**TRICOT encodes an AMP1-related carboxypeptidase that regulates root nodule development and shoot apical meristem maintenance in *Lotus japonicus***

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**SUMMARY**

During the course of evolution, mainly leguminous plants have acquired the ability to form de novo structures called root nodules. Recent studies on the autoregulation and hormonal controls of nodulation have identified key mechanisms and also indicated a possible link to other developmental processes, such as the formation of the shoot apical meristem (SAM). However, our understanding of nodulation is still limited by the low number of nodulation-related genes that have been identified. Here, we show that the induced mutation tricot (tco) can suppress the activity of spontaneous nodule formation 2, a gain-of-function mutation of the cytokinin receptor in *Lotus japonicus*. Our analyses of tco mutant plants demonstrate that TCO positively regulates rhizobial infection and nodule organogenesis. Defects in auxin regulation are also observed during nodule development in tco mutants. In addition to its role in nodulation, TCO is involved in the maintenance of the SAM. The TCO gene was isolated by a map-based cloning approach and found to encode a putative glutamate carboxypeptidase with greatest similarity to *Arabidopsis* ALTERED MERISTEM PROGRAM 1, which is involved in cell proliferation in the SAM. Taken together, our analyses have not only identified a novel gene for regulation of nodule organogenesis but also provide significant additional evidence for a common genetic regulatory mechanism in nodulation and SAM formation. These new data will contribute further to our understanding of the evolution and genetic basis of nodulation.

**KEY WORDS: AMP1, *Lotus japonicus*, Nodulation, Shoot apical meristem**

**INTRODUCTION**

The formation of root nodules (nodulation) is a form of de novo organogenesis that is particularly characteristic of leguminous plants. Nodule development involves the collaborative regulation of two processes: bacterial infection and nodule organogenesis. Following invasion of the root by soil bacteria (rhizobia), an ‘infection signal’ in the epidermis of the host induces de-differentiation of some cortical cells. These activated cortical cells then undergo a new developmental program that leads to formation of nodule primordia (Szczyglowski et al., 1998; Oldroyd and Downie, 2008; Oldroyd et al., 2011). During the course of nodule development, rhizobia invade the developing nodules via a specialized tubular structure called the infection thread, the successful formation of which is required for further nodule organogenesis (Murray, 2011). To date, a number of nodulation-related genes have been identified in *Lotus japonicus* and *Medicago truncatula* that act in the control of nodulation; however, most of these genes appear to be involved in the rhizobial infection process rather than nodule organogenesis (Madsen et al., 2010; Kouchi et al., 2010). The genetic factors that control specification and activities of nodule meristems in proliferating nodules have not yet been clarified.

The meristems of plants are specialized tissues containing stem cells that produce founder cells for the formation of lateral organs and other tissues (Stahl and Simon, 2005; Scheres, 2007). The molecular genetic mechanisms that regulate the shoot apical meristem (SAM) and the root apical meristem (located at the tips of the shoot and root, respectively) are well characterized. In *Arabidopsis*, the stem cell population in the SAM is maintained by a WUSCHEL (WUS)-CLAVATA (CLV) regulatory feedback loop (Brand et al., 2000; Schoof et al., 2000). Maintenance of the stem cells ceases in *wus* mutants (Laux et al., 1996); however, mutation in the *CLV* genes causes an enlargement of the stem cell region in the SAM (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). *WUS*, which encodes a transcription factor with a novel homeodomain, plays a key role in the specification of stem cells (Mayer et al., 1998), whereas CLV signaling negatively regulates the size of the stem cell population by repressing *WUS* (Brand et al., 2000; Schoof et al., 2000). *CLV3* encodes a signaling molecule termed the CLE (CLAVATA3/ESR) peptide (Fletcher et al., 1999), which is thought to function as a ligand for a receptor complex that includes CLV1 (Ohyama et al., 2009). Overproduction of *CLV3* or exogenous application of synthetic *CLV3* peptide causes death of stem cells; two receptor-like kinases, CORYNE and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), appear to mediate this *CLV3* effect (Müller et al., 2008; Kinoshita et al., 2010).

Legumes have a systemic negative regulatory mechanism called autoregulation of nodulation (AON), which prevents the production of an excess number of nodules (Caetano-Anollés and Gresshoff, 1991; Oka-Kira and Kawaguchi, 2006; Ferguson et al., 2010; Kouchi et al., 2010; Reid et al., 2011), and some of the genes involved in this control process have now been identified. In *L. japonicus*, mutation of the HYPERNODULATION ABERRANT
ROOT FORMATION 1 (HAR1) gene causes a hypernodulation phenotype (Wopereis et al., 2000; Krussel et al., 2002; Nishimura et al., 2002). Grafting experiments showed that this abnormal phenotype results from malfunction of the gene in the shoot. HAR1 encodes a putative leucine-rich repeat (LRR) receptor-like kinase and phylogenetic analysis indicates that the HAR1 protein belongs to a clade including Arabidopsis CLV1 and rice FLORAL ORGAN NUMBER 1, which is required for the maintenance of floral meristem (Suzuki et al., 2004; Oka-Kira and Kawaguchi, 2006). Recently, the AON-related KLAVER (KLV) gene was shown to function in the shoot and to encode another type of LRR receptor-like kinase that has similarity to Arabidopsis RPK2 (Miyazawa et al., 2010). In addition, mutation or knockdown of the CLV2-like gene in L. japonicus and in pea causes a hypernodulation phenotype (Krussel et al., 2011). Overall, the findings from the various studies described above indicate that AON in legumes is controlled by a series of genes that are orthologous to genes with essential roles for the regulation of the SAM in other plant species. Additionally, the findings suggest that there might be a common genetic mechanism in the regulation of nodulation and SAM formation.

The phytohormones cytokinin and auxin play fundamental roles in the control of many developmental regulatory processes, including the regulation of the SAM (Zhao et al., 2010; Durbak et al., 2012). Recent studies have shown that these hormones are also involved in the control of nodule development. In particular, analysis of the spontaneous nodule formation 2 (snf2) mutant line of L. japonicus showed that activation of cytokinin signaling is crucial to the control of nodule organogenesis (Tirichine et al., 2007). The snf2 phenotype was demonstrated to result from a gain-of-function mutation of the LATUS HISTIDINE KINASE 1 (LHK1) gene that encodes a putative cytokinin receptor. The mutation triggers formation of nodule-like structures (defined as spontaneous nodules) in the absence of rhizobia, owing to the constitutive activation of LHK1. It has also been shown that exogenous application of cytokinin to L. japonicus roots is sufficient to induce the formation of spontaneous nodules (Heckmann et al., 2011). Thus, activation of cytokinin signaling appears to be a pivotal event in nodule organogenesis. In addition to a role for cytokinin, there is evidence that a localized auxin response at the sites of incipient nodule primordia is essential for nodule organogenesis (Hirsch et al., 1989; Righthmyer and Long, 2011). These auxin responses are believed to occur in the downstream part of the cytokinin signaling pathway (Plet et al., 2011; Suzuki et al., 2012). Overall, the currently available data indicate that the two phytohormones play key roles in the regulation of nodule organogenesis. Despite these recent advances in our understanding of AON and the role of hormonal controls on nodule organogenesis, further insights into these processes will require the identification of other genes that contribute to the various molecular pathways relevant to nodule organogenesis.

The present study focused on the downstream part of the cytokinin signaling pathway. We identified a mutant tricot (tco) in a screen for mutants that could suppress the snf2 phenotype. The tco mutation prevents snf2-dependent spontaneous nodule formation, suggesting that TCO positively regulates nodule organogenesis in the downstream part of the cytokinin signaling pathway. Nodule development and the rhizobial infection process are also impaired in the tco mutant. Additionally, an auxin reporter analysis showed that there are defects in some aspects of auxin regulation in the tco mutant. The shoot phenotype of the tco mutant also indicates that TCO is involved in maintenance of the SAM.

We used map-based cloning and found that TCO encodes a putative glutamate carboxypeptidase with possible orthology to Arabidopsis ALTERED MERISTEM PROGRAM 1 (AMP1). Expression analysis showed that TCO is expressed in meristematic regions of nodules and in the SAM. Overall, our results provide further support for the proposed common genetic regulatory mechanism in nodule and SAM formation.

MATERIALS AND METHODS

Plant materials and growth conditions

The Miyakojima MG-20 ecotype of L. japonicus was used as the wild type (WT) in this study. The tco mutant was isolated from the M2 generation of snf2 plants (Miyazawa et al., 2010) that had been mutagenized with 0.4% ethylmethane sulfonate (EMS) for 6 hours. A description of the nin-9, har1-8 and DR5:GFP-NLS plants has been published previously (Suzuki et al., 2012). For the analyses of rhizobial-induced nodulation or spontaneous nodulation, plants were grown with or without Mesorhizobium loti MAFF 303099, respectively, on autoclaved vermiculite with Broughton and Dilworth (B and D) solution (Broughton and Dilworth, 1971) that does not contain a nitrogen source. The plants were grown under a 16-hour light/8-hour dark cycle. For analysis of shoot formation, plants were grown on a sterilized plate containing 1% agar (Wako, Japan) or in horticultural soil (Nihon Hiryo, Japan) under the same light conditions.

Analysis of the SAM

Plant materials were fixed in paraformaldehyde and dehydrated through an alcohol series (Suzuki et al., 2004). After replacement of the water with lemosol, the tissues were embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). Microtome sections (8 μm) were cut using a Microtome RM2255 (Leica) and stained with 0.05% Toluidine Blue. The sections were viewed using an Olympus BX-50 light microscope.

Map-based cloning of TCO

The tco locus was mapped using an F2 population derived from a cross of tco snf2 and Gifu B-129 plants. The locus was mapped to a region between the simple sequence repeat (SSR) markers BM2258 and TM0122 on chromosome 1 using a population of 28 F2 plants that exhibited nodulation-deficient phenotypes and had an increased number of cotyledons. Next, a gene highly similar to Arabidopsis AMP1 was found in this region in a search of the L. japonicus genomic database; the genomic sequence of this candidate gene in the tco mutant was determined by a direct sequencing method using the following primers: PCR-Fw1, 5’-CATACCC-ATGCCATAAGGC-3’; PCR-Rv1, 5’-CCACCGCTGAGTATGC-3’; Sequence-Fw1, 5’-TCACTTCCTCCAATCTCAC-3’; Sequence-Fw2, 5’-GGCTGATCCTTCTTGTGTTG-3’; Sequence-Rv1, 5’-ATGGGGAATGCAAGTGAG-3’; Sequence-Rv3, 5’-CTCCGATGCTTAGCAG-3’; and Sequence-Fw4, 5’-TGCTCCATCAATCACAGA-3’.

Constructs and transformation of L. japonicus

For the complementation analysis, a 1.7-kb gateway-cassette (GW) fragment was first excised from a previously reported vector (Suzuki et al., 2012) and inserted into the BamHI site of pCAMBIA1300-GFP. Next, the GFP moiety in the vector was removed using Xhol, and a PCR-amplified mCherry-NLS sequence (Suzuki et al., 2012) was inserted into the Xhol site to create the new binary vector pCAMBIA1300-GW-mCherry. The primer set 5’-AAACCTCGAGATGGTGAAGCGGCGAGGA-3’ and 5’-AACTCGAATGTTTCCTTCTGTCGAG-3’ was used for amplification of the mCherry-NLS sequence. A genomic DNA fragment including the TCO candidate gene was then amplified from WT genomic DNA using the primer set 5’-CACCGAACAAGGCTGTAGACCTT-3’ and 5’-TTGAAATTTGATCTTGC-3’. This amplification yielded a 7.8-kb fragment, including a 1.7-kb sequence directly upstream of the initiation codon, which was cloned into the pENTR/D-TOPO vector (Invitrogen). The genomic fragment was then transferred into the pCAMBIA1300-GW-mCherry vector using the LR recombination reaction (Invitrogen). For the development of calcium spiking, we created a construct in which nuclear-localized yellow-chameleon (YC2.60) was expressed under the control of the LjUBQ promoter (N.T. and M.K., unpublished).
Recombinant plasmids were introduced into Agrobacterium rhizogenes strain AR1193 and were transformed into the roots of WT or tco plants by the previously described hairy root transformation method (Suzaki et al., 2012). Purification of Nod factor and detection of calcium spiking were performed as reported previously (López-Lara et al., 1995; Ehrhardt et al., 1996; Maekawa et al., 2009). In order to achieve stable transformation of L. japonicus, the LR recombination reaction was used to insert the genomic fragment including the TCO candidate gene (see above) into the pGWB1 vector. This recombinant plasmid was then introduced into A. tumefaciens strain AGL1 and transformed into tco/TCO plants as described previously (Nishimura et al., 2002). After obtaining transgenic plants, the genotype of the tco locus in each plant was confirmed by direct sequencing of the PCR product from the endogenous TCO (data not shown).

**In situ hybridization**

The in situ hybridization probe for TCO was created by first amplifying 648-bp fragments by PCR from a template cDNA prepared from total RNA from inoculated roots. The primer set 5'-TCCACTCTGCCCCTCTAC-3' and 5'-CACCAGAGCACCTCAAGCCG-3' was used for the amplification. The fragments were inserted into the pGEM-T easy vector by TA cloning (Promega). Probe synthesis, preparation of sections and in situ hybridization were performed as described previously (Suzaki et al., 2004). In situ hybridization signals were analyzed by light microscopy using an Olympus BX-50.

**Expression analysis**

Total RNA was isolated from each plant tissue using the RNasy Plant Mini Kit (Qiagen). First-strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed using an ABI Prism 7000 (Applied Biosystems) with a THUNDERBIRD SYBR qPCR Mix (Toyobo) or with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer’s protocol. Expression of ubiquitin or ATP synthase was used as the reference. The following primers were used in the expression analysis: ubiquitin, 5'-ATGCAGATCTTTGTAAGACATG-3' and 5'-CTGCCCTTACGCAAATAC-3'; ATP synthase, 5'-CATCACATGCTTGACCAATCATAC-3' and 5'-TCCCAAACTGACACGTCAAAC-3'; NIN, 5'-CAATGCTTTGATCAGGCTGTTGA-3' and 5'-GAGTGCTAATGGCAAATTGTGTGTC-3'; Lj/RBR (chr3.LjT48P15.80), 5'-ATCAAGGATCAACAGGTGCC-3' and 5'-ATCAAGGATCAACAGGTGCC-3'; Lj/TAR1, 5'-AGGGCAATTGAAAGTGGTGAG-3' and 5'-CATCCAATGCAAATCATGGTA-3' (Okamoto et al., 2009; Suzaki et al., 2012). Data are shown as mean±s.d. of three biological replicates.

**Microscopy**

Brightfield and fluorescence microscopy were performed with an SZX12 stereomicroscope (Olympus) or with an A1 confocal laser-scanning microscope (Nikon). Detection of calcium spiking was performed with an Eclipse Ti inverted microscope (Nikon). Images were acquired and analyzed using DP Controller (Olympus), NIS elements (Nikon) or Adobe Photoshop (Adobe Systems).

**RESULTS**

**tco suppresses snf2-dependent spontaneous nodule formation**

In L. japonicus, the snf2 mutation of the LHK1 gene induces spontaneous nodule formation in the absence of rhizobia as a result of constitutive activation of cytokinin signaling (Tirichine et al., 2007). In order to elucidate the molecular genetic mechanism regulating nodule organogenesis, we performed a screen for genetic suppressors of the snf2 phenotype. In total, ~60,000 M2 plants derived from 3135 EMS-treated M1 snf2 plants were screened, and a candidate suppressor mutant was identified. This mutation was named tricot (tco) because of its predominately three cotyledon phenotype. When grown under nitrogen-deprived conditions, snf2 plants readily formed spontaneous nodules (Fig. 1A-C) as described previously (Tirichine et al., 2006). By contrast, spontaneous nodule formation was substantially reduced in tco plants, which formed approximately one-tenth of the number found in snf2 plants; moreover, the nodules formed in tco plants had an underdeveloped structure (Fig. 1A-C). This observation indicates that the tco mutation largely suppressed the ability for spontaneous nodule formation mediated by snf2.

**tco affects nodule formation**

We performed a genetic analysis and found that the tco mutation was inherited as a recessive trait and conferred a nodulation-deficient phenotype. F1 plants derived from a cross between tco snf2 and the L. japonicus MG-20 parental line showed the WT nodulation phenotype (data not shown). In the F2 population, the WT and tco nodulation phenotypes segregated in a 3:1 ratio (77 WT and 23 tco). The number of nodules was not only significantly reduced in tco (Fig. 2A,E) but those that formed were much smaller than the WT (Fig. 2B,C). These results indicate that TCO is essential for nodule formation; this conclusion is consistent with the observations described earlier for the tco snf2 double mutants (see above). Interestingly, increased lateral root formation was also induced in tco, which formed four times as many lateral roots as did WT plants (Fig. 2A,D). NIN is a key plant transcription factor that is required for both nodule formation and accommodation of bacteria within the host roots (Schauer et al., 1999). Expression of the NIN gene is strongly activated at an early stage after infection by rhizobia (Fig. 2F) (Schauer et al., 1999). To investigate the possible relationship between TCO and NIN, we examined the expression of NIN in tco plants and determined the nodulation phenotype of tco nin double mutants. We found that NIN expression was not induced in tco plants during the first 7 days after inoculation (dai) with M. loti and only became detectable by the first 7 and 14 days...
tco affects bacterial infection
During nodulation, rhizobia invade the dividing cortical cells via a specialized tubular structure called the infection thread (Murray, 2011). We examined the effect of the tco mutation on infection thread formation and found that, in contrast to the WT, a significantly smaller number of infection threads was observed in tco roots at 5 dai (Fig. 3A). At 14 dai, however, although the number of epidermal infection threads remained small, their progression through the root hair into the root cortex appeared normal and was indistinguishable from that observed in the WT (Fig. 3B-E).

During nodulation, a calcium spike is induced in root hairs infected by rhizobia and this induction is dependent on a Nod factor derived from the rhizobia (Ehrhardt et al., 1996; Oldroyd and Downie, 2008). Application of purified Nod factor to tco roots generated calcium spiking in 14 of 20 root hair cells compared with 23 of 28 cells in the WT, although the calcium spiking interval in tco was slightly longer than that in the WT (Fig. 3F,G). Overall, therefore, our findings show that the tco mutation affects both nodule organogenesis and rhizobial infection and indicate that the TCO protein is a positive regulator of these processes.

Interaction between TCO and auxin during nodulation
During nodule development in L. japonicus, a preferential auxin response in proliferating cortical cells occurs in the downstream part of the cytokinin signaling pathway (Fig. 4A,C,E) (Suzaki et al., 2012). We examined the interaction of TCO and auxin response during nodule development by analyzing auxin reporter expression patterns in tco mutant plants. DR5::GFP-NLS/tco plants were produced by crossing DR5::GFP-NLS transgenic plants with tco plants. In tco mutants, no GFP expression was observed in the cortical cells of 5 dai roots; by contrast, proliferation of the cortical cells in the WT was accompanied by GFP fluorescence in 20 whole root hairs examined (Fig. 4D). At 10 dai, GFP fluorescence was detectable in only three cortical cell regions in the 20 whole tco roots examined (Fig. 4D). Interestingly, GFP fluorescence was also present in the stele cells of 15 of 21 tco plant roots (Fig. 4D). This ectopic GFP expression persisted and was present in older tco roots that contained a few nodule primordia (Fig. 4F). Ectopic GFP expression in stele cells was observed in only two of 23 WT plant roots.

The L. japonicus TRYPTOPHAN AMINOTRANSFERASE RELATED 1 (TAR1) gene (LjTAR1), which has been suggested to be involved in auxin biosynthesis, is activated upon infection by rhizobia (Suzaki et al., 2012). In the tco mutant, a higher level of LjTAR1 expression was present than in the WT (Fig. 4G).

TCO is involved in the maintenance of the SAM
In addition to the effect on nodulation, the tco mutant displayed an abnormal shoot phenotype. Most L. japonicus WT MG-20 seedlings are dicotyledonous (99 of 100 plants screened) (Fig. 5A); seedlings with three cotyledons are rare (one of 100 plants

(Fig. 2F). The tco nin-9 double mutant plants showed a non-nodulating phenotype similar to that of the nin-9 single mutant (Fig. 2E). These results suggest that TCO is required for the normal induction of NIN and that the nin mutation is epistatic to tco.

The expression of LjRR4, which encodes a putative type-A cytokinin response regulator in L. japonicus, can be induced by application of Nod factor (Op den Camp et al., 2011). In tco plants, however, the ability to induce expression of the gene was abolished at 7 dai (Fig. 2G), suggesting that TCO is required for the normal induction of LjRR4 during nodule development. This finding is consistent with the observations described above that tco suppresses the constitutive cytokinin activation mediated by snf2.

tco suppresses har1-dependent hypernodulation
Nodule number in legumes is controlled by the systemic regulatory mechanism AON (Caetano-Anollés and Gresshoff, 1991; Oka-Kira and Kawaguchi, 2006). The L. japonicus HAR1 gene encodes a leucine-rich repeat receptor-like kinase and is a key component of AON (Krüssel et al., 2002; Nishimura et al., 2002). har1-8 mutant plants have a significantly increased number of nodules compared with WT (Fig. 2E) (Suzaki et al., 2012). In tco har1-8 double mutants, the hypernodulation phenotype is suppressed and nodulation returns to the WT level (Fig. 2E). This finding suggests that the tco effect is conserved in the har1 mutant background.
screened). By contrast, the majority of tco seedlings have three cotyledons (72 of 100 plants screened) (Fig. 5B), with a few forming four cotyledons (nine of 100 plants screened) (Fig. 5C). In addition, all tco plants examined showed a marked increase in the number of leaf primordia compared with WT plants at the same developmental stage (Fig. 5A-C), suggesting that the rate of leaf initiation (the plastochron) is shortened in the mutant. As leaf formation is closely related to the maintenance of the SAM (Steeves and Sussex, 1989), we compared SAM formation in mutant and WT plants. Shortly after germination, the width and height of the SAMs in tco mutants were ~1.5-fold larger than in the WT (Fig. 5D,E; width of 94.1±6.0 and height of 58.4±5.5 in tco mutants versus width of 62.2±4.4 and height of 41.6±3.7 in WT; n=12). Furthermore, the formation of a fasciated stem was observed in eight of 20 tco mutants (Fig. 5F). These mutant shoot phenotypes strongly suggest that the maintenance of the SAM is disrupted by the tco mutation. The tco shoot phenotypes co-segregated with the tco nodulation deficiency phenotype in 100 nodulation deficient F2 progeny from a cross between snf2 tco and WT plants. We therefore suggest that the tco mutation has a pleiotropic effect in L. japonicus.

**TCO encodes an AMP1-related putative glutamate carboxypeptidase**

To characterize the molecular function of TCO, we first sought to isolate the gene using a map-based cloning approach. The tco locus was mapped to 65 cM on L. japonicus chromosome 1. A search of the L. japonicus genomic sequence database identified a candidate gene, LjAMP1, which encodes a putative glutamate carboxypeptidase. This gene shows similarity to the Arabidopsis AMP1 gene. Arabidopsis amp1 mutants show an increased number of cotyledons and leaves and an enlarged SAM (Chaudhury et al., 1993; Vidaurre et al., 2007). These mutants therefore display similar shoot phenotypic changes as the tco mutant. We determined the nucleotide sequence of the LjAMP1 gene and identified a G2178 to A substitution that caused a nonsense mutation (Fig. 6A).
The mutant nodulation phenotype of tco was rescued when a 7.8-kb genomic fragment containing a normal LjAMP1 gene was introduced into the mutant by A. rhizogenes-mediated hairy root transformation (Fig. 6B-D). Moreover, this genomic fragment also rescued the mutant shoot phenotype in tco plants (Fig. 6E,F). Thus, these results clearly indicate that the tco phenotype is a result of mutation of the LjAMP1 gene. The hairy root transformation method produces hairy roots but leaves the shoot untransformed; thus, the result of the complementation analysis indicates that the nodulation-deficient phenotype in the tco mutant is due to a defect of TCO function in the root.

We determined the positions of the exons in the TCO locus by sequencing the RT-PCR product and predicted the open reading frame (Fig. 6A). The number of exons in TCO was identical to that of Arabidopsis AMP1 (Helliwell et al., 2001). TCO encodes a putative protein of 695 amino acids, including a protease-associated domain, an M28 peptidase domain and a transferrin receptor-like dimerization domain (Fig. 6A). The predicted structure of TCO closely resembles that of Arabidopsis AMP1 (Helliwell et al., 2001). In the tco mutant, a nonsense mutation occurred in the M28 peptidase domain, suggesting that a truncated form of the protein was probably translated in the mutant (Fig. 6A). To determine the evolutionary relationship of TCO and AMP1-related putative glutamate carboxypeptidases, we compared genes encoding proteins with high homology to TCO from other plants and from humans. The phylogenetic tree analysis showed that TCO belonged to a clade containing Arabidopsis AMP1 (Fig. 6G). This result strongly supports the supposition that TCO is a putative ortholog of Arabidopsis AMP1.

Spatial expression patterns of TCO
To clarify further the function of TCO, mRNA distribution patterns were investigated by in situ hybridization. During nodule development, TCO transcripts were detected in proliferating cortical cells (Fig. 7A). After rhizobial colonization of developing nodule primordia, transcripts were detected in the regions presumed to correspond to nodule parenchyma that surround the rhizobial infection zones (Fig. 7B) (Suzaki et al., 2012). In addition, TCO transcripts were present in vascular tissues and adjacent cells (Fig. 7C,D). In the shoot apex, TCO transcripts were predominantly located in vascular tissues, and weak signals were also observed throughout the SAM (Fig. 7F). In controls probed with sense RNA, no signals were detected (Fig. 7E).

DISCUSSION
Nodulation is achieved by interactive processes involving infection by rhizobia and nodule organogenesis; the complexity of these interactions has made it difficult to study regulation of these mechanisms separately. Although a number of nodulation-deficient mutants have been identified, most of these predominantly affect the rhizobial-infection process (Kouchi et al., 2010). As a consequence, the genetic mechanism regulating nodule organogenesis is relatively poorly characterized. In this study, in order to concentrate on nodule organogenesis, we performed a screen designed to identify mutations suppressing the snf2 (spontaneous nodulation) phenotype. This screen identified a locus, TCO, that has a novel action in the positive regulation of the nodulation process.

The putative cytokinin receptors L. japonicus LHK1 and M. truncatula CYTOKININ RESPONSE 1 function during nodule organogenesis (Gonzalez-Rizzo et al., 2006; Murray et al., 2007). In the downstream part of the cytokinin signaling pathway mediated by these proteins, the genes NIN, NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 have been identified as being required for nodule organogenesis (Tirichine et al., 2007; Madsen et al., 2010). Expression of NIN, in particular, is strongly upregulated during nodule organogenesis. In addition, MtHAP2-1 and MsZPT2-1 are known to play roles in nodule organogenesis in the genus Medicago (Fugier et al., 2000; Combier et al., 2006). Our analyses showed that the tco mutation suppressed snf2-dependent spontaneous nodule formation, and also downregulated and delayed NIN expression. Additionally, the nodulation phenotype of the tco nin double mutant suggested that the nin mutation was epistatic to tco. Thus, our results indicate that TCO might be involved in the induction of NIN in the downstream part of cytokinin signaling. The fact that the tco mutation also affects the rhizobial-infection process leads us to suggest that TCO is not a gene specifically involved in nodule organogenesis, but may rather play broader roles in the regulation of two regulatory processes required for proper nodule development. This is the case for NIN, NSP1 and NSP2, which are involved in the control of both nodule organogenesis and the rhizobial infection process (Schauer et al., 1999; Madsen et al., 2010).

Our map-based cloning approach and subsequent complementation analysis showed that TCO is likely to encode a glutamate carboxypeptidase that is orthologous to Arabidopsis AMP1. In the Arabidopsis amp1 mutant, the number of cotyledons is increased and the plastochron is shortened (Chaudhury et al., 1993). In addition, the mutation causes an enlargement of the SAM, in which the stem cell region is apparently enlarged as shown by an expanding CLV3 expression domain (Vidaurre et al., 2007). This indicates that AMP1 functions as a negative regulator of cell proliferation in the SAM. It has also been reported that the rice PLASTOCHRON 3 (PLA3) gene, a loss-of-function mutant that exhibits a shortened plastochron and enlarged SAM phenotype,
encodes an AMP1-like carboxypeptidase (Kawakatsu et al., 2009). As the shoot phenotype of tco is similar to those of Arabidopsis *amp1* and rice *pla3* mutants with respect to the increased number of leaves and enlarged SAM, it is probable that AMP1-related carboxypeptidases have a conserved role in the control of SAM maintenance in higher plants. Although this type of carboxypeptidase is thought to be involved in the processing step of a signaling molecule (Hellwell et al., 2001; Kawakatsu et al., 2009), its molecular function has yet to be fully characterized. Based on studies of *amp1* and *pla3* mutants, however, we speculate that carboxypeptidases may be involved in the homeostasis of some phytohormones. Our phenotypic analyses showed that the tco mutation is indicated by the arrowhead. (B, C) Complementation of the tco nodulation phenotype. Representative transgenic hairy roots of *L. japonicus* carrying a 7.8-kb genomic fragment encompassing the entire TCO locus (B) or the control GUS gene sequence (C). Note the presence of fully developed nodules in B; transgenic hairy roots were identified by mCherry-NLS red fluorescence signals. (D) Average number of nodules formed on transgenic hairy roots, as described in B and C (n=11 plants). Nodulation phenotypes were evaluated at 21 dai. Error bars indicate s.d. (E, F) Complementation of the TCO shoot phenotype. Representative stable transgenic tco plants carrying a 7.8-kb genomic fragment encompassing the entire TCO locus (E) or the control GUS gene sequence (F) at 7 dag. Note the formation of normal numbers of cotyledons and leaves in E. (G) Phylogenetic tree of TCO-related carboxypeptidases. Full-length amino acid sequences were compared and the tree was constructed by neighbor-joining methods (Saitou and Nei, 1987) using PLA3 and VP8 as the root. Numbers indicate bootstrap values. Accession numbers of the amino acid sequences of related proteins are as follows: TCO (AB743813), AMP1 (ABQ85084), At4g07670 (CAB81137), At5g19740 (AAP37682), PLA3 (AB447403), VP8 (ACA62934), NAALADase I (AAC53423), NAALADase II (Q9Y3Q0) and NAALADase L (Q9UQ11). Six amino acid sequences (chr2:CM0065.490 and chr2:CM0065.500 in *L. japonicus*; and Glyma01g01000.1, Glyma03g36430.1, Glyma10g11100.1 and Glyma19g39080.1 in *G. max*) were obtained by a BLAST search of the *L. japonicus* and *G. max* genomic sequence database, respectively, using the amino acid sequence of TCO as a query. Scale bars: 1 mm in B, C; 5 mm in E, F.

**Fig. 6. Structure of the TCO gene.** (A) Exon/intron structure of the TCO gene. Boxes indicate exons. The protease-associated domain (blue), the M28 peptidase domains (green) and the transferrin receptor-like dimerization domains (red) were predicted by a comparison of the amino acid sequence of TCO to those of TCO homologs in other plants. Location of the tco mutation is indicated by the arrowhead. (B, C) Complementation of the tco nodulation phenotype. Representative transgenic hairy roots of *L. japonicus* carrying a 7.8-kb genomic fragment encompassing the entire TCO locus (B) or the control GUS gene sequence (C). Note the presence of fully developed nodules in B; transgenic hairy roots were identified by mCherry-NLS red fluorescence signals. (D) Average number of nodules formed on transgenic hairy roots, as described in B and C (n=11 plants). Nodulation phenotypes were evaluated at 21 dai. Error bars indicate s.d. (E, F) Complementation of the tco shoot phenotype. Representative stable transgenic tco plants carrying a 7.8-kb genomic fragment encompassing the entire TCO locus (E) or the control GUS gene sequence (F) at 7 dag. Note the formation of normal numbers of cotyledons and leaves in E. (G) Phylogenetic tree of TCO-related carboxypeptidases. Full-length amino acid sequences were compared and the tree was constructed by neighbor-joining methods (Saitou and Nei, 1987) using PLA3 and VP8 as the root. Numbers indicate bootstrap values. Accession numbers of the amino acid sequences of related proteins are as follows: TCO (AB743813), AMP1 (ABQ85084), At4g07670 (CAB81137), At5g19740 (AAP37682), PLA3 (AB447403), VP8 (ACA62934), NAALADase I (AAC53423), NAALADase II (Q9Y3Q0) and NAALADase L (Q9UQ11). Six amino acid sequences (chr2:CM0065.490 and chr2:CM0065.500 in *L. japonicus*; and Glyma01g01000.1, Glyma03g36430.1, Glyma10g11100.1 and Glyma19g39080.1 in *G. max*) were obtained by a BLAST search of the *L. japonicus* and *G. max* genomic sequence database, respectively, using the amino acid sequence of TCO as a query. Scale bars: 1 mm in B, C; 5 mm in E, F.
However, we cannot rule out the possibility that upregulation of *LjTAR1* might be a secondary effect caused by the potential inhibition of auxin transport in the *tco* mutant. Further investigations will be needed to determine whether the link between TCO and auxin regulation is direct or indirect. According to the ATTED-II co-expression database, *Arabidopsis AMP1* is closely co-expressed with *TAR2* and *ARF1*, which are involved in auxin biosynthesis and signaling, respectively. In addition, *pla3* exhibits reduced root gravitropism, which is an auxin-related phenotype (Kawakatsu et al., 2009).

With respect to the origin of root nodule symbiosis, the most plausible current model suggests that it might have developed through the co-opting of genes involved in another plant soil microbe symbiosis system, such as that present in a wide range of plants and arbuscular mycorrhizal fungi (Parmiske, 2008). This hypothesis is supported by two lines of evidence: first, the establishment of the latter type of symbiosis is estimated to be more ancient than root nodule symbiosis; and second, a number of so-called common symbiosis pathway genes have been identified that are involved in the regulation of both types of symbiosis. During nodulation, these genes have roles in the epidermal rhizobial-infection signaling cascade that decodes calcium signaling (Kouchi et al., 2010), the ultimate trigger for the initiation of nodule organogenesis in cortical cells. In other words, the common symbiosis pathway genes predominantly function in the rhizobial infection process. It is therefore unlikely that the genes are directly involved in nodule organogenesis. Currently, therefore, there is still considerable uncertainty on the evolution of nodule production in legumes. Genetic studies of AON, which negatively regulates nodule organogenesis (Caetano-Anollés and Gresshoff, 1991; Oka-Kira and Kawaguchi, 2006), have identified a series of key genes, *HAR1*, *KLV* and *CLV2-like* genes, that are orthologous to genes that play essential roles in the regulation of SAM formation in other plants (Krusell et al., 2002; Miyazawa et al., 2010; Krussell et al., 2011). Among the AON genes, *KLV* and *CLV2-like* appear to retain the ability to control SAM formation; it is not known at present whether *HAR1* is involved in regulation of the SAM. In the present study, we have identified a novel factor, *TCO*, that is a potentially common regulator of both nodule organogenesis and SAM formation. Our findings therefore provide additional evidence for the existence of common genetic regulatory mechanisms for nodule organogenesis and SAM formation. It is possible that nodule organogenesis might have been achieved in part by co-opting genes involved in the formation of the SAM. Of note, although *TCO* has the same function as *KLV* and *CLV2-like* as a negative regulator of cell proliferation in the SAM, during nodule development *TCO* has a positive regulatory function that is the opposite of *KLV* and *CLV2-like*, which act to block cortical cell proliferation during nodulation. Further investigation of the potential interactions among *TCO*, *KLV* and *CLV2-like* will be needed to elucidate how *TCO* achieves an opposite function in the control of two meristematic tissues: nodules and the SAM.

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**Competing interests statement**

The authors declare no competing financial interests.

**References**


**Fig. 7. In situ localization of TCO transcripts in WT *L. japonicus*.

(A) Root region containing dividing cortical cells of a young nodule primordium at 5 dai. (B) A nodule at 8 dai. Asterisk indicates the central region of rhizobial colonization. (C,D) Root region containing nodules at 8 dai. D shows a magnification of the image shown in C. (E) A nodule at 8 dai. (F) Shoot apex. Arrow indicates shoot apical meristem. Arrowhead indicates vascular tissues. Longitudinal root/nodule and stem sections are shown. Antisense (A-D,F) or sense (E) probes were used for the detection of the TCO transcripts. Scale bars: 100 μm.**

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