiosis.

Hence, understanding the precise mechanism of the Legume-*Rhizobium* symbiosis is meaningful for an improvement of the environment, economically valuable, and scientifically important.

In this thesis, I used the model legume *Lotus japonicus* and its symbiont *Mesorhizobium loti*. They provide an excellent model system because their genomic information are available and biological resources are well organized.

1.2 Bacterial infection and nodule organogenesis

To fix the nitrogen efficiently, the host plants offer the symbiotic organs called nodules to let the rhizobium within. Forming nodules (i.e. nodulation) is a very important process because it enables the host plants and rhizobium to exchange nutrients with each other and to prevent oxygen from interfering with the nitrogen fixation as well. Therefore, to investigate the nodulation is the key to comprehend the precise mechanism of Legume-*Rhizobium* symbiosis. Studies on the symbiotic mutants of the model legumes (e.g. *L. japonicus, Medicago truncatula*, *P. sativum* and *G. max*) revealed that the nodulation is a complex process involving two developmental processes running synchronously in parallel [reviewed in (Madsen et al. 2010, Oldroyd et al. 2011)]. One is the bacterial infection process and the other is the nodule organogenesis (Figs. 1.1A-1.1B). Both of them are coordinated by the host genetic program and are triggered by plant recognition of a small organic compound referred to as Nod factor (NF). NF is a lipochitooligosaccharide derivative synthesized by rhizobia in response to the plant-derived flavonoids (Perret et al. 2000, D'Haeze et al. 2002). The modification types of the NF (such as the position of the acyl chain or the sulfate group) differs according to species, which determines the host specificity (D'Haeze et al. 2002). Loss-of-function mutants of the model legumes have shown that the recognition of NF requires the functional receptor-like kinases with extracellular LysM domains (e.g. *NFR1*/*NFR5* in *L. japonicus*) (Madsen et al. 2003, Radutoiu et al. 2003). NF perception by the receptors activates the physiological responses such as ion influx, membrane depolarization and Ca^{2+} spiking. These events are mediated by a leucine-rich repeat receptor kinase (*SymRK*) (Endre et al. 2002, Stracke et al. 2002), cation channels (*Castor* and *Pollux*) (Ane et al. 2004, Imaizumi-Anraku et al. 2005, Charpentier et al. 2008), nucleoporins

(*Nup85* and *Nup133*) (Kanamori et al. 2006, Saito et al. 2007, Groth et al. 2010), a calcium calmodulin-dependent protein kinase (*CCaMK*) (Levy et al. 2004, Mitra et al. 2004, Tirichine et al. 2006) and a nuclear localized coil–coil protein (*Cyclops*) (Messinese et al. 2007, Yano et al. 2008). This signaling pathway is termed "common sym pathway" because this genetic pathway is shared with mycorrhizal symbiosis. In this pathway, *SymRK*, *Castor*, *Pollux*, *Nup85* and *Nup133* act upstream of the Ca^{2+} spiking, whereas *CCaMK* and *Cyclops* are suggested to decode Ca^{2+} spiking signaling. Downstream of this pathway, several putative transcription factors [nodulation signalling pathway 1 (*NSP1*), *NSP2* (Kalo et al. 2005, Smit et al. 2005, Heckmann et al. 2006, Murakami et al. 2006), ERF required for nodulation 1 (*ERN1*) (Andriankaja et al. 2007, Middleton et al. 2007, Cerri et al. 2012), nodule inception (*NIN*) (Schauser et al. 1999, Marsh et al. 2007)] are required for upregulation of the NF-induced gene expression, which initiate the physiological responses for bacterial infection and nodule organogenesis.

The most common way for the bacterial infection into the nodule primordia is the formation of the intracellular tubes called infection threads (IT) in the dedifferentiated root hair cells [the genes involved in this process are reviewed in (Oldroyd et al. 2011)]. After perception of the NF, several morphological changes such as swelling and curing of the root hair tip are induced, resulting in the formation of the teaspoon-like structure so-called infection pocket in which rhizobia are entrapped (Geurts et al. 2005). The entrapped rhizobia form the colonies called infection foci. The formation of ITs in the root hair starts from the infection loci. When the progression of the IT formation reaches at the base of the root hair cell, the adjacent cortical cell starts to develop IT. By repeating this process, the bacterial infection is propagated into the nodule primodia.

Concurrently with IT formation, NF-induced nodule organogenesis proceeds in the root cortical cells. NF induces the cortical cell division (i.e. the dedifferentiation and proliferation of the root cortical cells), resulting in the formation of nodule primordia. Some mutants aseptically develop nodule-like structures called spontaneous nodules. Now I focus on two of them; *Snf1* and *Snf2*. *Snf1* is a gain-of-function allele of *CCaMK* that leads to the spontaneous formation of nodules. Interestingly, only the activation of a specific kinase is sufficient to trigger the preexisting developmental program for nodule organ formation, even if there are several components that are able to decode the Ca^{2+} spiking signature (e.g. *CDPK*, *CaMKII* and calcineurin (Sanders et al. 1999, Li et al. 2012)) Because NF application (Truchet et al. 1991) or the *Snf1* mutation (Tirichine et al. 2006) can induce the genuine nodule oraganogenesis, perception of NF at the root epidermal cells is likely to be sufficient for activation of the cell division in the cortical cell layers. *Snf2* is a gain-of-function allele of the cytokinin receptor gene *LHK1* of *L. japonicus* that leads to the formation of spontaneous nodules. In addition, the loss of function allele of *LHK1* in *L.japonicus* and its ortholog *CRE1 in M. truncatula* inhibit the nodule formation (Gonzalez-Rizzo et al. 2006, Murray et al. 2007). Taken together, after an unknown short-distance communication from the epidermal cells, cytokinin signaling plays a central role for the regulation of nodule organogenesis in the cortical cells (Cooper et al. 1994, Lohar et al. 2004). Recent study demonstrated that the nodulation-related Type-A RR (*MtRR4)*, the micro RNA (miR171h targeting *NSP2*) and a basic Helix-Loop-Helix TF (*bHLH476*) act downstream of the *LHK1*-mediated cytokinin signaling pathway (Ariel et al. 2012).

In conclusion, the two pathways (i.e. bacterial infection and nodule organogenesis) work synchronously in parallel so as to form a bacterially infected nodule.

1.3 Long-distance regulation of nodulation

As mentioned above, the interaction of legumes with N_2-fixing bacteria collectively called rhizobia results in root nodule development. Rhizobia supply the host legumes with ammonia fixed from the air, and host plants provide the rhizobia with photosynthates in return. Because the formation of nodules is energetically expensive, the number of nodules and the nodulation zone are tightly restricted by the negative feedback regulation of host legumes, which is termed the autoregulation of nodulation (AON) (Bhuvaneswari et al. 1980, Pierce et al. 1983, Caetano-Anolles et al. 1991, Ferguson et al. 2010)

The surgical excision of nodules in *T. pratense* led to an increase in the number of subsequently developed nodules, indicating that AON is activated by preceding nodulation events and negatively regulates the subsequent nodule formation (Nutman 1952). The pre-inoculated half of a split-root system inhibited the subsequent nodule formation on the other half in *G. max*, *Trifolium subterraneum*, *Medicago sativa*, *Phaseolus vulgaris* and *L. japonicus*. These findings suggest that AON is a systemic regulation and is common in a variety of legumes (Singleton 1983, Kosslak et al. 1984, Sargent et al. 1987, George 1992, Catford et al. 2003, Suzuki et al. 2008).

In addition, recent studies have suggested that AON is activated via a long-distance communication between the root and shoot. Several mutants (i.e., *Ljhar1/Mtsunn/Pssym29/Gmnark, Ljclv2/Pssym28* and *Ljklv)* were identified as hypernodulating mutants in which the number of nodules is increased (Sagan et al. 1996, Szczyglowski et al. 1998, Krusell et al. 2002, Nishimura et al. 2002, Penmetsa et al. 2003, Searle et al. 2003, Oka-Kira et al. 2005, Schnabel et al. 2005, Suzaki et al. 2008, Miyazawa et al. 2010, Schnabel et al. 2010, Krusell et al. 2011). Reciprocal grafting

experiments between these hypernodulating mutants and wild-type plants revealed that the shoot genotype rather than the root genotype determines the hypernodulation phenotype (Delves et al. 1986, Olsson et al. 1989, Sheng et al. 1997, Wopereis et al. 2000, Krusell et al. 2002, Nishimura et al. 2002, Penmetsa et al. 2003, Oka-Kira et al. 2005). Additionally, the genes responsible for the hypernodulation encode leucine-rich repeat receptor-like kinases (LRR-RLK) (Krusell et al. 2002, Nishimura et al. 2002, Searle et al. 2003, Oka-Kira et al. 2005, Schnabel et al. 2005, Krusell et al. 2011). These findings indicate that the activation of AON requires the LRR-RLKs acting in the shoot. The orthologs of these LRR-RLKs in *Arabidopsis thaliana* (*CLV1, CLV2* and *RPK2*) and *Oryza sativa* (*FON1*) have roles in maintaining the shoot apical meristem and are activated by receiving the short-distance signaling molecule, i.e., the CLE family of peptides CLV3 (Ogawa et al. 2008) and FON2/FON4 (Chu et al. 2006, Suzaki et al. 2008, Suzaki et al. 2009), respectively. In legumes, the CLE family of peptides are strong candidates for the root-derived signal molecules because the expression of *CLE* genes such as *LjCLE-RS1/RS2, MtCLE12/13* and *GmRIC1/2* is induced by rhizobial infection (Okamoto et al. 2009, Mortier et al. 2010, Mortier et al. 2011, Reid et al. 2011) and because the overexpression of these *CLE* genes drastically reduces or abolishes nodulation in a *HAR1/SUNN/NARK*- or *KLV*-dependent manner (Okamoto et al. 2009, Miyazawa et al. 2010, Mortier et al. 2010, Lim et al. 2011, Reid et al. 2011). Together with the relationship between the LRR-RLKs and the CLE peptides in *A. thaliana* and *O. sativa*, AON-related CLEs might be root-derived ligands for the AON-related LRR-RLKs in the shoot.

In addition, there have been several reports on the relationship between cytokinins and AON. The gain-of-function mutation of the cytokinin receptor *LjLHK1* in the *har1* or *klv*

mutant background develops an excessive number of spontaneous nodules without rhizobial infection, indicating that *HAR1* and *KLV* inhibit the nodule formation induced by the cytokinin signaling (Tirichine et al. 2007, Miyazawa et al. 2010). Moreover, a recent report has shown that a cytokinin regulates *MtCLE13* expression (Mortier et al. 2010). Therefore the *CLE*-, *HAR1/SUNN/NARK- and KLV*-dependent suppression of nodule formation may be induced by the cytokinin-induced nodule organogenesis itself, even without rhizobial infection.

To establish the systemic regulation of nodulation via long-distance communication, the AON needs both a root-to-shoot signal and a shoot-to-root signal (which is also called a shoot-derived inhibitor, SDI) (Lin et al. 2010). With respect to the shoot-to-root signal, some research attempting to isolate shoot-derived signals is in progress (Akashi et al. 2010, Lin et al. 2010, Yamaya et al. 2010, Yamaya et al. 2010, Lin et al. 2011). Recent studies suggest that the SDI is an amphiphilic and low-molecular-weight compound. However, it remains unsolved as to how nodulation is inhibited downstream of SDI in the final stage of AON in the root (Fig. 1.2).

Chapter 2

Molecular genetic analyses of the *tml* mutants

2.1 Introduction

Legume plants develop the specialized organs called nodules to establish the efficient nitrogen acquisition from the nitrogen-fixing rhizobia. Because the root nodule formation is an energy-consuming process, host plants have the mechanisms to keep the adequate number of nodules to sustain the proper symbiotic relation with the rhizobia. One of the nodule number regulation referred to as AON has been extensively studied. AON is the shoot-controlled regulation of nodulation in the roots. In *L. japonicus*, this regulatory pathway requires the functional LRR-RLKs such as *HAR1*, *CLV2* and *KLV*. Miyazawa et al. demonstrated that HAR1 and KLV form the heterodimeric complex to act the signaling center in shoots (Miyazawa et al. 2010). In addition, the genes encoding *CLE-RS1*/*RS2* are suggested to act upstream of the LRR-RLKs as the root-to-shoot signals. However, there is little understanding of the root factors involved in this long-distance regulation. A previous study has shown that *too much love-1* (*tml-1,* formerly *tml*), a hypernodulating mutant of *L. japonicus*, has a defect in the negative feedback regulation and that *TML* is a root factor acting downstream of *HAR1* (Magori et al. 2009). Therefore, *TML* must be a key factor in understanding what happens during the final stage of AON to maintain proper nodulation. Hence, it is crucial to investigate the function of *TML* and identify the *TML* gene. In this thesis, I clarify the acting point of *TML* during AON through the characterization of the *tml* mutant. Genetic and molecular analyses indicate that *TML* and *PLENTY* (another mutant of the root factor that regulates number of nodules) act in different genetic pathways; *TML* acts downstream of *LjCLE-RS1*/RS*2* and might suppress the nodulation signaling downstream of the cytokinin receptor *LHK1*/*CRE1*.

2.2 Results

TML and *PLENTY* function in different genetic pathways, whereas *TML* and *HAR1* function in the same genetic pathway

A previous study showed that the *har1* genotype did not enhance the *tml* hypernodulation phenotype in the grafting experiment or in the double mutant analysis, suggesting that *TML* functions downstream of *HAR1* (Magori et al. 2010). However, the results in the previous report did not exclude the possibility that the observations reflect that the root of the *tml-1* mutant does not have the potential ability to produce any more nodules. To clarify which explanation is reasonable, I examined the genetic interaction between *TML* and *PLENTY*, another root factor that regulates the number of nodules. First, I observed the nodulation phenotype of the *tml-1 plenty* double mutant. The *tml-1 plenty* double mutant showed an increased number of nodules compared to those of the respective single mutants, indicating that *TML* and *PLENTY* function in different genetic pathways (Figs. 2.1A-2.1E). This finding also indicates that if *TML* and the other gene that is also involved in the regulation of the nodule number act in different genetic pathways, the double mutant indeed illustrates the additional effect on the nodule number. I analyzed the *tml-1 har1-7* double mutants as well. In contrast, the *tml-1 har1-7* double mutant did not show an additive effect on nodulation (Figs. 2.1A-2.1C, 2.1F and 2.1G), which is consistent with previous results. Taken together, I conclude that *TML* and *HAR1* function in the same genetic pathway.

TML is required for the suppression of nodulation caused by *CLE-RS1*/*RS2*

The previous study demonstrated that either *CLE-RS1* or *CLE-RS2* systemically suppresses nodule formation in a *HAR1*-dependent manner. Therefore, to investigate the

genetic interaction between *TML* and *CLE-RS1*/*RS2,* I introduced the expression construct of *CLE-RS1*/*RS2* driven by the CaMV 35S promoter using the hairy root transformation method and examined whether the *tml* mutation affects the suppression by *CLE-RS1*/*RS2*. As has been reported, the roots overexpressing either *CLE-RS1* or *CLE-RS2* in the wild-type plants developed a drastically decreased number of nodules compared to the control roots overexpressing the GUS gene. In contrast, in the *tml* mutant background, the roots overexpressing either *CLE-RS1* or *CLE-RS2* developed an excessive number of nodules compared to that in the wild-type background (Fig. 2.2A). In addition, there is no statistically significant difference between the number of nodules in the CLE-overexpressing roots and the control GUS-overexpressing roots in the *tml* mutant background (Fig. 2.2B). This result indicates that *CLE-RS1*/*RS2* suppresses nodulation in a *TML*-dependent manner. Therefore, I conclude that *TML* functions in the regulation of the nodule development downstream of *CLE-RS1*/*RS2*.

TML inhibits the nodule organogenesis induced by the _LHK1_-mediated cytokinin signaling

In *L. japonicus*, the gain-of-function mutant of the cytokinin receptor *LHK1* (*Snf2*) develops nodule-like structures called “spontaneous nodules” in the absence of rhizobia (Tirichine et al. 2007). In the previous reports, an excessive number of spontaneous nodules was observed in the *har1 Snf2* and *klv Snf2* double mutants, suggesting that AON functions downstream of the cytokinin signaling–induced activation of root nodule founder cells (Tirichine et al. 2007, Miyazawa et al. 2010). To assess the role of *TML* in AON, I crossed the *tml* mutant with the *Snf2* mutant to generate the *tml-1 Snf2* double mutant. In the F2 population, the number of spontaneous nodules in the *Snf2* mutants that

were homozygous at the *TML* locus (*tml*/*tml* or *TML*/*TML*) was counted 5 weeks post-germination (Figs. 2.3A-2.3D). The *tml-1 Snf2* double mutant spontaneously developed many small nodules similar to those generally observed in the *tml* mutant upon rhizobial infection (Figs. 2.3B-2.3C), whereas the *Snf2* single mutant developed spontaneous nodules similar to those produced in the wild type upon infection (Fig. 2.3A). The *tml-1 Snf2* double mutant produced an average of 12.1 ± 6.6 spontaneous nodules, while the *Snf2* single mutant produced an average of 3.6 ± 1.7 (Fig. 2.3D), indicating that *TML* inhibits the nodule organogenesis induced by the *LHK1*-mediated cytokinin signaling. In addition, this result clearly indicates that the nodule formation itself, even without rhizobial infection, is enough to activate *TML*.

Additionally, the early symbiotic responses were analyzed in the *tml-1* mutant. Calcium (Ca^{2+}) spiking is defined as periodic calcium ion changes observed around the nucleus of the host root in response to the symbiotic signal Nod factor. The hypernodulating mutant *sickle*, as well as some mutants in the early symbiotic signaling pathway, showed a defect in or disturbance of the oscillation of Ca^{2+} (Wais et al. 2000, Walker et al. 2000, Oldroyd et al. 2001, Miwa et al. 2006, Sun et al. 2006). However, in the *tml-1* mutant, Ca^{2+} spiking was observed after treatment with Nod factor and the frequency and shape of spikes were normal compared to those in the wild-type plant (Figs. 2.4A-2.4B). This result suggests that *TML* does not affect the early signaling events in nodule symbiosis.

2.3 Discussion

***TML, HAR1* and *CLE-RS1*/*RS2* negatively regulate nodule organogenesis in the same genetic pathway**

In this study, I examined the detailed role of *TML* in AON using *tml* mutants. First, I demonstrated that the *tml* mutant still possesses the potential to develop more nodules whose formation is inhibited at least by another mechanism involving *PLENTY*. This result further supports my proposal that *TML* and *HAR1* act in the same genetic pathway. Because the results suggested that *TML* acts downstream of *HAR1*, I assumed that *TML* is the undiscovered root factor of AON that acts downstream of *CLE-RS1*/*RS2*. The overexpression of *CLE-RS1*/*RS2* in the roots of the *tml* mutant did not affect the number of nodules, indicating that the suppression of nodulation by *CLE-RS1/RS2* requires a functional *TML*.

Suzaki et al. reported that the overexpression of *CLE-RS1*/*RS2* affects auxin accumulation at root cortical cells, resulting in the abortion of cell division (Suzaki et al. 2012), suggesting the relationship among *TML*, auxin accumulation and cortical cell division as the factors downstream of *CLE-RS1*/*RS2*.

Additionally, the results demonstrated that *TML* inhibits the nodule organogenesis induced by the LHK1-mediated cytokinin signaling. Moreover, no abnormalities in the Ca^{2+} spiking were observed in the *tml* mutant. As the Nod factor-induced early symbiotic signaling involving the Ca^{2+} spiking activates the *LHK1*-mediated cytokinin signaling (reviewed in (Madsen et al. 2010, Oldroyd et al. 2011)), these results suggest that the target of AON is not the early symbiotic signaling for the bacterial infection but rather the nodule organ formation.

Taken together, I conclude that *TML* acts at the final stage of AON downstream of

CLE-RS1/*RS2* and *HAR1* to negatively regulate the nodule number by inhibiting the organogenesis induced by the cytokinin signaling.

2.4 Materials and methods

Plant materials and growth conditions

Lotus japonicus ecotype Miyakojima MG-20 was used as the wild type. After overnight water absorption, plants were grown with or without *Mesorhizobium loti* MAFF 30-3099 in autoclaved vermiculite supplemented with Broughton and Dilworth (B&D) solution (Broughton et al. 1971) containing 0.5 mM KNO_3 under 16 hour light/8 hour dark cycles at a light intensity of 150 mE/s/m2 at 22°C in a Biotron LH-300 growth cabinet (Nihon-ika, Osaka, Japan).

Double mutant analysis

For the double mutant analysis, *tml-1* and *plenty* mutants were crossed. From the F2 population, the homozygous double mutants were selected using PCR, which amplified the deleted region in *plenty* and *tml-1*. The number of nodules was counted at 21 dpi. To generate the *tml-1 Snf2* double mutant, I crossed the *tml-1* mutant with the *Snf2* mutant. For *tml-1 Snf2* double-mutant analysis, the *Snf2* mutants were selected from the F2 population based on spontaneous nodule formation and could have been either heterozygous or homozygous for the *snf2* mutation, as the mutant phenotype is dominant. The *tm-1l* genotypes were then checked using PCR, which amplified the region containing the junction of the deleted region in *tml-1*. The number of spontaneous nodules was counted 5 weeks post-germination. All experiments were performed in biological duplicates.

Hairy root transformation

Transgenic hairy roots were induced by Agrobacterium according to the method of

Okamoto (2009). The *CLE-RS1-, CLE-RS2-* or *GUS*-overexpression construct, the *TML* RNAi construct or the *ProTML-GUS* reporter construct was introduced to generate the transgenic hairy root. The GFP driven by the constitutive CaMV 35S promoter in the T-DNA region of the binary vector was used as the reporter for transformation. Plants with transformed roots were placed on vermiculite with B&D medium containing 1 mM KNO_3 in the growth cabinet at 24°C with a photoperiod of 16 hour light and 8 h dark for 6 days before the plants were inoculated with *M. loti.* The total number of nodules on the GFP-positive roots was counted at 21 dpi.

Measurement of Ca^{2+} spiking

Ca^{2+} spiking was measured using a transgenic plant containing the calcium indicator protein yellow cameleon 2.1. The *tml-1* mutant was crossed with the transgenic plant, and a *tml-1* mutant containing YC2.1 (F3) was obtained for calcium imaging. Calcium imaging was performed on the Nikon inverted microscope ECLIPSE Ti equipped with a 40x dry objective (numerical aperture 0.6) as described previously (Ehrhardt et al. 1996). The measurements were analyzed using NIS elements (Nikon, Tokyo, Japan) and Microsoft Excel.

Chapter 3

Molecular cloning and characterization of the *TML* gene

3.1 Introduction

In the model legumes, many genes involved in the legume-*Rhizobium* symbiosis have been identified (reviewed in (Kouchi et al. 2010, Oldroyd et al. 2011)). By 2008, the *L. japonicus* genome project has determined the 315.1 Mbp sequences, which correspond to 67% of the genome (472 Mb) and cover 91.3% of gene-coding regions (Sato et al. 2008). In addition, there are some useful high-density genetic linkage maps and a great number of mapping markers such as simple sequence repeat (SSR) and derived cleaved amplified polymorphic sequence (dCAPS) markers (Hayashi et al. 2001, Sandal et al. 2002, Wang et al. 2008). These infrastructures has accelerated the identification of the gene involved in the nodulation by facilitating the map-based gene cloning of the symbiotic mutants.

The previous study (Magori et al. 2009) and the results in the previous chapter of this thesis (chapter 2) demonstrated that the gene responsible for the hypernodulation phenotype in the *tml* mutant is a root regulator involved in the *HAR1*-mediated long-distance control of nodule numbers. However, it is still the hypothesis based on the observations of the genetic interactions. Therefore, the support to this hypothesis by the molecular biological approaches are necessary. In particular, to unveil its precise function, it is crucial to isolate the gene. In an attempt to localize the gene, the *TML* locus was mapped to the genomic region between the SSR markers TM0805 and TM0356 on chromosome 1 (Magori et al. 2009). In additon to the large deletion alleles produced by carbon-ion-beam irradiation (*tml-1/-2/-3*), I obtained the *tml-4* mutant that is generated by an ethyl methane sulfonate (EMS) mutagenesis. Here, I report the identification and the characterization of the *TML* gene using these alleles. *TML* gene encodes a Kelch repeat-containing F-box protein with two nuclear localization signals (NLSs) and potentially functions in proteasome-mediated degradation of its target protein.

3.2 Results

TML encodes a Kelch repeat-containing F-box protein with two NLSs

To narrow down the candidate region, inverse PCR was performed to identify the deleted regions in the large deletion alleles *tml-1* (formerly *tml* (Magori et al. 2009)), *tml-2* and *tml-3* (Fig. 3.1A). In all three alleles, the breakpoint was attached to repetitive regions. As a result, the deletions began at -2857 bp (*tml-1*), -10382 bp (*tml-2*) and -13822bp (*tml-3*) from the initial codon of the first ORF of the deleted region in *tml-1*. In addition, fine mapping was performed using the EMS allele *tml-4* (formerly *rdh1* (Yokota et al. 2009)). SSR and derived cleaved amplified polymorphic sequence (dCAPS) markers were assessed for co-segregation in 1958 F2 population. Consequently, the *TML* locus was confined to the region between the SSR markers TM0805 and TM2344. Together with the results of the fine mapping and inverse PCR, the *TML* gene locus was delimited to a region of approximately 117 kb (Fig. 3.1A). Because the region was newly assembled in the contig CM0064, I analyzed the sequence by GENSCAN. Of the 21 genes predicted in the region, the whole genome resequencing of *tml-4* using a next generation sequencer found only one non-synonymous single nucleotide alteration in the gene corresponding to the EST sequence (GenBank accession number AK339024), which results in a premature stop codon (Fig. 3.1B). Therefore, to determine whether the loss of the candidate gene is responsible for the *tml* hypernodulation phenotype, I knocked down the expression of the candidate gene in roots by introducing the 340 bp target sequence driven by the *LjUBQ* promoter. I checked the decreased expression of the candidate gene by quantitative real-time RT-PCR (qPCR) analysis. As expected, the number of nodules developed on the candidate gene-silenced roots increased approximately 7.5-fold compared to those on the control roots (Figs. 3.2A-3.2E). In

addition, to verify that this result was not due to an off-target effect, I performed gene silencing triggered by two other sequences of the candidate gene and obtained the same results (Figs. 3.2F-3.2G). These results clearly indicate that the gene corresponding to AK339024 is indeed responsible for the *tml* hypernodulating phenotype.

The sequence analysis revealed that *TML* encodes a Kelch repeat-containing F-box protein with three types of conserved domains: the F-box domain, the Kelch-repeat domain and two NLSs. An F-box domain is a motif that binds to the Skp1 family of proteins (e.g., ASK1 and ASK2 in *A. thaliana*), resulting in the formation of the Skp1 Cullin F-box (SCF) E3 ubiquitin ligase complex. A Kelch-repeat domain is a motif involved in protein-protein interactions. In the well-studied Kelch repeat-containing F-box proteins (i.e., *FKF1* and *LKP2* in *A. thaliana* and *JFK* in *Homo sapiens*), the Kelch-repeat domains are required for the physical interaction with their target proteins (Imaizumi et al. 2005, Sun et al. 2009). Therefore, in the *tml-4* mutant, the transcript is predicted to encode a nonfunctional protein lacking the Kelch-repeat domain (Fig. 3.1B). There are two NLSs predicted upstream and within the F-box domain (Fig. 3.1B). To analyze the subcellular localization of the TML protein, a TML-sGFP fusion protein was expressed in the roots of wild-type plants and the fluorescence of TML-sGFP was observed in the nucleus (Figs. 3.3H-3.3I). Therefore,I conclude that *TML* is a gene encoding a Kelch repeat-containing F-box protein that acts in the nucleus to regulate the nodule number.

To obtain the supplemental information from the orthologs, I conducted a phylogenetic analysis. A BLAST search using the full-length peptide sequence of *L. japonicus* TML (LjTML) as a query against the *G. max* proteome database in the phytozome database (http://www.phytozome.net/) revealed two closely related sequences, i.e.,

Glyma16g06160.1 and Glyma19g25770.1, with amino acid identities of 81 and 80% to LjTML, respectively. In addition, related sequences, including AT3G27150 and AT5G40680, were collected from the comparative gene analysis resource, GreenPhyl (http://greenphyl.cirad.fr/v2/cgi-bin/index.cgi). No orthologs of LjTML were found in *Cyanidioschyzon merolae*, *Ostreococcus tauri* or *Chlamydomonas reinhardtii*. A phylogenetic analysis using the F-box domains of the collected sequences revealed that the TML-related Kelch repeat-containing F-box proteins were widely conserved in embryophytes and were classified into three groups (i.e., the TML clade, the TML-like clade and the basal clade) (Fig. 3.4). LjTML, *G. max TML* orthologs (Glyma16g06160.1 and Glyma19g25770.1) and *A. thaliana TML* orthologs (AT3G27150 and AT5G40680) were included in the TML clade (Figs. 3.5). The phylogenetic tree showed that at least one close ortholog of TML in each legume plant exists in the TML clade, suggesting the conserved function of *TML* among legume species.

TML expression is regulated in both a _Mesorhizobium loti_ infection-dependent and -independent manner

To characterize the *TML* gene,I analyzed the spatial and temporal expression patterns of *TML* using samples from several tissues of wild-type plants 14 days post inoculation (dpi) with rhizobium. The total RNA was extracted from whole roots with nodules, nodules only, roots after the removal of nodules with a razor and shoots. The qPCR analysis showed that the expression was not detected in shoots from nodulated plants nor shoots from plants without nodules (Fig. 3.6A), demonstrating that *TML* is constitutively expressed in the roots and nodules (Fig. 3.6B). Furthermore, I investigated the detailed spatial expression patterns of *TML* using a *ProTML-GUS* reporter construct in which a 6 kb DNA fragment

from the putative translation initiation codon of *TML* was inserted upstream of the *GUS* reporter. I introduced this construct into *L. japonicus* roots by the Agrobacterium-mediated method and performed a histochemical GUS staining analysis. The GUS signal of *ProTML-GUS* was detected in the root apex transition zone (Figs. 3.7A-3.7C). The intensities of the GUS staining gradually reduced toward the basal parts of the root (Fig. 3.7D). This expression pattern at the root tip was not significantly changed by inoculation with *M. loti* or by nitrogen-rich conditions (Figs. 3.7A-3.7C). In addition, I found GUS staining in developing root nodules (Fig. 3.7E) and in nodule primordia before the emergence from roots (Fig. 3.7F). At the earlier stages of nodule organogenesis, GUS expression was not detected in the dividing cortical cells even beneath the epidermal cell with the infection thread (Fig. 3.7G). These results indicate that *TML* expression is regulated in both a *M. loti* infection-dependent and infection-independent manner.

3.3 Discussion

***TML* encodes a Kelch repeat-containing F-box protein**

I narrowed down the candidate region responsible for the *tml* phenotype through fine mapping and identified a candidate gene that has the nonsense mutation in the *tml-4* mutant. I then confirmed that the knockdown of the candidate gene induced the same phenotype as the *tml* mutants. Hence, I finally conclude that the Kelch repeat-containing F-box protein with two NLSs is the *TML* gene.

In *A. thaliana*, among more than 100 Kelch repeat-containing F-box proteins, only five members (i.e., *ZTL*, *FKF1*, *LKP2, AFR,* and *SON1*) are well-characterized. *ZTL*, *FKF1* and *LKP2* act as blue light receptors in the light-regulated growth and development (Ito et al. 2012), *AFR* is involved in the phytochrome A-mediated light signaling (Harmon et al. 2003) and *SON1* acts in the defense response (Kim et al. 2002). Therefore, the findings that *TML* acts in the long-distance control of organogenesis provide novel insight into the function of the Kelch repeat-containing F-box proteins. In this report, Dr. Soyano and I found that the TML protein localizes to the nucleus. The previous study has reported that FKF1 and LKP2 localize to the nucleus (Takase et al. 2011). In addition, ZTL, FKF1 and LKP2 bind to transcription factors (i.e., TOC1 or CDF1) and promote their degradation (Ito et al. 2012). Hence, it is inferred that TML acts in regulating the degradation of the transcription-associated factor involved in nodule formation. Furthermore, the translational products of the closest orthologs of *TML* in *A. thaliana* (AT3G27150 and AT5G40680) are localized to the nucleus and they are reported to bind to the Skp1 family of proteins, a subunit of the ubiquitin E3 ligase complex (Schumann et al. 2011). These data support my hypothesis that TML and its orthologs promote the degradation of transcription-associated factors.

An E3 ligase is involved in the degradation of a specific target protein by poly-ubiquitination. Therefore, *TML* might regulate the stability of a positive and/or negative regulator of nodule organogenesis by mediating poly-ubiquitination and 26S proteasome-dependent degradation.

***TML* is bifunctional in nodulation events.**

The quantitative estimation of the *TML* expression level demonstrated that *TML* is a root-specific gene. Although the *TML* expression level was not altered upon rhizobial infection when detected using qPCR, the detailed investigation of the promoter GUS assay revealed that *TML* is constitutively expressed not only in the root tip but also in the nodules and nodule primordia upon rhizobial infection. The expression pattern of *ProTML-GUS* in root nodule primordia suggests that TML might inhibit the nodule development after the initiation of cortical cell division. This hypothesis is consistent with the appearance of arrested root nodule primordia in roots overexpressing either *CLE-RS1* or *CLE-RS2* genes (Suzaki et al. 2012). However, the expression in root nodule primordia is not sufficient for explaining why the *tml* mutant develops an excessive number of infection threads (Magori et al. 2009). Dr. Soyano and I demonstrated that *TML* is constitutively expressed in the root apex transition zone, which is at the earlier developmental stage than the susceptible region for *M. loti* infection. One hypothesis is that *TML* expressing in the transition zone indirectly inhibits formation of infection threads prior to rhizobial infection. Therefore, *TML* may act bifunctionally, regulating the infection thread development and root nodule formation.

In conclusion, we identify the F-box protein TML as a key factor in maintaining proper nodulation during the final stage of AON.

3.4 Materials and methods

Map-based cloning of the *TML* gene.

The *tml-4* mutant was crossed with Gifu B-129, and F1 hybrids were selfed to obtain F2 seeds. A total of 1,958 F2 plants (614 mutant phenotype (nod++) plants and 1,344 wild-type phenotype (nod+) plants) were scored for the SSR markers TM0001 or TM0805 and BM1852. Among the 614 mutant (nod++) population, the recombinants were further scored for SSR or dCAPS markers C28_1, TM2344, SNP5 and TM2548.

The primers for the dCAPS markers used here are listed below.

C28_1(a 225 bp fragment (MG-20) or 17+208 bp fragment (Gifu B129) were obtained by *Mlu*I digestion)

C28_1_F; 5'-CACCTTAGGAATCAAAGAGCCCTC-3'

C28_1_R; 5'-GTTTGTTTCCGCGTCAACGCG-3'

SNP5 (a 199 bp fragment (MG-20) or 27+172 bp fragments (Gifu B129) were obtained by *Hinf*I digestion)

SNP5_F; 5'-CCTTCACTGTCAACCCCCTC-3'

SNP5_R; 5'-GGACTATGTCTCTTGAAGAGTGTGATT-3'

iPCR-based deletion detection in *tml* deletion alleles

The genomic DNA was isolated from 100 mg of fresh young leaves of each *tml* mutant plant using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) after grinding with a mortar and pestle. A total of 1 μg genomic DNA was digested with restriction enzymes (*Eco*RI or *Mse*I). For the self-ligation reaction, 0.5 μg digested DNA was diluted to 500 μl with T4 ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol and pH 7.5 at 25°C) containing 5 μl ligase (final 4 units/ μl). The reaction

mixture was then incubated overnight at 16°C. A nested PCR was performed using the ligated DNA as a template. The obtained fragment was sequenced to identify the junction sequence.

Constructs for RNA silencing

The 340 bp fragment of the *TML* gene and the 325 bp fragment of the GUS gene were amplified using PCR, cloned into pENTR d-TOPO (Invitrogen, Carlsbad, CA, USA) and subcloned into pUB-GWS-GFP (Maekawa et al. 2008). The hairy root transformation was performed as described above.

The primers used here were

TML RNAi_F; 5'-CACCATGGCCAATAAAAAAGCATT-3' and

TML RNAi_R; 5'-ACATGAACACTGAGGGCTCTTT-3',

which amplified nucleotides 1 - 340 of the *TML* gene, and

GUS RNAi_F; 5'-CACCTGAACCGTTATTACGGAT-3' and

GUS RNAi_R; 5'-CGAGTGAAGATCCCTTTCTTG-3',

which amplified nucleotides 1392-1718 of the GUS gene.

Constructs for the *ProTML-GUS* reporter assay

The 6 kb DNA fragment from the putative translation initiation codon of *TML* was inserted upstream of the *GUS* reporter in pGWB3 (Nakagawa et al. 2007).

The primers used here were

*ProTML*_F; 5'-GAAACACAAACCTCGACAACCACCA-3' and

*ProTML*_R; 5'-CATAATAAGTCAGGTACAGGCAAATGCTTC-3'.

Expression analysis

The total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). First strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). The real-time RT-PCR was performed using an ABI Prism 7000 (Applied Biosystems) with a QuantiTect SYBR Green RT-PCR Kit (Qiagen). Transcript amounts in different samples were normalized to those of *ubiquitin (UBQ)*. The results are provided as the means ± SD of results for independent biological duplicates with technical triplicates.

The primers used here were

TML_qPCR_F; 5'-ACAAACAGCTGGAGCCTAATTC-3',

TML_qPCR_R; 5'-AGAAGCATCAAGCGAGTAAAGC-3',

UBQ_qPCR_F; 5'-TTCACCTTGTGCTCCGTCTTC-3' and

UBQ_qPCR_R; 5'-AACAACAGCACACACAGACAATCC-3'.

Phylogenetic analysis

To examine the phylogenetic relationships, the amino acid sequences of Kelch repeat-containing F-box proteins were downloaded from http://greenphyl.cirad.fr/v2/cgi-bin/index.cgi (family 36739) and http://www.phytozome.net/ (2 EST sequences from *M. truncatula*, 1 genomic sequence from *P. vulgaris* and 2 genomic sequences from *Physcomitrella patens*). All nucleotide data were translated into peptide sequence. The pseudogenes and fragmented ORFs were removed, resulting in 7 genes from *A. thaliana*, 3 from *Brachypodium distachyon*, 3 from *Carica papaya*, 17 from *G. max*, 4 from *M. truncatula*, 4 from *O. sativa*, 5 from *Populus trichocarpa*, 5 from *Ricinus communis*, 5 from *Sorghum bicolor*, 7 from *Selaginella moellendorffii*, 6 from *Vitis vinifera* and 7 from *Zea mays*. The peptide sequences were

analyzed using ClustalW multiple sequence alignment programs. The phylogenic tree was calculated using the neighbor-joining method after the alignment of the sequences. A bootstrap analysis was performed on 1,000 random samples taken from the multiple alignment. The tree is shown with bootstrap confidence values expressed in percentage.

Chapter 4

General discussion

4.1 Mode of action of AON

Legumes develop the root nodules to establish and to sustain proper symbiotic interactions with the nitrogen-fixing soil bacteria collectively called rhizobia. Rhizobia provide the host legumes with the ammonium as a nitrogen source fixed from the air. In return, host legumes provide rhizobia with the carbon source produced through the photosynthesis. Therefore, this relationship is mutually beneficial symbiosis.

There are increasing evidences that the legumes have evolved the preprogrammed signaling pathways to establish and to keep the adequate symbiotic balance. NF is the signaling molecule synthesized and secreted by rhizobia, which triggers the nodulation signaling pathways in the host legumes. Studies on the symbiotic mutants have revealed that NF-induced nodulation signaling pathway is mediated by several components such as the putative receptor-like kinases with extracellular LysM domains (e.g. *NFR1*/*NFR5* in *L. japonicus*) (Madsen et al. 2003, Radutoiu et al. 2003), the leucine-rich repeat receptor kinase (*SymRK*) (Endre et al. 2002, Stracke et al. 2002), cation channels (*Castor* and *Pollux*) (Ane et al. 2004, Imaizumi-Anraku et al. 2005, Charpentier et al. 2008), nucleoporins (*Nup85* and *Nup133*) (Kanamori et al. 2006, Saito et al. 2007, Groth et al. 2010), a calcium calmodulin-dependent protein kinase (*CCaMK*) (Levy et al. 2004, Mitra et al. 2004, Tirichine et al. 2006) and a nuclear localized coil–coil protein (*Cyclops*) (Messinese et al. 2007, Yano et al. 2008). Downstream of the signaling pathway, putative transcription factors (nodulation signalling pathway 1 (*NSP1*), *NSP2* (Kalo et al. 2005, Smit et al. 2005, Heckmann et al. 2006, Murakami et al. 2006), ERF required for nodulation 1 (*ERN1*) (Andriankaja et al. 2007, Middleton et al. 2007, Cerri et al. 2012), nodule inception (*NIN*) (Schauser et al. 1999, Marsh et al. 2007)) are activated to initiate the morphological changes for bacterial infection and nodule organogenesis. Even if this

symbiosis is of advantage for the host legumes, the nodule formation is an enegetically expensive process. Hence, overproducing nodules is harmful for the host growth, which is corresponding to the dwarf phenotype of the mutants that lack the nodule-number regulation (Sagan et al. 1996, Szczyglowski et al. 1998, Krusell et al. 2002, Nishimura et al. 2002, Penmetsa et al. 2003, Searle et al. 2003, Oka-Kira et al. 2005, Schnabel et al. 2005, Magori et al. 2009, Miyazawa et al. 2010, Schnabel et al. 2010, Krusell et al. 2011). To avoid this unfavorable effect, legumes developed the negative feedback regulation called AON. In *L. japonicus*, AON is primarily mediated by CLE-family peptides named CLE-RS1/RS2, which are the putative root-derived signals transporting to the shoot (Okamoto et al. 2009). Subsequently, the signal is mediated by the LRR-RKs (i.e. *HAR1*, *CLV2* and *KLV* (Krusell et al. 2002, Nishimura et al. 2002, Miyazawa et al. 2010, Krusell et al. 2011)) These LRR-RKs are orthologous to the *CLV1*, *CLV2* and *RPK2* in *A. thaliana*, which act to maintain stem cells and the receptors for the CLE peptides (Ogawa et al. 2008, Kinoshita et al. 2010).

The grafting experiments have shown that the LRR-RKs function in shoots, indicating that there are long-distance communications between roots and shoots in the course of this negative feedback regulation of the nodulation. It is a possible idea that legumes regulate the number of nodules in tune with their body size or in response to the environment stresses. However, the precise function of AON is largely unknown because the root regulator is not molecularly isolated.

In this thesis, I characterized the *TML* gene by the molecular genetic analyses and identified the *TML* gene by the map-based cloning and the gene knock-down analysis. The detailed analysis of double mutants has shown that *TML* and *HAR1* act in the same genetic pathway. In addition, overexpression of *CLE-RS1*/*RS2* in the *tml* mutant

background did not affect the number of nodules compared to the control GUS overexpressing root in the *tml* mutant background, indicating that the suppression of nodulation by *CLE-RS1/RS2* requires a functional *TML*. Furthermore, double mutant analysis using the *tml-1 Snf2* mutant revealed that *TML* inhibits the nodule organogenesis induced by the LHK1-mediated cytokinin signaling. In addition to these results, Suzaki et al. has shown that *CLE-RS1*/*RS2* affects auxin accumulation at root cortical cells, resulting in the abortion of cell division. Taken together, AON inhibits the nodule organogenesis by activating the *CLE-RS1/RS2*, *HAR1* and *TML* (Fig. 3.8A).

Through the fine mapping, the deep sequencing by the NGS and the RNAi experiments, I found that the Kelch repeat-containing F-box protein with two NLSs is the *TML* gene. An E3 ligase is involved in the degradation of a specific target protein by poly-ubiquitination. Therefore, *TML* might regulate the stability of a positive and/or negative regulator of nodule organogenesis by mediating poly-ubiquitination and 26S proteasome-dependent degradation (Fig. 3.8B). Because the 26S proteasome degrades the ubiquitinated proteins even if the process consumes ATP as an energy source, the ubiquitin proteasome-mediated degradation is often a biologically meaningful process. Indeed, in most of the known phytohormones (e.g., auxin, jasmonate, gibberellin, strigolactone and salicylic acid) the signalings are mediated by the components of E3 ligase-substrate complexes (Gomi et al. 2004, Dharmasiri et al. 2005, Kepinski et al. 2005, Umehara et al. 2008, Yan et al. 2009, Fu et al. 2012, Yoshida et al. 2012). Intriguingly, the strigolactone signaling that is mediated by the LRR-type F-box protein has recently been reported to positively regulate the number of nodules, contrary to *TML* (Foo et al. 2012). As it has been suggested that SDI is an amphiphilic and low-molecular-weight compound, extrapolating from the relation between

phytohormones and F-box proteins, it is possible that SDI reception triggers the activation of the TML protein. As TML is an F-box protein that functions during the final stage of AON, identifying its target will unveil a detailed mechanism for establishing the well-suited symbiotic relationship. In conclusion, I concluded that *TML* encoding the the Kelch repeat-containing F-box protein with two NLSs, which negatively regulates the nodule number downstream of *CLE-RS1/RS2* and *HAR1* by suppressing the LHK1-mediated cytokinin signaling for nodule organogenesis.

4.2 The function of *TML* in nodule number regulation might be co-opted from another systemic regulation

TML-related F-box genes are conserved among land plants and are classified into three groups: the basal clade, the TML clade and the TML-like clade. In *L. japonicus*, *TML* acts to coordinate the legume-rhizobium symbiosis. A phylogenetic analysis revealed that other legumes have at least one *TML* ortholog that belongs to the TML clade, indicating that these orthologs might play a role in nodule number regulation. However, angiosperms other than legumes have *TML* orthologs that belong to TML clade, suggesting that *TML* orthologs have a general function.

A recent study reported that the *TML* ortholog in *A. thaliana*, AT3G27150, is regulated by the microRNA miR2111 in response to the nutrient status. Interestingly, though AT3G27150 is expressed specifically in the roots, a large quantity of miR2111 was detected in the phloem sap during Pi limitation, suggesting that AT3G27150 acts in the systemic regulation responsive to environmental stresses (Pant et al. 2009). In addition, a recent study has shown that root development is systemically regulated in response to the NO_3^- status in the root (Ruffel et al. 2011). Moreover, several reports have demonstrated that AON is activated in response to nitrogen as an environmental cue (Okamoto et al. 2009, Jeudy et al. 2010, Reid et al. 2011). Furthermore, *LjTML* is constitutively expressed in the root tips. Taken together, *TML* orthologs in angiosperms might act in the root in the systemic environmental response.

Interestingly, no *TML* orthologs belonging to the TML clade were found in monocots (Fig. 3.4). The root system architecture differs between monocots and eudicots, especially with regard to the existence of lateral roots. Several reports on the relation

between lateral root formation and nodule organogenesis (Mathesius et al. 2000, Gonzalez-Rizzo et al. 2006, Bishopp et al. 2009, Kuppusamy et al. 2009, Yendrek et al. 2010) suggest that the *TML* clade genes might regulate the root system architecture that is required in eudicots.

Considering these instances, it has been suggested that the systemic and long-distance regulation of root development via *TML* orthologs has been co-opted to root nodule organogenesis in legume plants. Thus, I propose a hypothesis in which AON regulates nodule organogenesis through the 26S proteasome pathway and that this mechanism has evolved by co-opting the systemic regulation of root development responsive to environmental stress.

Concluding remarks

In this dissertation, I characterized and identified the gene of the hypernodulating mutant *too much love* (*tml*) of *Lotus japonicus*. In the future, the identification of the proteins physically interacting with TML will clarify the function of *TML* at the molecular level. And I sincerely hope that this research will facilitate the big progress in this field.

References

Akashi, K., H. Yamaya and Y. Arima (2010). Shoot-synthesized nodulation-restricting substances of wild-type soybean present in two different high-performance liquid chromatography peaks of the ethanol-soluble medium-polarity fraction. *Soil Sci. Plant Nutr.* 56: 399-406.

Andriankaja, A., A. Boisson-Dernier, L. Frances, L. Sauviac, A. Jauneau, D. G. Barker, et al. (2007). AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. *Plant Cell* 19: 2866-2885.

Ane, J. M., G. B. Kiss, B. K. Riely, R. V. Penmetsa, G. E. Oldroyd, C. Ayax, et al. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. *Science* 303: 1364-1367.

Ariel, F., M. Brault-Hernandez, C. Laffont, E. Huault, M. Brault, J. Plet, et al. (2012). Two direct targets of cytokinin signaling regulate symbiotic nodulation in *Medicago truncatula*. *Plant Cell* 24: 3838-3852.

Bhuvaneswari, T. V., B. G. Turgeon and W. D. Bauer (1980). Early Events in the Infection of Soybean (*Glycine max* L. Merr) by *Rhizobium japonicum*: I. LOCALIZATION OF INFECTIBLE ROOT CELLS. *Plant Physiol* 66: 1027-1031.

Bishopp, A., H. Help and Y. Helariutta (2009). Cytokinin signaling during root development. *Int Rev Cell Mol Biol* 276: 1-48.

Broughton, W. J. and M. J. Dilworth (1971). Control of leghaemoglobin synthesis in snake beans. *Biochem J* 125: 1075-1080.

Caetano-Anolles, G. and P. M. Gresshoff (1991). Plant genetic control of nodulation. *Annu Rev Microbiol* 45: 345-382.

Canfield, D. E., A. N. Glazer and P. G. Falkowski (2010). The evolution and future of Earth's nitrogen cycle. *Science* 330: 192-196.

Catford, J. G., C. Staehelin, S. Lerat, Y. Piche and H. Vierheilig (2003). Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. *J Exp Bot* 54: 1481-1487.

Cerri, M. R., L. Frances, T. Laloum, M. C. Auriac, A. Niebel, G. E. Oldroyd, et al. (2012). *Medicago truncatula* ERN Transcription Factors: Regulatory Interplay with NSP1/NSP2 GRAS Factors and Expression Dynamics throughout Rhizobial Infection. *Plant Physiol* 160: 2155-2172.

Charpentier, M., R. Bredemeier, G. Wanner, N. Takeda, E. Schleiff and M. Parniske (2008). Lotus japonicus CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. *Plant Cell* 20: 3467-3479.

Chu, H., Q. Qian, W. Liang, C. Yin, H. Tan, X. Yao, et al. (2006). The *floral organ number4* gene encoding a putative ortholog of Arabidopsis CLAVATA3 regulates apical meristem size in rice. *Plant Physiol* 142: 1039-1052.

Cooper, J. B. and S. R. Long (1994). Morphogenetic Rescue of Rhizobium meliloti Nodulation Mutants by trans-Zeatin Secretion. *Plant Cell* 6: 215-225.

D'Haeze, W. and M. Holsters (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* 12: 79R-105R.

Delves, A. C., A. Mathews, D. A. Day, A. S. Carter, B. J. Carroll and P. M. Gresshoff (1986). Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors. *Plant Physiol* 82: 588-590.

Dharmasiri, N., S. Dharmasiri and M. Estelle (2005). The F-box protein TIR1 is an auxin receptor. *Nature* 435: 441-445.

Ehrhardt, D. W., R. Wais and S. R. Long (1996). Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* 85: 673-681.

Endre, G., A. Kereszt, Z. Kevei, S. Mihacea, P. Kalo and G. B. Kiss (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature* 417: 962-966.

Ferguson, B. J., A. Indrasumunar, S. Hayashi, M. H. Lin, Y. H. Lin, D. E. Reid, et al. (2010). Molecular analysis of legume nodule development and autoregulation. *J Integr Plant Biol* 52: 61-76.

Foo, E., K. Yoneyama, C. J. Hugill, L. J. Quittenden and J. B. Reid (2012). Strigolactones and the Regulation of Pea Symbioses in Response to Nitrate and Phosphate Deficiency. *Mol Plant* 6: 76-87.

Fu, Z. Q., S. Yan, A. Saleh, W. Wang, J. Ruble, N. Oka, et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486: 228-232.

Galloway, J. N., F. J. Dentener, D. G. Capone, E. W. Boyer, R. W. Howarth, S. P. Seitzinger, et al. (2004). Nitrogen Cycles: Past, Present, and Future. *Biogeochemistry* 70: 153-226.

George, M. L. C. (1992). Nodulation Suppression by Rhizobium leguminosarum bv. phaseoli in Bean Split-root Systems. *Symbiosis* 12: 95-105.

Geurts, R., E. Fedorova and T. Bisseling (2005). Nod factor signaling genes and their function in the early stages of Rhizobium infection. *Curr Opin Plant Biol* 8: 346-352.

Gomi, K., A. Sasaki, H. Itoh, M. Ueguchi-Tanaka, M. Ashikari, H. Kitano, et al. (2004). GID2, an F-box subunit of the SCF E3 complex, specifically interacts with phosphorylated SLR1 protein and regulates the gibberellin-dependent degradation of SLR1 in rice. *Plant J* 37: 626-634.

Gonzalez-Rizzo, S., M. Crespi and F. Frugier (2006). The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. *Plant Cell* 18: 2680-2693.

Graham, P. H. and C. P. Vance (2003). Legumes: importance and constraints to greater use. *Plant Physiol* 131: 872-877.

Groth, M., N. Takeda, J. Perry, H. Uchida, S. Draxl, A. Brachmann, et al. (2010). NENA, a Lotus japonicus homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. *Plant Cell* 22: 2509-2526.

Harmon, F. G. and S. A. Kay (2003). The F box protein AFR is a positive regulator of phytochrome A-mediated light signaling. *Curr Biol* 13: 2091-2096.

Hayashi, M., A. Miyahara, S. Sato, T. Kato, M. Yoshikawa, M. Taketa, et al. (2001). Construction of a genetic linkage map of the model legume Lotus japonicus using an intraspecific F2 population. *DNA Res* 8: 301-310.

Heckmann, A. B., F. Lombardo, H. Miwa, J. A. Perry, S. Bunnewell, M. Parniske, et al. (2006). Lotus japonicus nodulation requires two GRAS domain regulators, one of which

is functionally conserved in a non-legume. *Plant Physiol* 142: 1739-1750.

Imaizumi, T., T. F. Schultz, F. G. Harmon, L. A. Ho and S. A. Kay (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of *CONSTANS* in *Arabidopsis*. *Science* 309: 293-297.

Imaizumi-Anraku, H., N. Takeda, M. Charpentier, J. Perry, H. Miwa, Y. Umehara, et al. (2005). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433: 527-531.

Ito, S., Y. H. Song and T. Imaizumi (2012). LOV domain-containing F-box proteins: light-dependent protein degradation modules in *Arabidopsis*. *Mol Plant* 5: 573-582.

Jeudy, C., S. Ruffel, S. Freixes, P. Tillard, A. L. Santoni, S. Morel, et al. (2010). Adaptation of *Medicago truncatula* to nitrogen limitation is modulated via local and systemic nodule developmental responses. *New Phytol* 185: 817-828.

Kalo, P., C. Gleason, A. Edwards, J. Marsh, R. M. Mitra, S. Hirsch, et al. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science* 308: 1786-1789.

Kanamori, N., L. H. Madsen, S. Radutoiu, M. Frantescu, E. M. Quistgaard, H. Miwa, et al. (2006). A nucleoporin is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proc Natl Acad Sci U S A* 103: 359-364.

Kepinski, S. and O. Leyser (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* 435: 446-451.

Kim, H. S. and T. P. Delaney (2002). *Arabidopsis* SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *Plant Cell* 14: 1469-1482.

Kinoshita, A., S. Betsuyaku, Y. Osakabe, S. Mizuno, S. Nagawa, Y. Stahl, et al. (2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis. *Development* 137: 3911-3920.

Kosslak, R. M. and B. B. Bohlool (1984). Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side.

Plant Physiol 75: 125-130.

Kouchi, H., H. Imaizumi-Anraku, M. Hayashi, T. Hakoyama, T. Nakagawa, Y. Umehara, et al. (2010). How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. *Plant Cell Physiol* 51: 1381-1397.

Kraiser, T., D. E. Gras, A. G. Gutierrez, B. Gonzalez and R. A. Gutierrez (2011). A holistic view of nitrogen acquisition in plants. *J Exp Bot* 62: 1455-1466.

Krusell, L., L. H. Madsen, S. Sato, G. Aubert, A. Genua, K. Szczyglowski, et al. (2002). Shoot control of root development and nodulation is mediated by a receptor-like kinase. *Nature* 420: 422-426.

Krusell, L., N. Sato, I. Fukuhara, B. E. Koch, C. Grossmann, S. Okamoto, et al. (2011). The Clavata2 genes of pea and Lotus japonicus affect autoregulation of nodulation. *Plant J* 65: 861-871.

Kuppusamy, K. T., S. Ivashuta, B. Bucciarelli, C. P. Vance, J. S. Gantt and K. A. Vandenbosch (2009). Knockdown of *CELL DIVISION CYCLE16* reveals an inverse relationship between lateral root and nodule numbers and a link to auxin in *Medicago truncatula*. *Plant Physiol* 151: 1155-1166.

Levy, J., C. Bres, R. Geurts, B. Chalhoub, O. Kulikova, G. Duc, et al. (2004). A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303: 1361-1364.

Li, L., M. I. Stefan and N. Le Novere (2012). Calcium input frequency, duration and amplitude differentially modulate the relative activation of calcineurin and CaMKII. *PLoS One* 7: e43810.

Lim, C. W., Y. W. Lee and C. H. Hwang (2011). Soybean nodule-enhanced CLE peptides in roots act as signals in GmNARK-mediated nodulation suppression. *Plant Cell Physiol* 52: 1613-1627.

Lin, Y. H., B. J. Ferguson, A. Kereszt and P. M. Gresshoff (2010). Suppression of hypernodulation in soybean by a leaf-extracted, NARK- and Nod factor-dependent, low molecular mass fraction. *New Phytol* 185: 1074-1086.

Lin, Y. H., M. H. Lin, P. M. Gresshoff and B. J. Ferguson (2011). An efficient

petiole-feeding bioassay for introducing aqueous solutions into dicotyledonous plants. *Nat Protoc* 6: 36-45.

Lohar, D. P., J. E. Schaff, J. G. Laskey, J. J. Kieber, K. D. Bilyeu and D. M. Bird (2004). Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. *Plant J* 38: 203-214.

Madsen, E. B., L. H. Madsen, S. Radutoiu, M. Olbryt, M. Rakwalska, K. Szczyglowski, et al. (2003). A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425: 637-640.

Madsen, L. H., L. Tirichine, A. Jurkiewicz, J. T. Sullivan, A. B. Heckmann, A. S. Bek, et al. (2010). The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. *Nat Commun* 1: 10.

Maekawa, T., M. Kusakabe, Y. Shimoda, S. Sato, S. Tabata, Y. Murooka, et al. (2008). Polyubiquitin promoter-based binary vectors for overexpression and gene silencing in Lotus japonicus. *Mol Plant Microbe Interact* 21: 375-382.

Magori, S. and M. Kawaguchi (2010). Analysis of two potential long-distance signaling molecules, LjCLE-RS1/2 and jasmonic acid, in a hypernodulating mutant too much love. *Plant Signal Behav* 5: 403-405.

Magori, S., E. Oka-Kira, S. Shibata, Y. Umehara, H. Kouchi, Y. Hase, et al. (2009). *Too much love*, a root regulator associated with the long-distance control of nodulation in Lotus japonicus. *Mol Plant Microbe Interact* 22: 259-268.

Marsh, J. F., A. Rakocevic, R. M. Mitra, L. Brocard, J. Sun, A. Eschstruth, et al. (2007). *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol* 144: 324-335.

Marui, H., T. Nagafuku, Y. Shinogi, N. Yasufuku, K. Omine, T. Kobayashi, et al. (2012). Soil Physical Properties to Grow the Wild Licorice at Semi-arid Area in Mongolia. *J Arid Land Stud* 22: 33-36.

Mathesius, U., J. J. Weinman, B. G. Rolfe and M. A. Djordjevic (2000). Rhizobia can induce nodules in white clover by "hijacking" mature cortical cells activated during lateral root development. *Mol Plant Microbe Interact* 13: 170-182.

Maunoury, N., M. Redondo-Nieto, M. Bourcy, W. Van de Velde, B. Alunni, P. Laporte, et al. (2010). Differentiation of symbiotic cells and endosymbionts in Medicago truncatula nodulation are coupled to two transcriptome-switches. *PLoS One* 5: e9519.

Messinese, E., J. H. Mun, L. H. Yeun, D. Jayaraman, P. Rouge, A. Barre, et al. (2007). A novel nuclear protein interacts with the symbiotic DMI3 calcium- and calmodulin-dependent protein kinase of *Medicago truncatula*. *Mol Plant Microbe Interact* 20: 912-921.

Middleton, P. H., J. Jakab, R. V. Penmetsa, C. G. Starker, J. Doll, P. Kalo, et al. (2007). An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. *Plant Cell* 19: 1221-1234.

Mitra, R. M., C. A. Gleason, A. Edwards, J. Hadfield, J. A. Downie, G. E. Oldroyd, et al. (2004). A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. *Proc Natl Acad Sci U S A* 101: 4701-4705.

Miwa, H., J. Sun, G. E. Oldroyd and J. A. Downie (2006). Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of *Lotus japonicus*. *Mol Plant Microbe Interact* 19: 914-923.

Miyazawa, H., E. Oka-Kira, N. Sato, H. Takahashi, G. J. Wu, S. Sato, et al. (2010). The receptor-like kinase KLAVIER mediates systemic regulation of nodulation and non-symbiotic shoot development in *Lotus japonicus*. *Development* 137: 4317-4325.

Mortier, V., G. Den Herder, R. Whitford, W. Van de Velde, S. Rombauts, K. D'Haeseleer, et al. (2010). CLE peptides control *Medicago truncatula* nodulation locally and systemically. *Plant Physiol* 153: 222-237.

Mortier, V., B. A. Fenta, C. Martens, S. Rombauts, M. Holsters, K. Kunert, et al. (2011). Search for nodulation-related CLE genes in the genome of *Glycine max*. *J Exp Bot* 62: 2571-2583.

Murakami, Y., H. Miwa, H. Imaizumi-Anraku, H. Kouchi, J. A. Downie, M. Kawaguchi, et al. (2006). Positional cloning identifies Lotus japonicus NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. *DNA Res* 13: 255-265.

Murray, J. D., B. J. Karas, S. Sato, S. Tabata, L. Amyot and K. Szczyglowski (2007). A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. *Science* 315: 101-104.

Nakagawa, T., T. Kurose, T. Hino, K. Tanaka, M. Kawamukai, Y. Niwa, et al. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 104: 34-41.

Nishimura, R., M. Hayashi, G. J. Wu, H. Kouchi, H. Imaizumi-Anraku, Y. Murakami, et al. (2002). HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420: 426-429.

Nutman, P. S. (1952). Studies on the Physiology of Nodule Formation. *Annals of Botany* 16: 79-103.

Ogawa, M., H. Shinohara, Y. Sakagami and Y. Matsubayashi (2008). Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. *Science* 319: 294.

Oka-Kira, E., K. Tateno, K. Miura, T. Haga, M. Hayashi, K. Harada, et al. (2005). *klavier* (*klv*), a novel hypernodulation mutant of *Lotus japonicus* affected in vascular tissue organization and floral induction. *Plant J* 44: 505-515.

Okamoto, S., E. Ohnishi, S. Sato, H. Takahashi, M. Nakazono, S. Tabata, et al. (2009). Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. *Plant Cell Physiol* 50: 67-77.

Oldroyd, G. E., E. M. Engstrom and S. R. Long (2001). Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *Plant Cell* 13: 1835-1849.

Oldroyd, G. E., J. D. Murray, P. S. Poole and J. A. Downie (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45: 119-144.

Olsson, J. E., P. Nakao, B. B. Bohlool and P. M. Gresshoff (1989). Lack of Systemic Suppression of Nodulation in Split Root Systems of Supernodulating Soybean (*Glycine max* [L.] Merr.) Mutants. *Plant Physiol* 90: 1347-1352.

Pant, B. D., M. Musialak-Lange, P. Nuc, P. May, A. Buhtz, J. Kehr, et al. (2009). Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing.

Plant Physiol 150: 1541-1555.

Penmetsa, R. V., J. A. Frugoli, L. S. Smith, S. R. Long and D. R. Cook (2003). Dual genetic pathways controlling nodule number in *Medicago truncatula*. *Plant Physiol* 131: 998-1008.

Perret, X., C. Staehelin and W. J. Broughton (2000). Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* 64: 180-201.

Pierce, M. and W. D. Bauer (1983). A rapid regulatory response governing nodulation in soybean. *Plant Physiol* 73: 286-290.

Radutoiu, S., L. H. Madsen, E. B. Madsen, H. H. Felle, Y. Umehara, M. Gronlund, et al. (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585-592.

Reid, D. E., B. J. Ferguson and P. M. Gresshoff (2011). Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. *Mol Plant Microbe Interact* 24: 606-618.

Ruffel, S., G. Krouk, D. Ristova, D. Shasha, K. D. Birnbaum and G. M. Coruzzi (2011). Nitrogen economics of root foraging: transitive closure of the nitrate-cytokinin relay and distinct systemic signaling for N supply vs. demand. *Proc Natl Acad Sci U S A* 108: 18524-18529.

Sagan, M. and G. Duc (1996). *Sym28* and *Sym29*, two new genes involved in regulation of nodulation in pea (*Pisum sativum* L.). *Symbiosis* 20: 229-245.

Saito, K., M. Yoshikawa, K. Yano, H. Miwa, H. Uchida, E. Asamizu, et al. (2007). NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus. *Plant Cell* 19: 610-624.

Sandal, N., L. Krusell, S. Radutoiu, M. Olbryt, A. Pedrosa, S. Stracke, et al. (2002). A genetic linkage map of the model legume Lotus japonicus and strategies for fast mapping of new loci. *Genetics* 161: 1673-1683.

Sanders, D., C. Brownlee and J. F. Harper (1999). Communicating with calcium. *Plant Cell* 11: 691-706.

Sargent, L., S. Z. Huang, B. G. Rolfe and M. A. Djordjevic (1987). Split-Root Assays

Using Trifolium subterraneum Show that Rhizobium Infection Induces a Systemic Response That Can Inhibit Nodulation of Another Invasive Rhizobium Strain. *Appl Environ Microbiol* 53: 1611-1619.

Sato, S., Y. Nakamura, T. Kaneko, E. Asamizu, T. Kato, M. Nakao, et al. (2008). Genome structure of the legume, Lotus japonicus. *DNA Res* 15: 227-239.

Schauser, L., A. Roussis, J. Stiller and J. Stougaard (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402: 191-195.

Schnabel, E., E. P. Journet, F. de Carvalho-Niebel, G. Duc and J. Frugoli (2005). The *Medicago truncatula SUNN* gene encodes a *CLV1*-like leucine-rich repeat receptor kinase that regulates nodule number and root length. *Plant Mol Biol* 58: 809-822.

Schnabel, E., A. Mukherjee, L. Smith, T. Kassaw, S. Long and J. Frugoli (2010). The *lss* supernodulation mutant of *Medicago truncatula* reduces expression of the *SUNN* gene. *Plant Physiol* 154: 1390-1402.

Schumann, N., A. Navarro-Quezada, K. Ullrich, C. Kuhl and M. Quint (2011). Molecular evolution and selection patterns of plant F-box proteins with C-terminal kelch repeats. *Plant Physiol* 155: 835-850.

Searle, I. R., A. E. Men, T. S. Laniya, D. M. Buzas, I. Iturbe-Ormaetxe, B. J. Carroll, et al. (2003). Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. *Science* 299: 109-112.

Sheng, C. and J. E. Harper (1997). Shoot versus Root Signal Involvement in Nodulation and Vegetative Growth in Wild-Type and Hypernodulating Soybean Genotypes. *Plant Physiol* 113: 825-831.

Singleton, P. W. (1983). A Split-root Growth System For Evaluating The Effect Of Salinity On Components Of The Soybean *Rhizobium Japonicum* Symbiosis. *Crop Sci.* 23: 259-262.

Smit, P., J. Raedts, V. Portyanko, F. Debelle, C. Gough, T. Bisseling, et al. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. *Science* 308: 1789-1791.

Stracke, S., C. Kistner, S. Yoshida, L. Mulder, S. Sato, T. Kaneko, et al. (2002). A plant

receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417: 959-962.

Sun, J., V. Cardoza, D. M. Mitchell, L. Bright, G. Oldroyd and J. M. Harris (2006). Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. *Plant J* 46: 961-970.

Sun, L., L. Shi, W. Li, W. Yu, J. Liang, H. Zhang, et al. (2009). JFK, a Kelch domain-containing F-box protein, links the SCF complex to p53 regulation. *Proc Natl Acad Sci U S A* 106: 10195-10200.

Suzaki, T., M. Ohneda, T. Toriba, A. Yoshida and H. Y. Hirano (2009). *FON2 SPARE1* redundantly regulates floral meristem maintenance with *FLORAL ORGAN NUMBER2* in rice. *PLoS Genet* 5: e1000693.

Suzaki, T., K. Yano, M. Ito, Y. Umehara, N. Suganuma and M. Kawaguchi (2012). Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. *Development* 139: 3997-4006.

Suzaki, T., A. Yoshida and H. Y. Hirano (2008). Functional diversification of CLAVATA3-related CLE proteins in meristem maintenance in rice. *Plant Cell* 20: 2049-2058.

Suzuki, A., H. Hara, T. Kinoue, M. Abe, T. Uchiumi, K. Kucho, et al. (2008). Split-root study of autoregulation of nodulation in the model legume *Lotus japonicus*. *J Plant Res* 121: 245-249.

Szczyglowski, K., R. S. Shaw, J. Wopereis, S. Copeland, D. Hamburger, B. Kasiborski, et al. (1998). Nodule Organogenesis and Symbiotic Mutants of the Model Legume *Lotus japonicus*. *Molecular Plant-Microbe Interactions* 11: 684-697.

Takase, T., Y. Nishiyama, H. Tanihigashi, Y. Ogura, Y. Miyazaki, Y. Yamada, et al. (2011). *LOV KELCH PROTEIN2* and *ZEITLUPE* repress Arabidopsis photoperiodic flowering under non-inductive conditions, dependent on *FLAVIN-BINDING KELCH REPEAT F-BOX1*. *Plant J* 67: 608-621.

Tirichine, L., H. Imaizumi-Anraku, S. Yoshida, Y. Murakami, L. H. Madsen, H. Miwa, et al. (2006). Deregulation of a Ca2+/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441: 1153-1156.

Tirichine, L., N. Sandal, L. H. Madsen, S. Radutoiu, A. S. Albrektsen, S. Sato, et al. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* 315: 104-107.

Truchet, G., P. Roche, P. Lerouge, J. Vasse, S. Camut, F. de Billy, et al. (1991). Sulphated lipo-oligosaccharide signals of Rhizobium meliloti elicit root nodule organogenesis in alfalfa. *Nature* 351: 670-673.

Umehara, M., A. Hanada, S. Yoshida, K. Akiyama, T. Arite, N. Takeda-Kamiya, et al. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature* 455: 195-200.

Wais, R. J., C. Galera, G. Oldroyd, R. Catoira, R. V. Penmetsa, D. Cook, et al. (2000). Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc Natl Acad Sci U S A* 97: 13407-13412.

Walker, S. A., V. Viprey and J. A. Downie (2000). Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by nod factors and chitin oligomers. *Proc Natl Acad Sci U S A* 97: 13413-13418.

Wang, X., S. Sato, S. Tabata and S. Kawasaki (2008). A high-density linkage map of Lotus japonicus based on AFLP and SSR markers. *DNA Res* 15: 323-332.

Wopereis, J., E. Pajuelo, F. B. Dazzo, Q. Jiang, P. M. Gresshoff, F. J. De Bruijn, et al. (2000). Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *Plant J* 23: 97-114.

Yamaya, H. and Y. Arima (2010). Evidence that a shoot-derived substance is involved in regulation of the super-nodulation trait in soybean. *Soil Sci. Plant Nutr.* 56: 115-122.

Yamaya, H. and Y. Arima (2010). Shoot-synthesized nodulation-restricting substances are present in the medium-polarity fraction of shoot extracts from wild-type soybean plants *Soil Sci. Plant Nutr.* 56: 418-421.

Yan, J., C. Zhang, M. Gu, Z. Bai, W. Zhang, T. Qi, et al. (2009). The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell* 21: 2220-2236.

Yano, K., S. Yoshida, J. Muller, S. Singh, M. Banba, K. Vickers, et al. (2008).

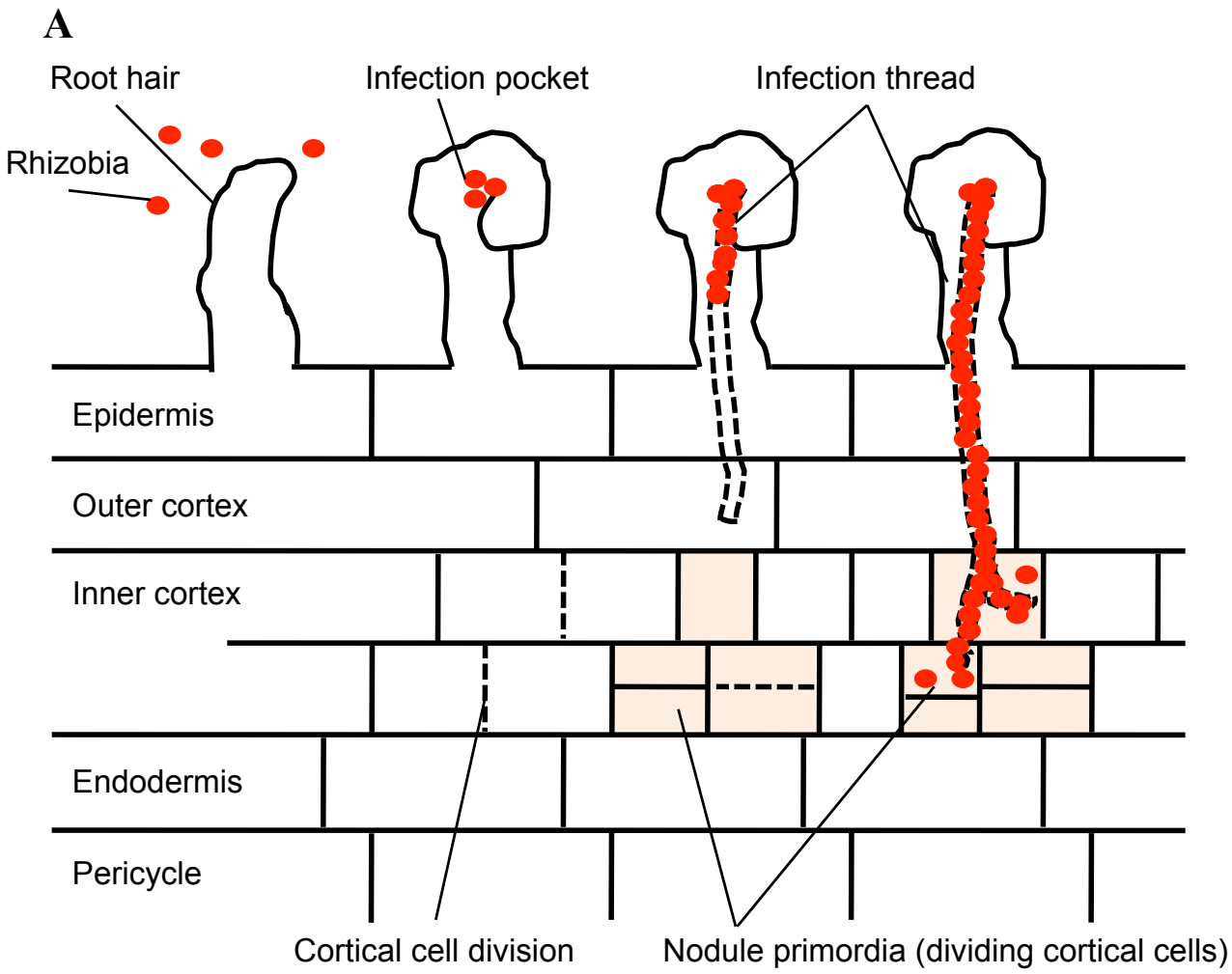
CYCLOPS, a mediator of symbiotic intracellular accommodation. *Proc Natl Acad Sci U S A* 105: 20540-20545.

Yendrek, C. R., Y. C. Lee, V. Morris, Y. Liang, C. I. Pislariu, G. Burkart, et al. (2010). A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. *Plant J* 62: 100-112.

Yokota, K., Y. Y. Li, M. Hisatomi, Y. Wang, K. Ishikawa, C. T. Liu, et al. (2009). Root-determined hypernodulation mutant of *Lotus japonicus* shows high-yielding characteristics. *Biosci Biotechnol Biochem* 73: 1690-1692.

Yoshida, S., H. Kameoka, M. Tempo, K. Akiyama, M. Umehara, S. Yamaguchi, et al. (2012). The D3 F-box protein is a key component in host strigolactone responses essential for arbuscular mycorrhizal symbiosis. *New Phytol* 196: 1208-1216.

Figures



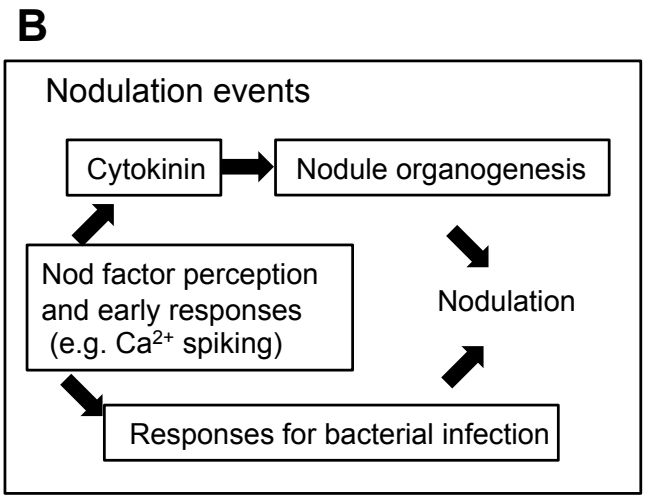


Fig. 1.1 Overview of the Nod factor signaling pathway
(A) Schematic illustration of the NF-induced nodulation events. (B) The signal flow chart for the NF-induced nodulation events.

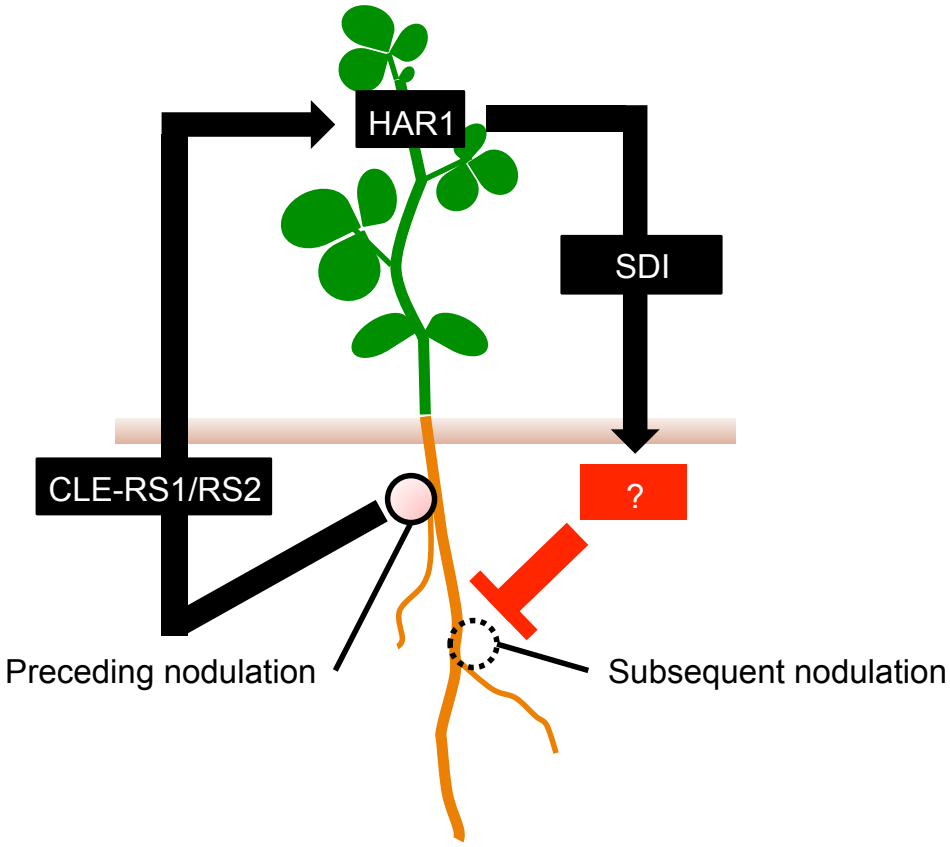


Fig. 1.2 HAR1-mediated long-distance control of nodulation
Preceding nodulation event rapidly induces the root-derived signals (CLE-RS1/RS2). The root-derived signals transport to the shoot and activated the LRR-RLK (*HAR1*). The activated LRR-RLK send the shoot-derived inhibitor (SDI) to the root. SDI transports to the roots and, through an unknown mechanism, subsequent nodulation is inhibited.

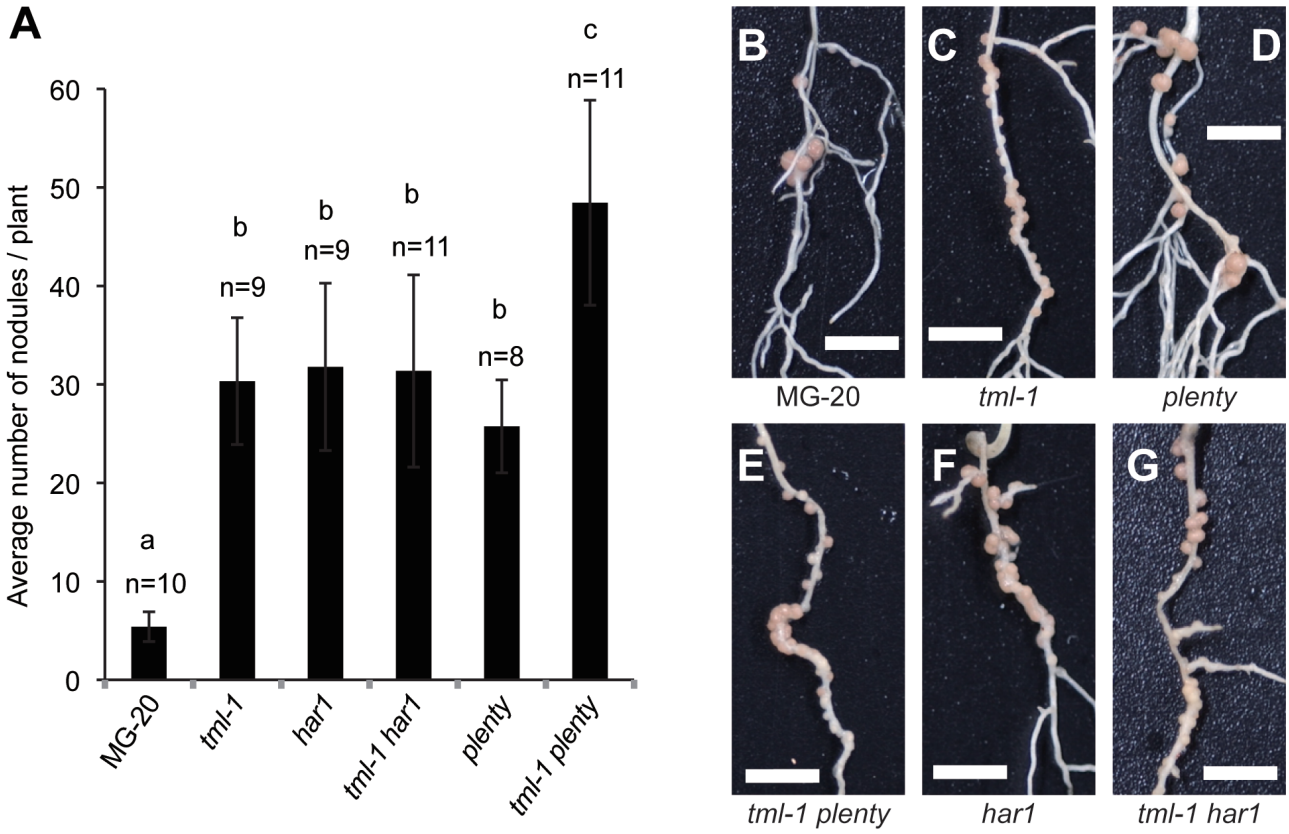


Fig. 2.1 The nodulation phenotypes in the *tml-1 har1-7* and *tml-1 plenty* double mutants, each single mutant and wild type MG-20.
(A) The number of nodules and nodule primordia counted 21 dpi. The results are represented as the means ± SD. Columns with the same lower-case characters are not significantly different (Tukey's test after an analysis of variance (ANOVA) ($P<0.001$)). (B-G) The nodulation phenotype of each mutant observed 21 dpi.

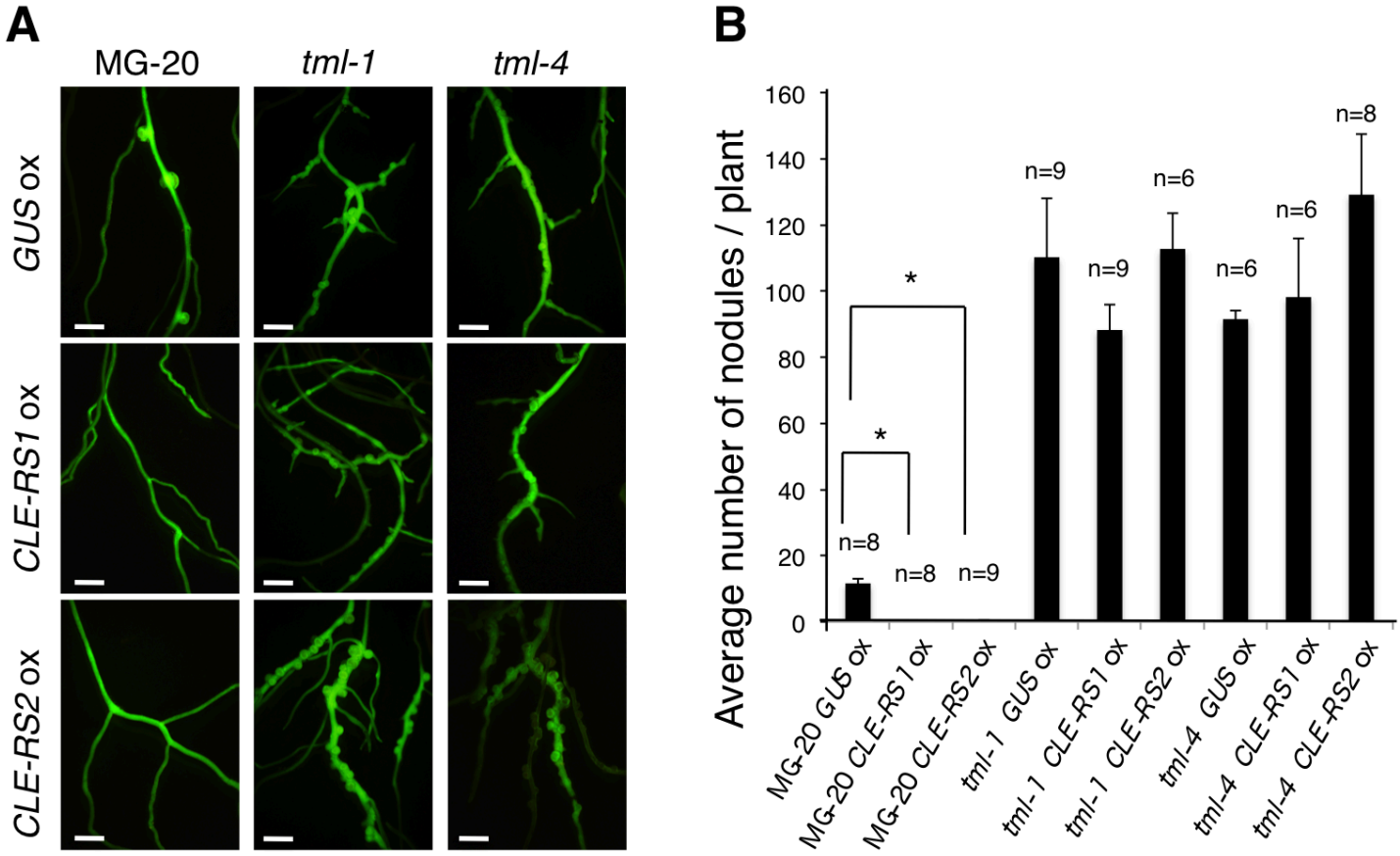
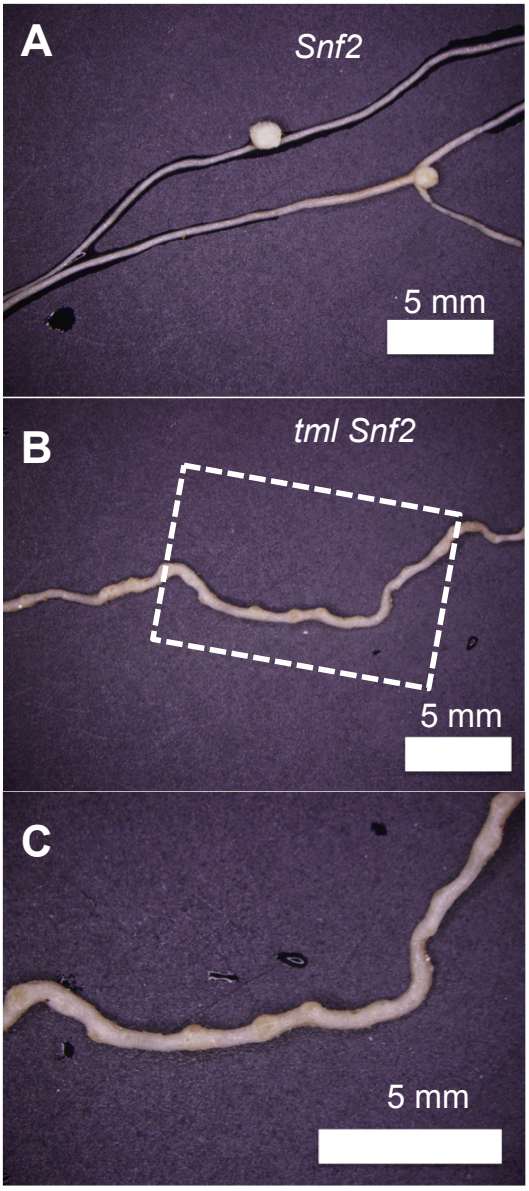


Fig. 2.2 Nodulation phenotypes of *CLE-RS1*/*RS2*-overexpressing roots.
(A) Nodulation phenotypes of *GUS-, CLE-RS1*- and *CLE-RS2*-overexpressing roots in MG-20, *tml-1* and *tml-4*. Bars indicate 2 mm. (B) Number of nodules on *CLE-RS1*-, *CLE-RS2*- and *GUS*-overexpressing roots. The results are represented as the means ± SD. Asterisks indicate the statistically significant difference at $P < 0.05$ (*) using Student's *t*-test.



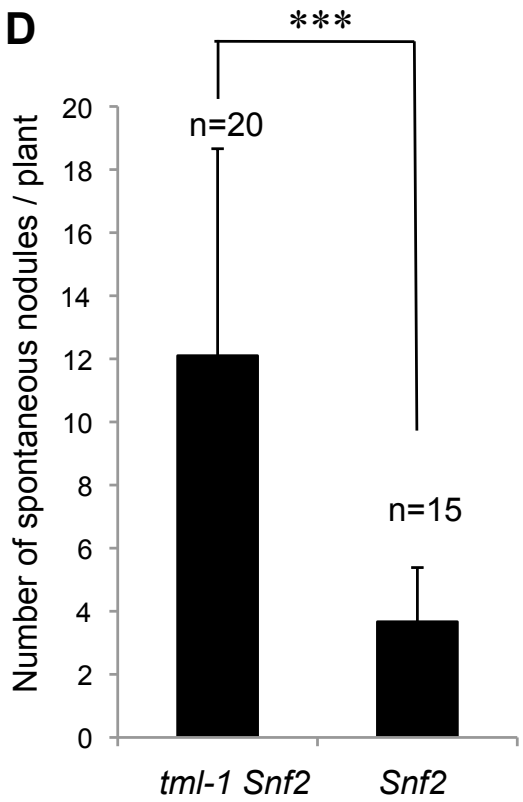


Fig. 2.3 Analyses of the genetic interactions between *TML* and *LHK1*
(A) Appearance of the spontaneous nodules on *Snf2* single mutants. (B) Appearance of spontaneous nodules on *tml-1 Snf2* double mutants. (C) The magnified image of (B). (D) The number of spontaneous nodules on *tml-1 Snf2* double mutants and *Snf2* single mutants counted 5 weeks post-inoculation. The 96 plants that were genotyped from the F2 progeny of F1 plants of *tml-1* crossed with *Snf2*. *tml-1 Snf2* double mutants and S*nf2* single mutants were selected to assess the phenotype. Bars in (A-C) indicate 5 mm. Error bars in (D) represent SD. Asterisks indicate the statistically significant difference at $P < 0.001$ (***) using Student's *t*-test.

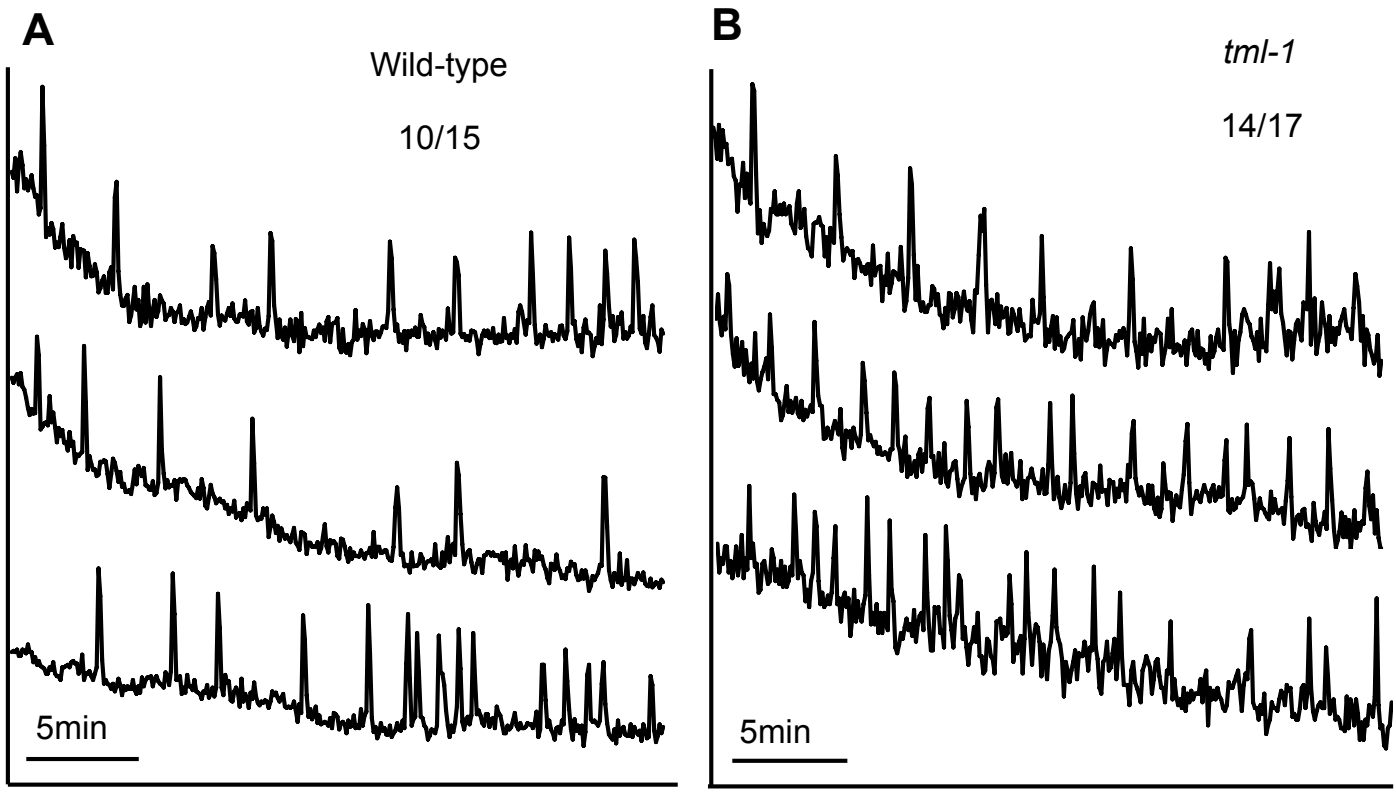


Fig. 2.4 Measurement of Ca^{2+} spiking in the wild type and the *tml* mutant.
Ca^{2+} spiking was measured after Nod factor (10^{-8} M) treatment in root hair cells of the wild type (A) and the *tml-1* (B). Three representative measurements are shown in the graph. The numbers indicate the ratio of root hair cells that showed Ca^{2+} spiking to the total number of cells.

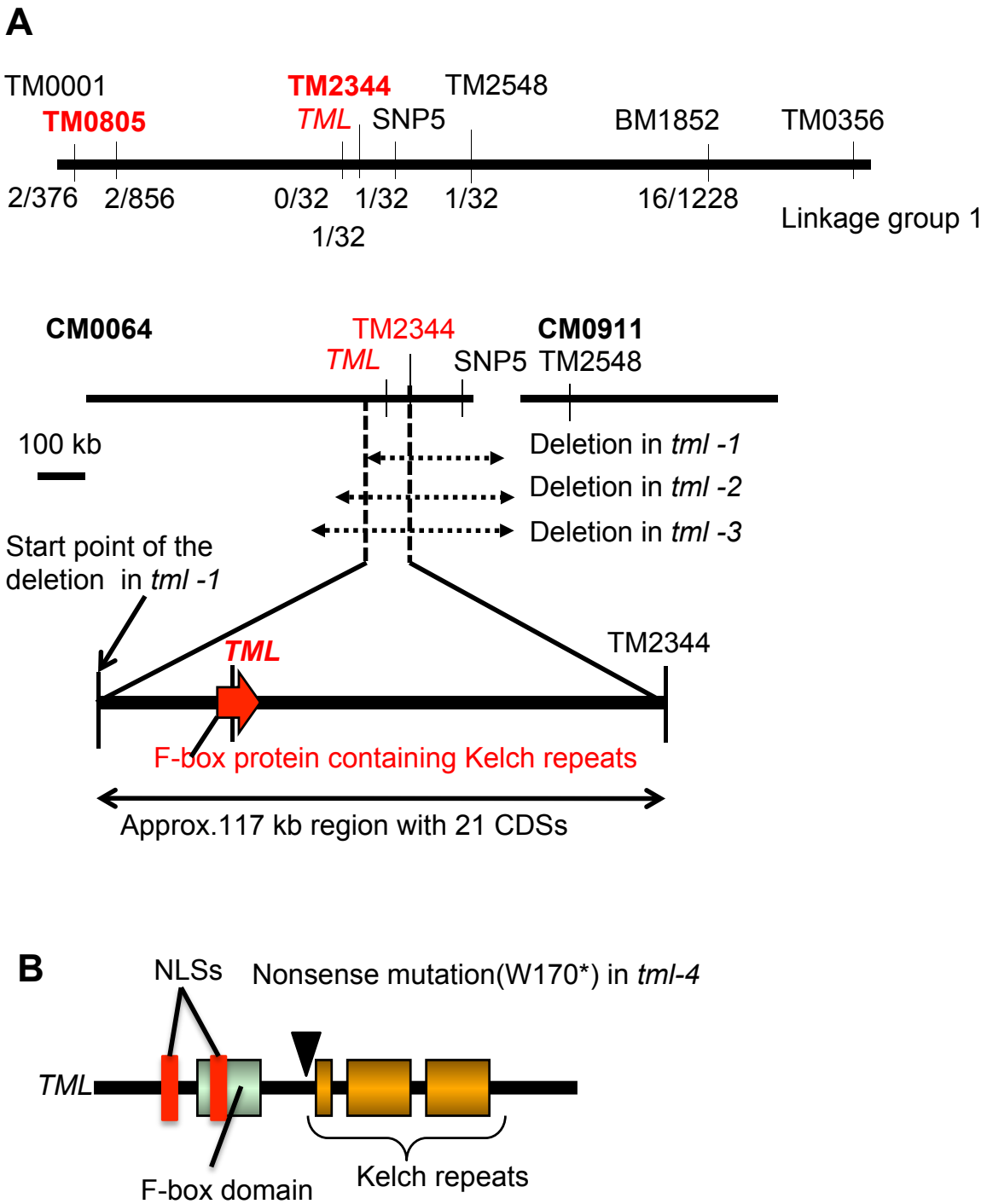


Fig. 3.1 Identification of the *TML* gene.
(A) Map-based cloning of the *TML* gene. (B) Annotated domains of the *TML* gene product.

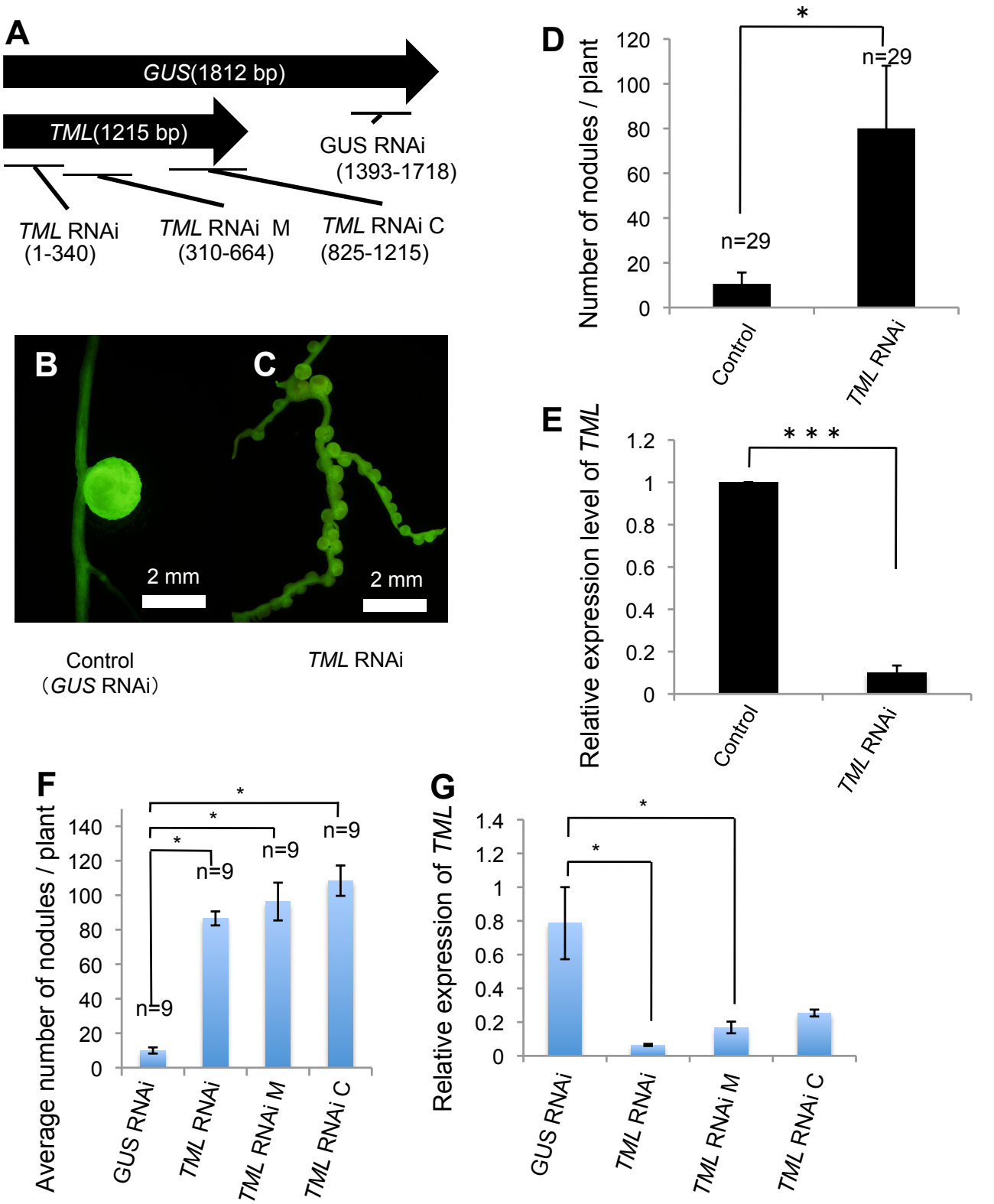


Fig. 3.2 Nodule number and relative expression level of *TML* in *TML*-silenced roots.
(A) The schematic illustration of loci of trigger sequences in the *GUS* gene and the *TML* gene. (B-C) Nodulation phenotypes of *GUS*- and *TML*-silenced roots. Trigger sequences were driven under the ubiquitin promoter. (D) Number of nodules on *TML*-silenced roots. (E) Relative expression level of *TML* in the *TML*-silenced roots. (F) Number of nodules on GUS or *TML* RNAi roots induced in wild type MG-20 plants. Nodules were counted at 21 dpi. Error bars indicate SE. Asterisks indicate the statistically significant differences at $P < 0.05$ (*) using Student's *t*-test. (G) The relative expression level of *TML* was examined. Error bars indicate SE. Asterisks indicate the statistically significant differences at $P < 0.05$ (*) using Student's *t*-test.

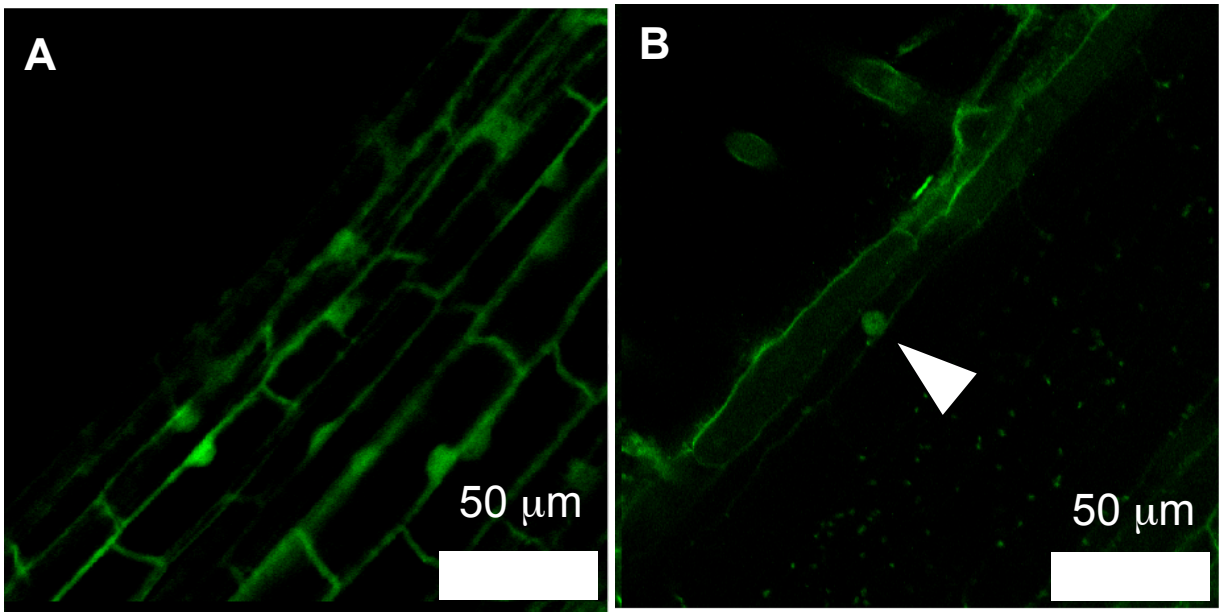


Fig. 3.3 Identification of the *TML* gene.
(A-B) Subcellular localization of the TML protein. Arrowheads indicate TML localization in the nucleus. Fluorescence of GFP (A) and GFP-TML (B) was observed.

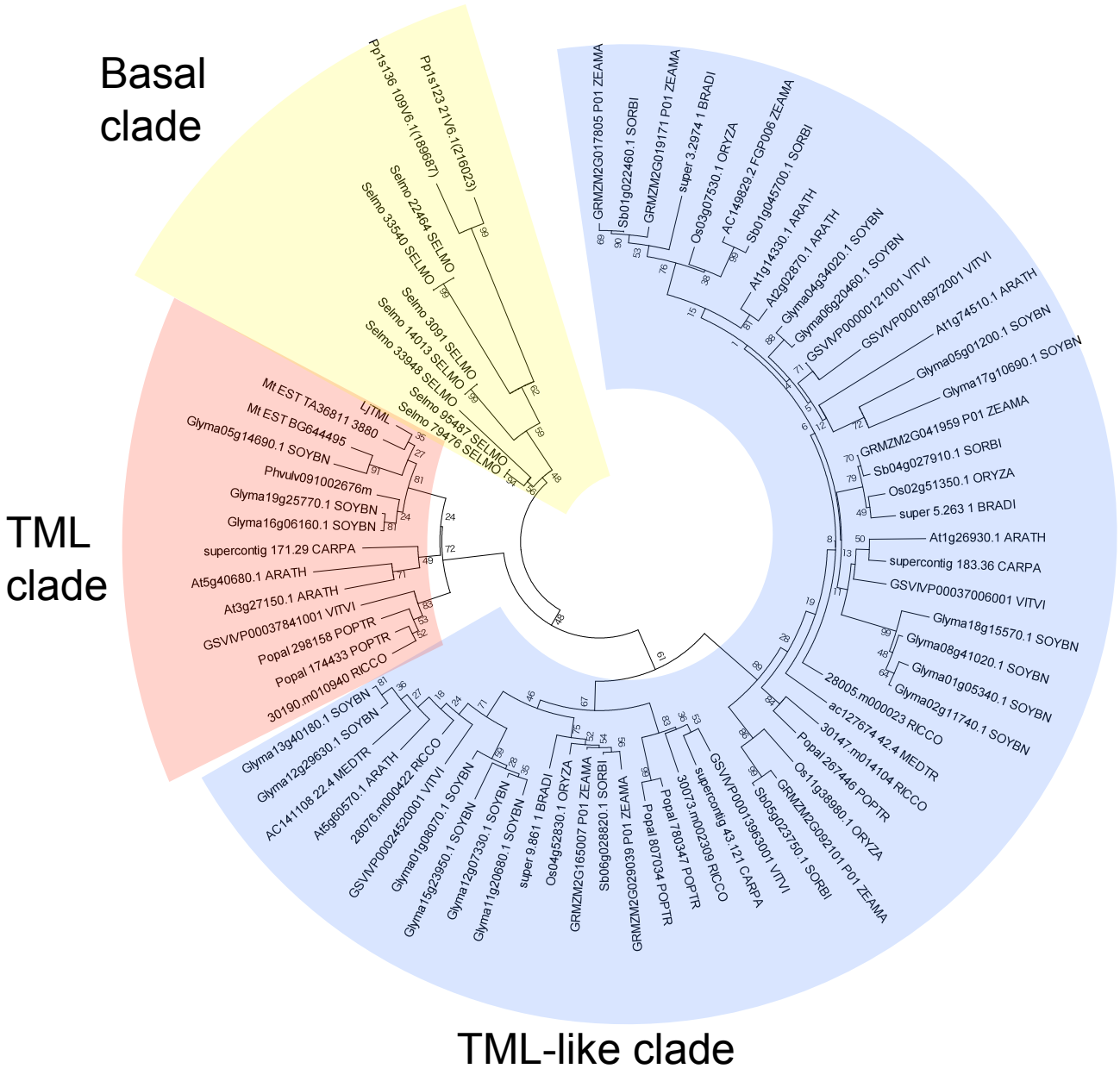


Fig. 3.4 Phylogenetic analysis of *TML*-related genes.
Deduced amino acid sequences of F-box domain in *TML*-related proteins were aligned and the phylogenetic tree was constructed by neighbor-joining. A node was supported in 1000 bootstrap pseudoreplications. Tree is shown with bootstrap confidence values expressed in percentage.

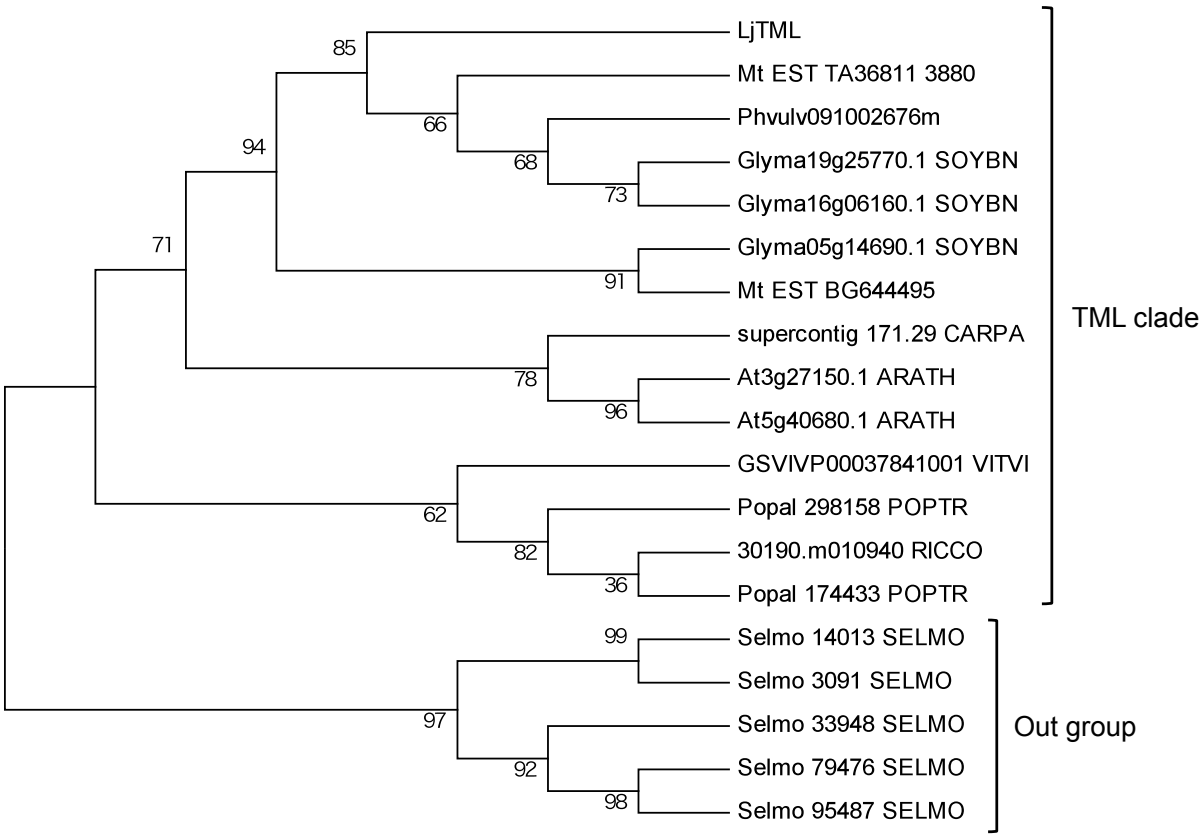


Fig. 3.5 The phylogenetic tree of the TML clade.
Deduced amino acid sequences of the F-box domains in *TML*-related proteins were aligned and the phylogenetic tree was constructed using the neighbor-joining method. A node was supported in 1000 bootstrap pseudoreplications. The tree is shown with bootstrap confidence values expressed in percentage.

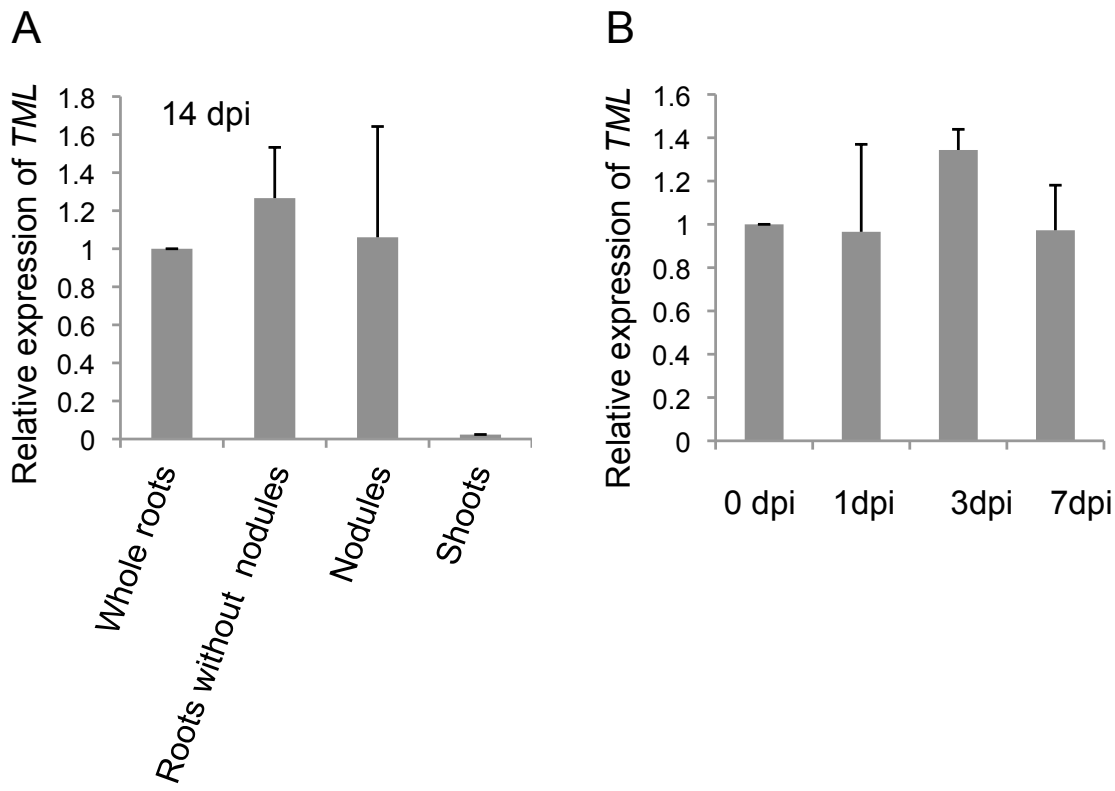


Fig. 3.6 Expression analyses of *TML* transcripts.
(A) The relative expression levels of *TML* in various organs of *L. japonicus*. (B) The relative expression levels of *TML* in the whole root 0-7 dpi. The results are represented as the means ± SD.

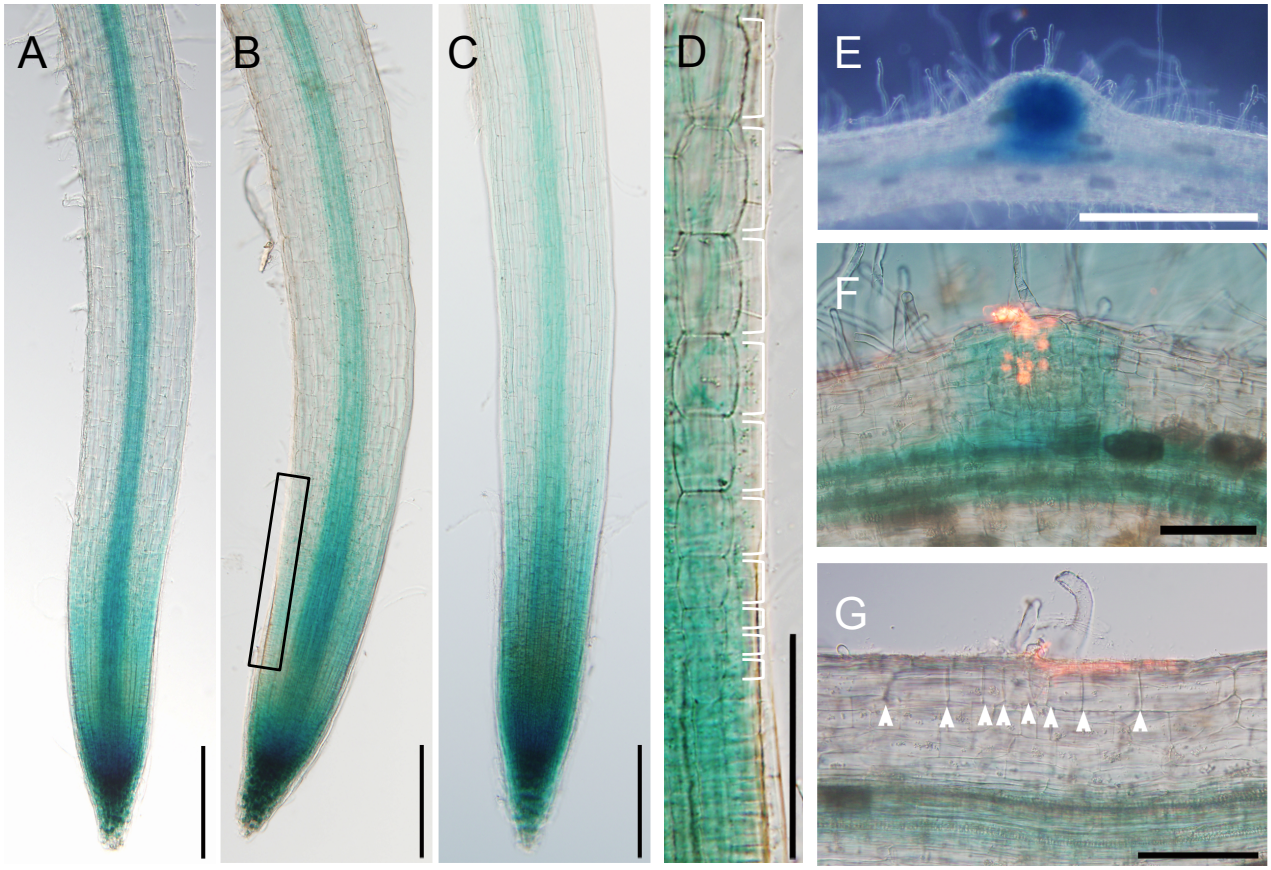
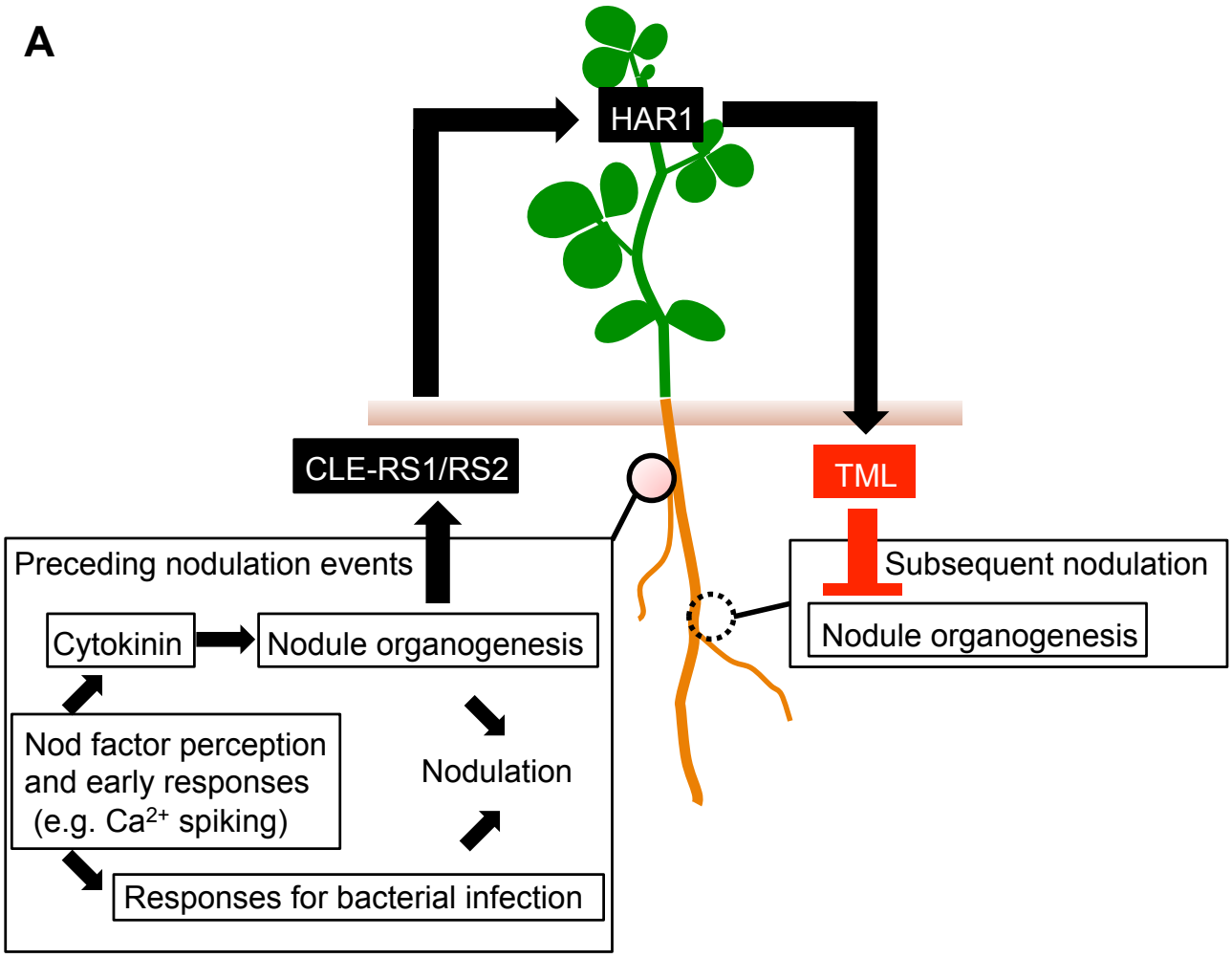


Fig. 3.7 GUS expression in roots transformed with *ProTML-GUS*.
(A-C) GUS staining in root tips. Roots were cultured in 1/2 B&D medium with (B) or without (A) *M. loti* for 7 days. The medium was supplemented with 10 mM NH_4NO_3 (C). (D) A magnified image of the boxed region in (B). White lines represent sizes of cortical cells. (E-G) GUS expression in a developing root nodule (E) and in root nodule primordia (F, G). (E) and (F) show merged images of GUS staining with fluorescence from DsRed that is constitutively expressed in *M. loti*. Arrowheads in (G) indicate division planes of cortical cells. Bars indicate 0.1 mm in (A-C), 0.5 mm in (E) and 50 mm in (D, F, G).



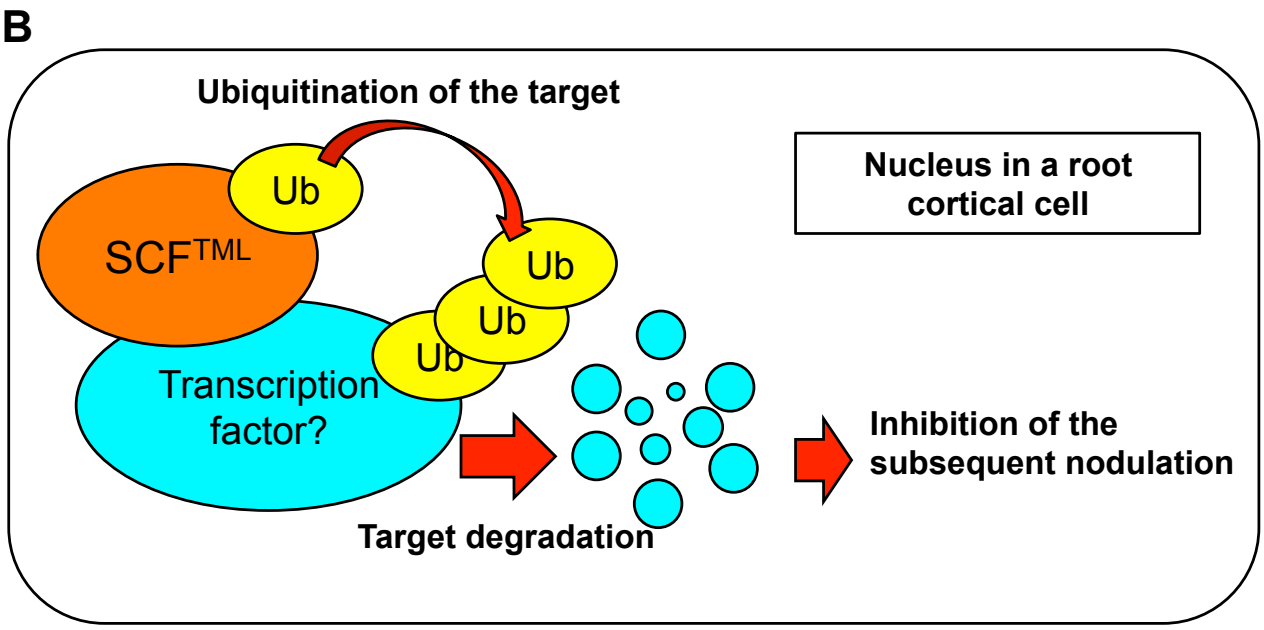


Fig. 3.8 Schematic illustration of the proposed models.
(A) A model for the TML-mediated nodule number regulation. (B) A proposed mode of action of TML.