RESEARCH REPORT

Endoreduplication-mediated initiation of symbiotic organ development in *Lotus japonicus*

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ABSTRACT

Many leguminous plants have a unique ability to reset and alter the fate of differentiated root cortical cells to form new organs of nitrogen-fixing root nodules during legume-Rhizobium symbiosis. Recent genetic studies on the role of cytokinin signaling reveal that activation of cytokinin signaling is crucial to the nodule organogenesis process. However, the genetic mechanism underlying the initiation of nodule organogenesis is poorly understood due to the low number of genes that have been identified. Here, we have identified a novel nodulation-deficient mutant named *vagrant infection thread 1* (*vag1*) after suppressor mutant screening of spontaneous nodule formation 2, a cytokinin receptor gain-of-function mutant in *Lotus japonicus*. The *VAG1* gene encodes a protein that is putatively orthologous to *Arabidopsis* ROOT HAIRLESS 1/HYPOCOTYL 7, a component of the plant DNA topoisomerase VI that is involved in the control of endoreduplication. Nodule phenotype of the *vag1* mutant shows that *VAG1* is required for the ploidy-dependent cell growth of rhizobial-infected cells. Furthermore, *VAG1* mediates the onset of endoreduplication in cortical cells during early nodule development, which may be essential for the initiation of cortical cell proliferation that leads to nodule primordium formation. In addition, cortical infection is severely impaired in the *vag1* mutants, whereas the epidermal infection threads formation is normal. This suggests that the *VAG1*-mediated endoreduplication of cortical cells may be required for the guidance of symbiotic bacteria to host meristematic cells.

KEY WORDS: Endoreduplication, *Lotus japonicus*, Root nodule development, Topoisomerase VI

INTRODUCTION

In response to appropriate inductive conditions, plants have the capacity to form new organs from differentiated cells. Root nodulation is a form of such *de novo* organogenesis that occurs predominantly in leguminous plants (Crespi and Frugier, 2008). During early nodule development, a rhizobia-derived nodulation factor induces the dedifferentiation of root cortical cells. The activated cortical cells then proliferate to form the primordium of the symbiotic nitrogen-fixing root nodule (Yang et al., 1994; Geurts and Bisseling, 2002; Oldroyd et al., 2011). Recent identification and functional analyses of the putative cytokinin receptors *Lotus japonicus* LOTUS HISTIDINE KINASE 1 (*LHK1*) and *Medicago truncatula* CYTOKININ RESPONSE 1 have led to a greater understanding of how the activation of cytokinin signaling is crucial to the initiation of nodule organogenesis (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). In particular, it has been shown that in the *L. japonicus* spontaneous nodule formation 2 (*snf2*) mutant, which possesses a gain-of-function form of *LHK1*, confers the constitutive activation of cytokinin signaling, resulting in the formation of spontaneous nodule-like structures in the absence of rhizobia (Tirichine et al., 2007).

Normal nodulation is achieved by interactive processes involving infection by rhizobia and nodule organogenesis (Madsen et al., 2010); the complexity of these interactions has made it difficult to study the regulation of these mechanisms separately. The use of spontaneous nodules, however, appears to be an efficient approach for focusing on nodule organogenesis because the effect of the rhizobial infection process can be excluded from spontaneous nodule development (Tirichine et al., 2006). Spontaneous nodule formation is suppressed by the mutation of any of the three putative transcription factors NODULE INCEPTION, NODULATION SIGNALING PATHWAY 1 (*NSP1*) and *NSP2*; these factors are involved in nodule organogenesis (Tirichine et al., 2007). But our understanding of the nodule initiation process is still limited because of the limited number of genes that have been identified.

RESULTS AND DISCUSSION

*vag1* suppresses *snf2*-dependent spontaneous nodule formation

To investigate the genetic mechanism involved in the onset of nodule organogenesis, we performed a screen for genetic suppressors of the *snf2* spontaneous nodulation phenotype. A recessive semi-dwarf mutant named *vagrant infection thread 1* (*vag1*) was identified, and the number of spontaneous nodules reduced in the *vag1* *snf2* double mutant compared with the *snf2* single mutant (Fig. 1A). The number of spontaneous nodules of *snf2* increased in the presence of the *hyp* mutation as previously shown (Tirichine et al., 2007), but the enhanced spontaneous nodulation was strikingly suppressed by the *vag1* mutation (Fig. 1A). In the *vag1* single mutant, the initiation of nodule organogenesis was severely attenuated after inoculation of its rhizobial symbiont *Mesorhizobium loti* (Fig. 1B,C), without substantially altering overall root architecture (supplementary material Fig. S1A,B). The *vag1* mutant appeared to respond normally to cytokinin, as shoot and root growth of the mutant, as well as of the wild type (WT), were inhibited in the presence of high cytokinin concentrations (supplementary material Fig. S2), suggesting that the *vag1* mutant acts as a suppressor of *snf2* without affecting cytokinin signaling in shoot and root growth.

During nodule development, rhizobia invade host cortical cells via a specialized structure called the infection thread (IT), which originates in a root hair cell (Murray, 2011). In WT, ITs penetrated the root hair and ramified within inner cortical cells (Fig. 1D;
supplementary material Fig. S3). By contrast, ITs that formed in the vag1 mutant appeared to penetrate root hair cells normally but failed to ramify and reach cortical cells (Fig. 1E,F; supplementary material Fig. S3), suggesting that the mutant infection threads are blocked at the epidermal-cortical interface. Normal nodule development is achieved by interactive processes involving nodule organogenesis and IT formation (Madsen et al., 2010). To examine the two processes separately, we focused on the cyclops mutant, in which, despite a premature arrest of IT elongation in root hairs, the initiation of nodule organogenesis is nonetheless induced (Yano et al., 2008). In the vag1 cyclops-6 double mutant, although ITs showed the cyclops-type premature arrest phenotype, the initiation of nodule organogenesis was significantly suppressed (supplementary material Fig. S4), suggesting that the impairment of nodule initiation in the vag1 mutant is due to a defect in the nodule organogenesis process rather than its aberrant IT progression.

VAG1 encodes a putative component of plant DNA topoisomerase VI

Map-based cloning identified a gene that is responsible for the vag1 mutation (supplementary material Fig. S5). The vag1 mutation carries a G-to-A nucleotide substitution in the splice donor site of intron 9 in the gene, causing intron mis-splicing (Fig. 2A-C). The mutant nodulation phenotype of vag1 was rescued when a genomic fragment containing the wild-type gene was introduced into the mutant by hairy root transformation (Fig. 2D-F). Moreover, we isolated another mutant carrying a mutation in the VAG1 gene (hereafter we denote the mutant vag1-2). The expression of VAG1 seemed to be unaffected by M. loti infection (supplementary material Fig. S6A). To determine the spatial expression pattern of VAG1, we first identified a functional VAG1 promoter that could rescue the vag1 mutation when VAG1-coding sequence was expressed under the control of the promoter (Fig. 2F). Reporter gene analysis using the promoter (pVAG1::GFP-NLS) showed that VAG1 was expressed throughout the root, including proliferating cortical cells, to form nodules (supplementary material Fig. S6B-E).

VAG1 encodes a protein that is putatively orthologous to Arabidopsis ROOT HAIRLESS 1 (RHL1)/HYPOCOTYL 7 (HYP7) (Schneider et al., 1998; Sugimoto-Shirasu et al., 2005), a component of the plant DNA topoisomerase VI. In Arabidopsis, loss-of-function mutants of factors constituting topoisomerase VI have general defects in ploidy-dependent cell growth, and the number of highly endoreduplicated cells is reduced in the mutants (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002, 2005; Yin et al., 2002), indicating that topoisomerase VI has a function as a positive regulator of endoreduplication. Although the rhl1 mutation prevents the formation of root hairs, they were formed normally in the vag1 mutants (supplementary material Fig. S1C,D). RHL1 could rescue the vag1 mutation when it was expressed under the control of the VAG1 promoter (Fig. 2F), suggesting that the VAG1 protein has a function similar to RHL1.

As represented by trichomes of the leaf epidermis in Arabidopsis and by endosperm of the embryo in maize, it is believed that endoreduplication contributes to increasing plant cell sizes, in particular to producing terminal differentiated cells (Lee et al., 2009). In legume-Rhizobium symbiosis, rhizobial-infected cells of nodules are terminal differentiated as a consequence of endoreduplication (Kondorosi and Kondorosi, 2004). To assess the involvement of VAG1 in controlling endoreduplication, we next investigated the nodule phenotype of vag1. The vag1-I mutant could form nodules, although their numbers were small (Fig. 1B). The size of the nodules formed in the mutant was largely indistinguishable from those of the WT (Fig. 3A,B). An observation of vag1-I mutant nodule sections showed that the sizes of cells located in the inner region of nodules were smaller and the numbers of potential rhizobia-colonized infected cells were reduced compared with the WT (Fig. 3C-F). In addition, the inner region of vag1-I nodules comprised large numbers of small rhizobia-infected (as yet uncolonized) cells that resembled those located at surrounding regions of rhizobia-colonized infected cells in WT (supplementary material Fig. S7). In wild-type nodules, flow cytometry revealed that few diploid cells were detected, and cells underwent up to four rounds of endoreduplication to reach 32C (Fig. 3G1). By contrast, vag1-I nodules comprised increased proportions of diploid cells, whereas the proportions of endoreduplicated cells (>4C) were significantly reduced (Fig. 3H1). The ploidy level of uninoculated vag1 roots was indistinguishable from that of WT (supplementary material Fig. S1E-G).
VAG1-mediated endoreduplication is crucial to the initiation of nodule organogenesis

An observation of the attenuation of nodule initiation in the vag1 mutant and VAG1 implication in endoreduplication of infected cells led us to postulate that VAG1-mediated endoreduplication is required for the onset of nodule organogenesis. We previously produced transgenic L. japonicus plants, in which nuclear localized GFP is expressed under the control of a synthetic auxin-responsive element, DR5 (Suzaki et al., 2012). Use of these plants enabled us to monitor the nuclei of cortical cells relevant to the nodulation cell lineage. As a result, we observed that nuclei of the outermost cortical cells immediately before initiation of division were larger than those of the surrounding cortical cells in WT (Fig. 4A). The enlarged nuclei were continuously observed after the initiation of cortical cell proliferation (Fig. 4B,C). In addition, ITs appeared to elongate towards the cortical cells with the enlarged nuclei (Fig. 4A-C). By contrast, in the vag1-1 mutants there were no enlarged nuclei in cortical cells beneath root hairs with ITs and no cortical cell division was induced (Fig. 4D,E), despite the induction of auxin response (supplementary material Fig. S8). As DNA size is reflected in nuclear size (Bourdon et al., 2011; Iwata et al., 2011), we next estimated the DNA size of the enlarged nuclei by quantifying the size of DAPI-labeled nuclei. In WT, the size of the enlarged nuclei was more than 8-fold larger compared with those of root cap cells (2C control) (supplementary material Fig. S9), suggesting that their DNA size reaches at least 16C. In the vag1 mutants, the size of nuclei in cortical cells beneath root hairs with IT was largely comparable to that of cortical cells of uninoculated roots (supplementary material Fig. S9).

In plants, CCS52A isoforms are necessary and sufficient for the progress of endocycling (Cebolla et al., 1999; Larson-Rabin et al., 2009; Mathieu-Rivet et al., 2010; Baloban et al., 2013), which skips the mitotic phase and re-enters the S phase without cytokinesis, resulting in the duplication of the nuclear DNA content. In M. truncatula, Mccs52A is shown to be expressed before endoreduplication and to regulate endoreduplication of infected cells within nodules (Vinardell et al., 2003). We isolated two L. japonicus CCSS2A1 genes (LjCCSS2A1-LIKE1 and LjCCSS2A1-LIKE2) with high similarity to Arabidopsis CCSS2A1. Promoter-GUS reporter analysis and in situ hybridization revealed that the two genes were expressed within nodules (supplementary material Fig. S9). The genes were also expressed in dividing cortical cells during early nodule development.
The Miyakojima MG-20 ecotype of *L. japonicus* was used as WT in this study. The *vag1-1* mutant was isolated from the M₄ generation of *snf2* plants that had been mutagenized with ethylmethane sulfonate (EMS) (Miyazawa et al., 2010; Suzuki et al., 2013). The *vag1*-2 mutants were isolated by a screening of nodulation-deficient mutants using EMS-treated MG-20 plants, which were provided by Legume Base. *DR5::GFP-NLS/vag1-1* plants were produced by crossing *DR5::GFP-NLS* transgenic plants (Suzaki et al., 2012) with *vag1-1* plants. For the analyses of rhizobial-induced nodulation or spontaneous nodulation, plants were grown with or without *M. loti* MAFF 303099, respectively, as previously described (Suzaki et al., 2013).

**Constructs and transformation of *L. japonicus***

The primers used for PCR are listed in supplementary material Table S1. For the complementation analysis, a 10.9 kb genomic DNA fragment including the *VAG1* candidate gene was amplified by PCR from wild-type genomic DNA. This fragment included 3.0 kb of sequence directly upstream of the initiation codon, and was cloned into pCAMBIA1300-GFP-LjLTI6b (Suzaki et al., 2012). For the *VAG1* expression analysis, a gateway-cassette (GW) fragment was cloned into pCAMBIA1300-GFP-LjLTI6b to create the new binary vector pCAMBIA1300-GFP-LjLTI6b. Next, 3.0 kb of sequence directly upstream of the initiation codon of *VAG1* was amplified by PCR and cloned into pDONR221 (Suzaki et al., 2012) were inserted downstream of the *VAG1* promoter by LR recombination reactions. To create the
Flow cytometry

Nodules were removed from wild-type or vag-I-I roots, cut into pieces with razor blades in extraction buffer (Partec) and incubated for 2 min at room temperature. The suspension was filtered through a 30 μm Celltrix filter (Partec) and stained with staining buffer (Partec). Flow cytometry was performed using a Ploidy Analyzer PA (Partec). Data are shown as mean±s.e.m. of three biological replicates.

Accession numbers

Sequence data from this report can be found in the GenBank/EMBL data libraries under the following accession numbers: VAG1, ABB71650; RHL1, At1g48380; LjCCS52A1-LIKE1, ABB71651; LjCCS52A1-LIKE2, ABB71652; CCS52A1, At4g22910.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.S. and M.I. designed research and analyzed data; T.S., M.I., E.Y., S.S., H.H. and Byers, B. performed research; and Makoto Hayashi for providing pLjCCS52A1-LIKE2::GUS expression is a determinant of endoreduplication and cell expansion in Arabidopsis. Plant Physiol. 149, 874-884.


