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学位論文題目	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*.
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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 exhibits a long distance communication between the shoot and the root. This long-distance regulation of nodulation is termed auto regulation of nodulation (AON). However, the large part of the mechanism remains to be elucidated. A previous study has shown that *too much love-1* (*tml-1*, formerly *tml*), 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 the number of nodules, the author performed molecular biological and genetic analyses using *tml* mutant alleles.

Firstly, the author examined the genetic interaction between *TML* and *PLENTY*, another root factor that regulates the number of nodules. 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 and that if *TML* and the other genes act in different genetic pathways, the double mutant indeed illustrates the additional effect on the nodule number. In contrast, the *tml-1 har1-7* double mutant did not show an additive effect on nodulation. Taken together, he concluded that *TML* and *HAR1* function in the same genetic pathway.

Secondly, the genetic interaction between *TML* and *CLE-RS1/RS2* (the genes encoding the putative root-derived signals transporting to the shoot) was investigated. In the *tml* mutant background, the roots overexpressing either *CLE-RS1* or *CLE-RS2* developed as many nodules as the control GUS expressing roots. This result indicates that *CLE-RS1/RS2* suppresses nodulation in a *TML*-dependent manner. Therefore, he concluded that *TML* functions in nodule development downstream of *CLE-RS1/RS2*.

Thirdly, the author investigated the genetic interaction between *TML* and *Snf2* (the gain-of-function mutant of the cytokinin receptor *LHK1*) to assess the role of *TML* in AON. The *tml-1 Snf2* double mutant spontaneously developed many small nodules similar to those generally observed in the *tml* mutant upon rhizobial infection, whereas the *Snf2* single mutant developed spontaneous nodules similar to those produced in the wild type upon infection, indicating that *TML* inhibits the nodule organogenesis induced by the *LHK1*-mediated cytokinin signaling.

Taken together, he concluded 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.

In an attempt to localize the *TML* gene, inverse PCR was performed and the deleted regions in the large deletion alleles *tml-1*, *tml-2* and *tml-3* were determined. In addition, the fine mapping was performed using the EMS allele *tml-4* (formerly *rdh1*). Together with the results, the *TML* gene locus was delimited to a region of approximately 117 kb. Of the 21 genes predicted in the region, the whole genome resequencing of *tml-4* using a next generation sequencer revealed 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. The number of nodules developed on the candidate gene-silenced roots increased approximately 7.5-fold compared to those on the control roots, indicating 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 nuclear localization signals (NLSs). The F-box domain is a conserved domain in the component of E3 ubiquitin ligase that is involved in the proteasome-mediated protein degradation. The fluorescence of TML-sGFP was observed in the nucleus in the transgenic hairy roots in the wild-type plants. Taken together, TML might function by degrading its target protein in the nucleus. A phylogenetic analysis revealed that the TML-related Kelch repeat-containing F-box proteins were widely conserved in embryophytes. It also showed that at least one ortholog of *TML* in each legume plant exists in the TML clade, which supported the possibility that there is the conserved function of *TML* among legume species.

The qPCR analysis showed that *TML* is constitutively expressed in the roots and nodules and the expression was not detected in shoots from the plants with nodules nor shoots from the plants without infection, suggesting that *TML* is a root-specific gene. In addition, the author and his collaborator demonstrated that *TML* is constitutively expressed in the root tip region, including the meristematic region and elongation zone, which might explain why the *tml* mutant develops an excessive number of infection threads. Moreover, the expression pattern of *ProTML-GUS* was detected in root nodule primordia after several cell divisions of the cortical cells but not soon after the initial cell division, suggesting that *TML* inhibits 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.

In this thesis, the author clarified the acting point of *TML* during AON through the characterization of the *tml* mutant. Genetic and molecular analyses indicated that *TML* and *PLENTY* act in different genetic pathways and *TML* acts downstream of *LjCLE-RS1/2* and *HAR1* to suppress the nodulation signaling downstream of the cytokinin receptor *LHK1/CRE1*. He also revealed that the *TML* gene encodes a Kelch repeat-containing F-box protein with two NLSs and potentially functions in proteasome-mediated degradation of its target protein. In conclusion, the author identified the F-box protein TML as a key factor in maintaining proper nodulation at the final stage during AON.

マメ科植物は根粒菌による窒素固定により窒素源の乏しい環境でも生育することが可能であるが、過剰な根粒の形成は宿主植物の成長を阻害する。そのため根粒の着生数は宿主植物により厳密に制御されており、特にオートレギュレーション（AON）と呼ばれる制御が良く研究されている。AONは根で発現する *CLE-RS1/RS2*、地上部で働く *HAR1* を介した全身的な制御であることが示されているが、最終的に根で根粒形成を抑制している機構については大部分が未解明である。ミヤコグサの *too much love*（*tml*）は根粒が過剰に着生する変異体で、その原因遺伝子 *TML* は *HAR1* の下流かつ根で働く因子であることが示唆されている。本研究ではAONによる根粒数制御機構をより良く理解するために *tml* 変異体を使った分子遺伝学的解析と *TML* 遺伝子の特定を行った。

既に報告されているミヤコグサの2つの根粒過剰着生変異体 *har1, plenty* との遺伝学的関係を知るために、二重変異体を作成し表現型の解析を行った。*tml-1 har1* では根粒数に総加的な影響がみられなかったが、*plenty*（別の根粒数制御因子の変異体）との二重変異体 *tml-1 plenty* には各々の単一変異体よりも根粒を過剰に着生したことから、*TML* と *PLENTY* が異なる遺伝学的経路で働くこと、*TML* と *HAR1* が同一の遺伝学的経路で働くことが示唆された。次に *CLE-RS1/RS2* の過剰発現による根粒形成の抑制が *tml* 変異体背景では見られなくなることから *TML* が *CLE-RS1/RS2* を介した根粒形成の抑制に必要であることが示唆された。さらに、*Snf2*（サイトカイニンレセプター *LHK1* の機能獲得変異体、根粒菌の感染なしで自発的に根粒様器官を形成する）との二重変異体 *tml-1 Snf2* が *Snf2* と比較して過剰な数の自発的根粒形成をしたことから、*TML* が *LHK1* を介して起こる根粒器官形成を阻害していることが示唆された。

tml-1/-2/-3 の欠失領域の特定と、*tml-4* の fine mapping、次世代シーケンサーを使ったSNP検索から、候補遺伝子が一つに絞られた。この遺伝子をノックダウンした形質転換根が *tml* 変異体と同じ表現型を示したことから、この遺伝子が *TML* であると結論づけた。*TML* は Kelch-repeat domain、F-box domain および 2 つの核移行シグナルを持ち、TML-sGFP の局在解析から核局在することが示唆された。*TML* と相同性のある遺伝子は陸上植物のみに見つかり、マメ科植物は少なくとも1つのオルソログを持つことが示された。発現解析から *TML* は根特異的に発現し、構成的に根端、伸長領域で発現していること、根粒菌感染依存的に根粒原基と根粒で発現していることが示された。
これらから *TML* が *CLE-RS1/RS2* と *HAR1* の下流で働き、ユビキチン-プロテアソーム系を介しターゲットを分解することで、サイトカイニンシグナル伝達によって引き起こされる根粒器官形成を抑制していると結論づけた。

以上の結果は、根粒形成の遠距離シグナル伝達を介した全身制御機構において鍵となる遺伝子を特定したもので、その成果は国際誌に発表されている。適切な実験方法とともに、得られた成果は新規性が高く、本論文は博士論文として十分であると審査員全員一致で結論した。