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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.

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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; Krusell 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 (Suzaki et al., 2004; Oka-Kira and Kawaguchi, 2006). Recently, the AON-related *KLAVIER* (*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 (Krusell 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 *LOTUS 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; Rightmyer and Long, 2011). These auxin responses are believed to occur in the downstream part of the cytokinin signaling pathway (Plet et al., 2011; Suzaki 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 M_2 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 (Suzaki 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 (Suzaki 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 F_2 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 F_2 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′-CATAACCC-ATGCCATAAGCC-3′; PCR-Rv1, 5′-CCACCGCTCGAGTAGTTAGC-3′; Sequence-Fw1, 5′-TCCACTTCCTCCAACTCCAC-3′; Sequence-Fw2, 5′-GCCTGTACTTCCTTTGTTTGG-3′; Sequence-Rv1, 5′-ATGGGGAA-AATGCAAGTGAG-3′; Sequence-Fw3, 5′-CCTCGATGCTTAGCAG-CTTT-3′; and Sequence-Fw4, 5′-TTGCTCCCATCAATCACAGA-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 (Suzaki et al., 2012) and inserted into the *Bam*HI site of pCAMBIA1300-GFP. Next, the *GFP* moiety in the vector was removed using *Xho*I, and a PCR-amplified *mCherry-NLS* sequence (Suzaki et al., 2012) was inserted into the *Xho*I site to create the new binary vector pCAMBIA1300-GW-mCherry. The primer set 5′-AAACTCGAGATGGTGAGCAAGGGCGAGGA-3′ and 5′-AAACTCGAGTTACTCTTCTTCTTGATCAG-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′-CACCCACAAGGCCATTGAATCCTT-3′ and 5′-TTGAAATTGGATCCTCTGCC-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 analysis 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′-TCCTACCTCCGCGCCCTCAC-3′ and 5′-CACCGGAGCACCTCCAAGCG-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 RNeasy 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′-ATGCAGATCTTCGTCAAGACCTTG-3′ and 5′-CCTCCCCTCAGACGAAG-3′; *ATP synthase*, 5′-ACATGCTTGCACCATACCAA-3′ and 5′-TCCCCAACTCCAGCAAATAC-3′; *NIN*, 5′-CAATGCTCTTGATCAGGCTGTTGA-3′ and 5′-GAGTGCTAATGGCAAATTGTGTGTC-3′; *LjRR4* (chr3.LjT48P15.80), 5′-ATCAAGGATCAACAGGTGCC-3′ and 5′-ATGGGTTGTTGGTCTTGCTC-3′; *LjTAR1*, 5′-AGGGCATTGAAAATGGTGAG-3′ and 5′-CATCCATGCCAATCATGGTA-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 M_2 plants derived from 3135 EMS-treated M_1 *snf2* plants were screened, and a candidate suppressor mutant was identified. This mutation was named *tricot* (*tco*) because of its predominantly 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*.

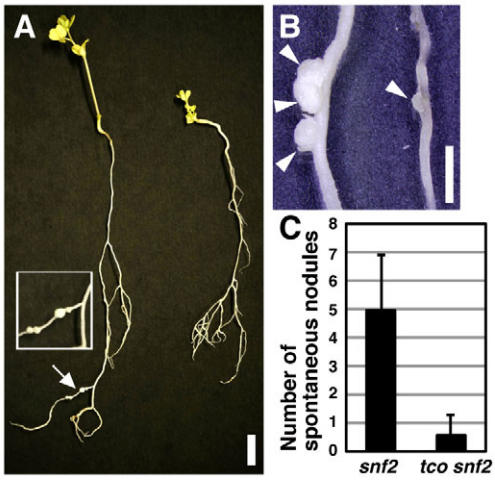

Fig. 1. The effect of the *tco* mutation on *snf2*-dependent spontaneous nodule formation in *L. japonicus*. (**A**) Shoot and root phenotype of the *snf2* mutant (left) and the *tco snf2* double mutant (right). The inset shows an enlarged segment of the *snf2* mutant (indicated by the arrow) that contains spontaneous nodules. (**B**) Representative examples of spontaneous nodules (arrowheads) in the *snf2* mutant (left) and *tco snf2* double mutant (right); note the size difference between the two genotypes. (**C**) The numbers of spontaneous nodules in the *snf2* and *tco snf2* double mutants. Roots from 20 plants per genotype were analyzed at 35 days after germination in the absence of rhizobia. Error bars indicate s.d. Scale bars: 1 cm in A; 1 mm in B.

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. F_1 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 F_2 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 underdeveloped and 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 (Schauser et al., 1999). Expression of the *NIN* gene is strongly activated at an early stage after infection by rhizobia (Fig. 2F) (Schauser 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 between 7 and 14 days

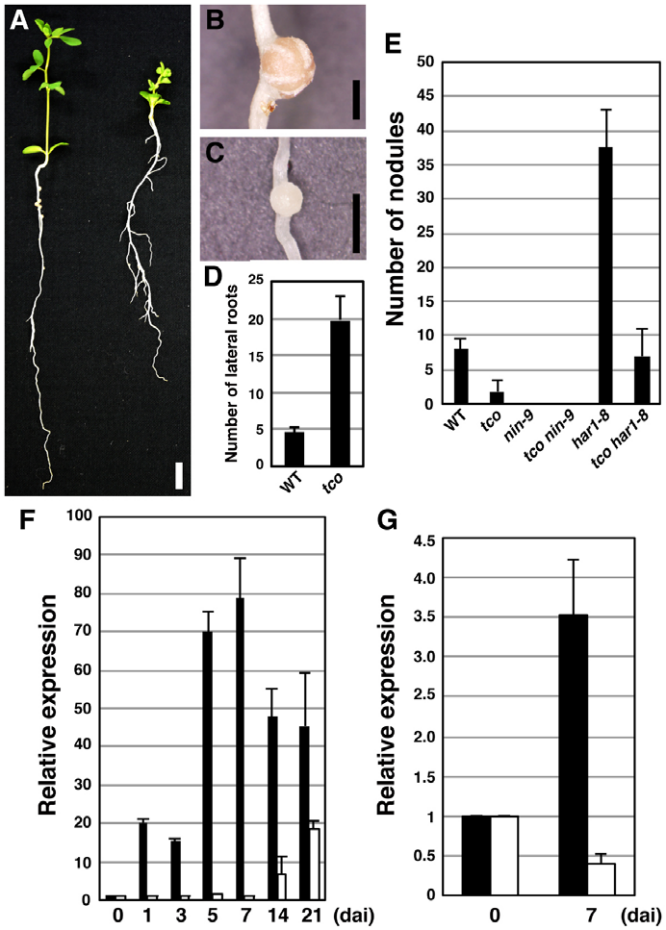

Fig. 2. The effect of the *tco* mutation on nodulation. (**A**) Shoot and root phenotype of *L. japonicus* WT MG-20 (left) and *tco* (right) plants. (**B**,**C**) Enlarged images of nodules in WT (B) and *tco* (C) roots. (**D**) Number of lateral roots in *L. japonicus* WT MG-20 and *tco* plants. Roots from 14 plants per genotype were analyzed at 21 dai with *M. loti* MAFF 303099. (**E**) Number of nodules in different *L. japonicus* MG-20 mutant lines, including *tco* (*n*=14-19). Nodulation phenotypes were observed at 21 dai. (**F**,**G**) Real-time RT-PCR analysis of *NIN* (F) and *LjRR4* (G) expression in uninoculated WT (black bars) and *tco* (white bars) roots (0), and in inoculated roots at 1, 3, 5, 7, 14 and 21 dai. cDNAs were prepared from total RNAs from whole roots. The relative (fold) changes in expression are shown compared with roots at 0 dai. Error bars indicate s.d. Scale bars: 1 cm in A; 1 mm in B,C.

(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 (Krusell 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.

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 by a signaling cascade 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* expression (Fig. 4A,B). 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

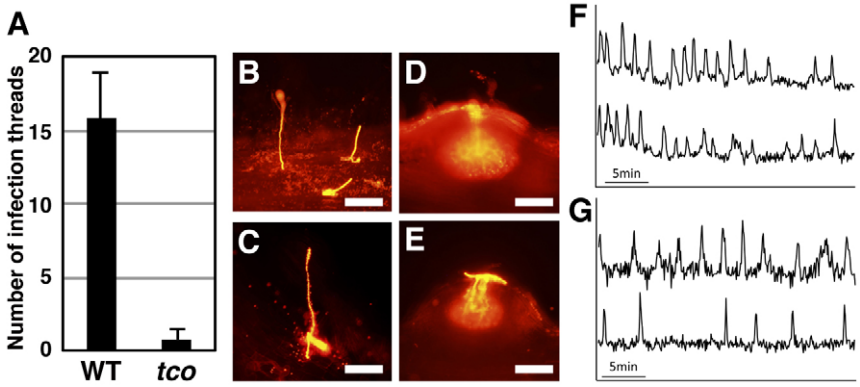

Fig. 3. The effect of the *tco* mutation on infection by rhizobia. (**A**) Number of infection threads at 5 dai. The roots of 20 *L. japonicus* plants per genotype were analyzed. (**B-E**) The formation of epidermal and cortical infection threads in WT (B,D) and *tco* (C,E) roots at 14 dai. For the observation of infection threads, plants were inoculated with *M. loti* MAFF303099 that constitutively express a *DsRED* reporter gene. (**F**,**G**) Calcium spiking caused by Nod-factor application to WT (F) and *tco* (G) roots; transgenic hairy roots containing the nuclear-localized yellow-chameleon (YC2.60) construct were analyzed. Two examples of calcium spiking pattern are shown for each genotype. The bottom trace in G indicates a low-frequency calcium spiking pattern. Error bars indicate s.d. Scale bars: 100 μm.

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 F_2 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).

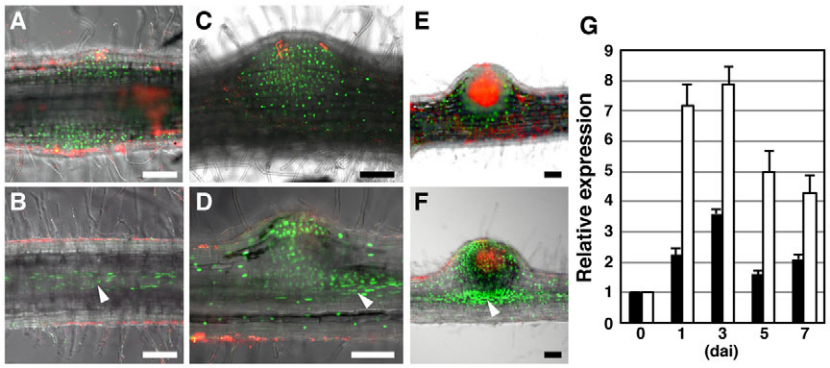

Fig. 4. Auxin response pattern and *LjTAR1* expression in the *tco* mutant during nodulation. (**A-F**) Auxin response patterns during nodulation visualized by GFP fluorescence (green) in *DR5::GFP-NLS*/WT (A,C,E) and in *DR5::GFP-NLS*/*tco* transgenic *L. japonicus* plants (B,D,F). Red areas (DsRED fluorescence) indicate the presence of rhizobia. Arrowheads point to ectopic auxin response in root stele cells in *tco* roots. Roots were observed at 5 (A,B), 8 (C), 10 (D,E) and 14 (F) dai. At least 20 plants were examined at each developmental stage in three independent experiments. (**G**) Real-time RT-PCR analysis of *LjTAR1* expression in uninoculated WT (black bars) and *tco* (white bars) roots (0), and at 1, 3, 5 and 7 dai. cDNAs were prepared from total RNAs derived from whole roots. Relative (fold) changes in expression are shown compared with roots at 0 dai. Error bars indicate s.d. Scale bars: 100 μm.

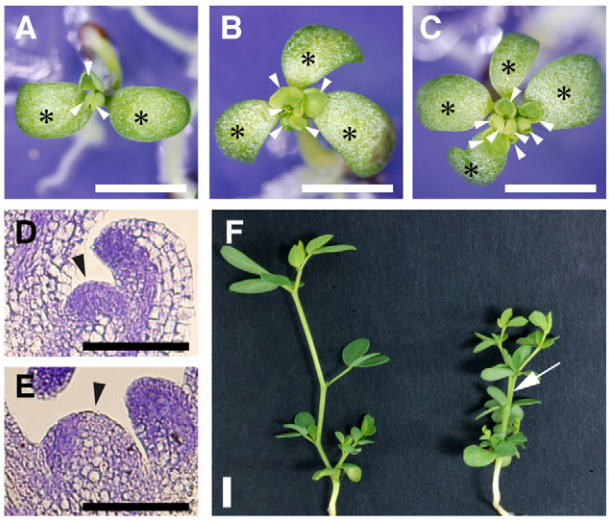

Fig. 5. Shoot phenotype of the *tco* mutant. (**A-C**) Young shoots of WT (A) and *tco* (B,C) *L. japonicus* plants showing embryonic leaves (cotyledons) at 7 days after germination (dag). Asterisks and white arrowheads indicate cotyledons and leaf primordia, respectively. (**D**,**E**) Longitudinal sections through shoot apex of WT (D) and *tco* (E) plants at 4 dag. Black arrowheads point to the shoot apical meristem. (**F**) Shoots from WT (left) and *tco* (right) plants at 28 dag. Arrow indicates the formation of a fasciated stem. Scale bars: 5 mm in A-C; 100 μm in D,E; 1 cm in F.

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* (Frugier 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 process 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 (Schauser 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,

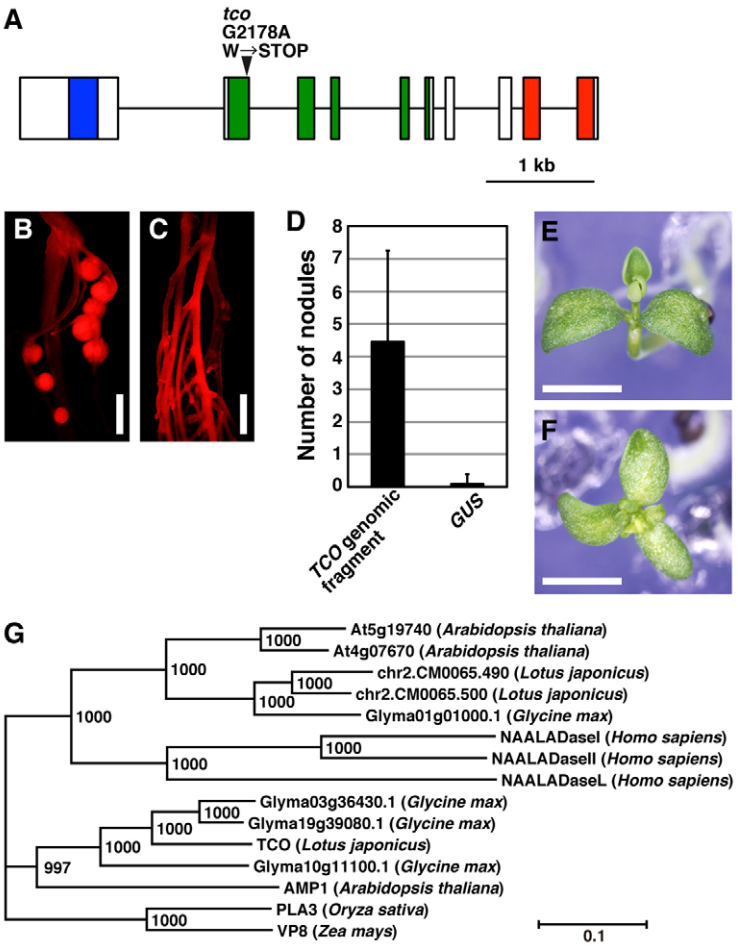

Fig. 6. Structure of the *TCO* gene. (**A**) Exon/intron structure of the *TCO* gene. Boxes indicate exons. The protease-associated domain (blue), the M28 peptidase domains (green) and the transferrin receptor-like dimerization domains (red) were predicted by a comparison of the amino acid sequence of 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 (Q9UQQ1). 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 *Glycine 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.

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 (Helliwell 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 suppressed not only the effects of constitutive activation of cytokinin signaling but also the activation of the nodulation-related cytokinin response regulator. These results indicate that the cytokinin response might be reduced in the *tco* mutant, in contrast to the *amp1* and *pla3* mutants, in which cytokinin levels are elevated (Chaudhury et al., 1993; Kawakatsu et al., 2009). Further analysis, such as determining cytokinin levels and detailing the cytokinin response in *tco*, will be needed to clarify the diverse functions of this carboxypeptidase with respect to control of cytokinin action.

Auxin responses are predominantly induced in the downstream part of the cytokinin signaling pathway in proliferating cortical cells (Suzaki et al., 2012). The establishment and maintenance of auxin response are closely related to the control of further nodule organogenesis. Our fluorescence analysis of *DR5::GFP-NLS* plants showed that auxin response rates were lower in cortical cells of the *tco* mutant than in the WT. In addition, it is interesting that an auxin response occurred ectopically in root stele cells of the mutant. During nodule development, auxin is thought to be transported to the sites of incipient nodule primordia from root vasculatures through control of the levels of expression of some *PIN* genes (Plet et al., 2011). Our *in situ* hybridization analysis showed that the distribution of *TCO* transcripts was similar to that of auxin-responsive cells during nodule development (Suzaki et al., 2012). Moreover, *TCO* was expressed in root vascular tissues and adjacent cells through which auxin is presumed to be transported to the cortical cells. Thus, based on these results, we propose that the *tco* mutation might affect the transport of auxin from the root vasculature to the cortical cells. Under this hypothesis, auxin flow would be slow in the stele cells in the *tco* mutant whereas it would be smoothly transported to the cortical cells in the WT and would therefore not accumulate at a detectable level in the stele cells. The ectopic auxin response in the stele cells might underlie the increased lateral roots formation in the *tco* mutant. We recently found that expression of the *LjTAR1* gene, which is involved in auxin biosynthesis, is contemporaneous with the onset of local auxin response (Suzaki et al., 2012). In contrast to the WT, the *LjTAR1* gene is activated in the *tco* mutant. Despite such high expression of a gene involved in auxin biosynthesis, the *tco* mutant nevertheless displays a nodulation-deficient phenotype, suggesting that auxin is not linked to nodule development in the mutant. This might indicate that auxin is not properly utilized in the *tco* mutant.

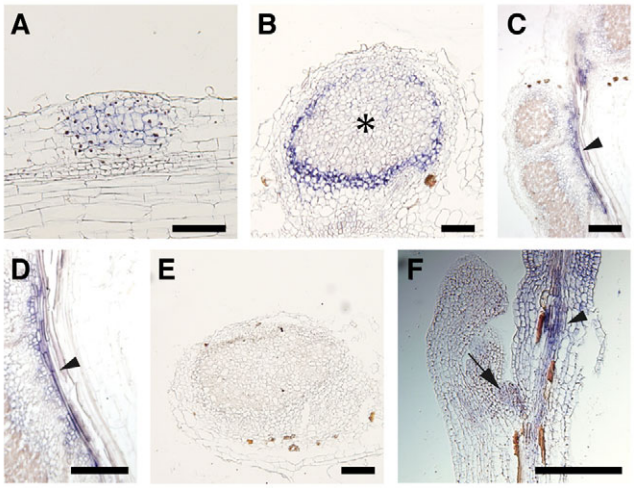

Fig. 7. *In situ* localization of *TCO* transcripts in WT *L. japonicus*. (**A**) Root region containing dividing cortical cells of a young nodule primordium at 5 dai. (**B**) A nodule at 8 dai. Asterisk indicates the central region of rhizobial colonization. (**C**,**D**) Root region containing nodules 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.

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 (Parniske, 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; Nishimura et al., 2002; Miyazawa et al., 2010; Krusell 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.

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