Identification of genes involved in *Meloidogyne incognita*-induced gall formation processes in *Arabidopsis thaliana*

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Abstract  Root-knot nematodes (RKN; *Meloidogyne incognita*) are plant-parasitic nematodes that cause significant damage to crop plants worldwide. Recent studies have revealed that RKNs disrupt various physiological processes in host plant cells to induce gall formation. However, little is known about the molecular mechanisms of gall formation induced by nematodes. We have previously found that RNA expression levels of some genes related to micro-RNA, cell division, membrane traffic, vascular formation, and meristem maintenance system were modified by nematode infection. Here we evaluated these genes importance during nematode infection by using Arabidopsis mutants and/or β-glucuronidase (GUS) marker genes, particularly after inoculation with nematodes, to identify the genes involved in successful nematode infection. Our results provide new insights not only for the basic biology of plant–nematode interactions but also to improve nematode control in an agricultural setting.

Key words: gall formation, plant–microorganism interaction, root-knot nematodes.

Introduction

Plants exhibit a wide range of interactions with a variety of organisms. Symbiosis and parasitism are two extreme types of interactions between associated species. Arbuscular mycorrhizal (AM) symbiosis improves plant nutrition; in turn, the plant supplies the fungus with lipids and sugars derived from photosynthesis (Smith and Read 2008). In the legume–rhizobia symbiotic association, biological nitrogen fixation is carried out in the nodules produced on roots. In mature nodules, rhizobia convert atmospheric nitrogen (N2) into ammonia (NH3), which is essential for plant growth. In turn, the bacteria obtain photosynthesis-derived carbon from the plant (Burris and Roberts 1993). In contrast, several obligate parasites, such as witchweeds (Striga) and plant-parasitic nematodes, are harmful agricultural pests that can affect important crops, severely affecting host growth and yields by depriving them of water and nutrients. For example, *Striga*, a parasitic plant that parasitizes other plants, connects its own vascular bundle to the vascular bundle of the host plant and deprives it of nutrients (Ishida et al. 2016). Plant-parasitic nematodes, such as cyst nematodes (*Heterodera* spp., *Globodera* spp.), root-knot nematodes (*Meloidogyne* spp.), and root lesion nematodes (*Pratylenchus* spp.), are known to damage a broad spectrum of crops. Of these parasites, cyst nematodes and root-knot nematodes (RKN) feed on cells in host plant roots and cause problems for plant development. The second-stage juveniles (J2) of cyst nematodes invade plant roots and migrate through cortical cells. When they reach the vascular bundle, they induce cell fusion of vascular cells to form a syncytium of feeding cells. RKNs invade their host plant roots and migrate to the vasculature, where they induce galls. It is thought that RKNs inject various effector proteins into vasculature cells, which convert these cells into specialized feeding cells, known as giant cells (GCs).

Nematodes inject various effector proteins from their esophageal gland into procambium cells to promote re-differentiation of vascular cells into multinucleate GCs with endoreduplication, which causes DNA...
amplification without mitosis (Bird 1961; de Almeida Engler et al. 2012). In Arabidopsis thaliana, knockout of CELL CYCLE SWITCH PROTEIN 52, which has been characterized as an enhancer of endoreduplication, or overexpression of KIP-RELATED PROTEIN6 (KRP6), which is known to inhibit the endocyte, attenuate the hypertrophy of GCs. It has been suggested that a DNA amplification mechanism, such as cytokinetic mitosis or endoreduplication, is involved in the increase in cell volume of GCs (de Almeida Engler et al. 2012; Vieira et al. 2011, 2012). Previously, we showed that the expression of procambium-related genes was increased (Yamaguchi et al. 2017) and the expression of secondary cell wall-related genes was decreased (Ishida et al. 2020) during gall development. In addition, LATERAL ORGAN BOUNDARIES-DOMAIN 16, which is responsible for lateral root formation, is involved in gall formation (Cabrera et al. 2014). The auxin transporter, ARABIDOPSIS THALIANA PIN-FORMED, is also involved in gall formation (Kyndt et al. 2016). It is clear that various plant responses and developmental mechanisms are involved in gall formation.

Here, we evaluated the nematode susceptibility of various Arabidopsis mutants and their spatial gene expression patterns, particularly after inoculation with nematodes, to identify the genes involved in gall formation.

Materials and methods

Plant materials and growth conditions

The data of mutant line information were detailed in Supplementary Table S1. Arabidopsis thaliana Columbia-0 (Col-0) was used as the control. Mutant seeds were provided by other researchers: myb3r1, myb3r2, myb3r3, myb3r4, myb3r5, myb3r7/14, myb3r9/35, myb3r11/35, gig1 uvi4, gig1 myb3r4, MYB3R3::GUS, MYB3R4::GUS, MYB3R5::GUS (Prof. Masaki Ito), ara6, ara7, raba(a–d), vamp727, vpp9a-2, vpp42, vpp43, vam3-1, rha1, vpp42-pv43 (Prof. Takashi Ueda), CPT::GUS (Dr. Takuji Wada), ago1-27, ago2-1, ago3-1, ago4-6, ago5-2, ago6-3, ago7-1, ago9-2, and ago10-1 (Prof. Yuichiro Watanabe); bgul21-1, pyk10-1, and mamm1 (Prof. Eiko Hara-Nishimura); jar1 and coi1-1 (Prof. Shinobu Satoh); max1-4, max2-4, max3-12, and max4-7 (Prof. Mikihisa Umehara); CCA1 ox, lhy12 CCA1 LUC, and cca1-1 lhy12 CCA1 LUC (Prof. Elaine Tobin); CUC2::GUS, KNAT1::GUS, STM::GUS, QC25::GUS and QC184::GUS (Prof. Massao Tasaka). Arabidopsis seeds were surface-sterilized and sown on a plate (0.25× Murashige and Skoog (MS) salt mixture (Sigma), 0.5% (w/v) sucrose, and 0.6% (w/v) phytargel or gellan gum at pH 6.4), placed in the dark for 2 days at 4°C, then allowed to germinate and grow under continuous light at 23°C.

Nematode preparation and inoculation

RKN were prepared as previously described (Nishiyama et al. 2015). Briefly, 6- to 7-week-old tomato plants were inoculated with juvenile nematodes at 3-day intervals for a total of four inoculations. Approximately 80,000 juveniles were used to inoculate each plant. The inoculated tomato plants were then transferred to a hydroponic system, and at 2- to 4-day intervals infective juveniles were collected from the hydroponic culture media. Six Arabidopsis seeds were sown on an MS plate. Five-day-old Arabidopsis seedlings were inoculated with approximately 80 sterilized nematodes per plant and incubated under short-day conditions (8 h light/16 h dark) at 25°C. Their roots were covered with black paper to mimic the dark underground environment found in nature.

Evaluation of gall formation efficiency

Gall formation efficiency was evaluated as reported (Nakagami et al. 2020). In brief, Gall number and germinated seedlings number were counted for each petri dish of six seedlings. Galls/ seedling value was calculated as “total number of galls per plate/ germinated seedling number.” The galls/seedling values from WT and mutants were normalized to the basal value for each petri dishes to calculate relative galls number.

β-glucuronidase (GUS) staining

Arabidopsis roots with or without galls were fixed in 90% acetone for 24 h at −30°C and stained in GUS buffer (100 mM NaPO4 buffer (pH 7), 10 mM EDTA (pH 8), 0.1% Triton X-100, 3 mM K3Fe(CN)6, and 3 mM K4Fe(CN)6) with 0.5 mg ml−1, 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (Wako) for 4 h. The reaction was stopped with Carnoy’s solution (90% (v/v) methanol, 10% (v/v) acetic acid), and the roots were mounted in a chloral hydrate solution (8 g chloral hydrate, 2 ml water, and 1 ml glycerol). They were then observed using an Axio Imager M1 microscope (Carl Zeiss Microscopy) and photographed using a DP71 digital camera (Olympus). Here we have used Arabidopsis wild type with GUS reporter gene construct without any other mutation to examine the spatial expression after nematode inoculation.

Results

Evaluation of nematode susceptibility of Arabidopsis mutants

We have previously found that RNA expression levels of some genes related to micro-RNA, cell division, membrane traffic, vascular formation, and meristem maintenance system were modified by nematode infection (Figure 1; Yamaguchi et al. 2017). Furthermore, it is reported that jasmonate, salicylic acid, and strigolactone signaling pathway modulate regulate nematode infection in plant (López-Raez et al. 2017; Molinari et al. 2014; Nahar et al. 2011).

To identify candidate genes that may be responsible for successful RKN infection, 39 different Arabidopsis mutants were examined for their susceptibility to M. incognita. The spatial promoter activity of 19 different genes in galls following nematode inoculation was
also examined, using a GUS reporter to decipher their possible function during nematode infection of Arabidopsis roots.

In the first screening, two independent infection experiments were conducted. Supplementary Figures S2 and S3 summarize some plants with no differences compared with that of wild-type. We examined nematode resistance using 15 genotypes, categorized as cell cycle, membrane trafficking, plant hormones and clock, as shown in Supplementary Figure S2. However, these mutants showed no obvious abnormalities resulting from nematode infection. Furthermore, spatial expression was examined using MYB DOMAIN PROTEIN 3R3 (MYB3R3::GUS), QC25::GUS, QC184::GUS, WUSCHEL (WUS::GUS), RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2::GUS), and CAPRICE (CPC::GUS) following nematode inoculation, and we did not observe any GUS signals in the galls (Supplementary Figure S3).

Some other plants with features of interest were selected for additional analyses (Figures 1–5).

**Cell proliferation-related genes**

It has been reported that the balanced cell cycle gene expression which occurs during gall development is characterized by two major cell cycle mechanisms: cytokinesis and endoreduplication (de Almeida Engler et al. 2012). The decrease in cyclin-dependent kinase (CDK) activity that acts in M phase is involved in the
switch from normal cell division to endoreplication. For the regulation of CDK activity, the synthesis and degradation of its activating subunit, cyclin (CYC), play an important role. Many plant G2/M-specific genes are controlled by three Myb repeat-containing transcription factors, named R1R2R3-Myb (MYB3R) (Ito 2005). The Arabidopsis genome has five MYB3R genes, MYB3R1 to MYB3R5. MYB3R1 and MYB3R4 act as transcriptional activators, while MYB3R1 both as an activator and as a repressor (Kobayashi et al. 2015), MYB3R3, and MYB3R5 are transcriptional repressors. The expression of these genes is controlled temporally and spatially during the cell cycle (Haga et al. 2007, 2011; Kobayashi et al. 2015).

Seven different genotypes of cell cycle-related mutants were examined to evaluate resistance to *M. incognita*. Five-day-old plants were inoculated with sterilized RKNs. At 14 days post-inoculation (dpi), the number of galls was counted and compared with that of wild-type. The *myb3r1*, *myb3r5* and *myb3r1/3/5* mutants showed almost the same susceptibility as the wild-type, whereas the *myb3r3*, *myb3r4*, *myb3r1/4*, and *myb3r3/5* mutants showed significant reductions in the number of galls, to ∼26%, ∼31%, ∼37%, and ∼41%, respectively (Figure 1A).

Spatial expression patterns were examined for B1-type cyclins (CYCB1;1), MYB3R4 and MYB3R5, using GUS as a reporter gene. CYCB1;1::GUS showed patchy activity both in the root tips and lateral root primordia (LRP). MYB3R4 and MYB3R5 promoter activity was detected in the vascular region of Arabidopsis roots (Figure 1B). Following inoculation with nematodes, the CYCB1;1::GUS signal was detected around gall regions, and the signal became more obvious in late stage (Figure 1B). Together with the vascular region, the GUS signal became stronger in the whole central cylinder region. A similar spatial signal pattern for GUS was observed in the MYB3R4::GUS transgenic plants. The expression levels of MYB3R4::GUS were higher than those of CYCB1;1::GUS at 3 dpi. MYB3R5::GUS signal was detected in the vascular region, and weak expression was also observed in the central cylinder (Figure 1B). In total, all of the myb reporter gene expression was dominant in the vascular region, and the MYB3R4::GUS expression level was the highest in the MYB3 genes after nematode inoculation.

GUS reporter signal patterns of the plant-specific B1-type CDKs (CDKB), CDKB1;1::CDKB1;1-GUS and CDKB2;1-GUS were also examined. GUS activity was observed at the root tips and LRP in the non-infected roots. Following inoculation with nematodes, the GUS signal was observed in vascular regions from 1 dpi. The signal became stronger even in the central cylinder at 2 dpi and became stronger still at 3 dpi. After 3 dpi, the GUS signal gradually weakened during gall development; however, some patchy but strong
signals were still observed. This may indicate that both CDKB1;1 and CDKB2;1 function in restricted cells that are actively proliferating, even during the later stages of gall development, and that gall formation may rely on the cell proliferation activity of host plant cells (Figure 1C).

**Micro RNA-related genes**

AGO1 facilitates the induction of genes in jasmonate (JA) signaling pathways and activates plant responses to hormonal, biotic, and abiotic stimuli (Liu et al. 2018). miRNA binds AGO proteins, forming RNA-induced silencing complexes (RISCs) that silence the expression of complementary mRNAs (Iwakawa and Tomari 2013). Phylogenetic analysis grouped AGO proteins into three clades. In Arabidopsis, the ten members are equally distributed among the three clades: AGO1/AGO5/AGO10 are in the first clade; AGO2/AGO3/AGO7 are in the second clade; and AGO4/AGO6/AGO8/AGO9 (AGO8 is a pseudogene) are in the third clade (Vacheret 2008). Nine different argonaute (ago) mutants were examined to evaluate their susceptibility to nematodes (Figure 2). ago1, ago2, and ago9 showed no abnormalities in gall formation rates. ago3, ago4, ago5, ago6, ago7, and ago10 mutants showed reductions in the number of galls to ~48%, ~32%, ~27%, ~54%, ~49%, and ~43%, respectively (Figure 2).

**Membrane trafficking-related genes**

It has been reported that the membrane trafficking system plays a pivotal role in rapid responses to environmental stimuli, such as challenges by microorganisms (Inada and Ueda 2014). RABs and SNAREs (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) are conserved regulatory molecules involved in membrane trafficking. Three RAB5 homologs have been identified in A. thaliana: conventional-type ATRABF2B (ARA7) and ATRABF2A (RHA1) and plant-specific ATRABF1 (ARA6). These GTPases are activated by ARABIDOPSIS THALIANA VACUOLAR PROTEIN SORTING 9A (VPS9A) protein (Goh et al. 2007). The RAB11 group of the RAB/RAB11, which is uniquely expanded during land plant evolution (Minamino et al. 2018), acts in the secretory pathway and consists of four members (RAB1A to D) (Asaoka et al. 2013; Choi et al. 2013). The SNAREs in Arabidopsis can be classified into the Q-group, containing VACUOLAR MORPHOLOGY 5 (VAM3) also known as SYNTAXIN OF PLANTS 22, and the R-group, containing VESICLE-ASSOCIATED MEMBRANE PROTEIN 727 (VAMP727) (Sandefoot 2007). To examine whether the membrane trafficking system is involved in gall formation, eight genes were examined with mutants. As a result, ara7, vam727, syntaxin of plants 43 (syx43), vam3-1, and rha1 showed similar susceptibilities to that of the wild-type. ara6, raba(a–d), and vps9a-2 mutants showed reduction in the number of galls to ~30%, ~18%, and ~37%, respectively (Figure 3).

**Shoot apical meristem-related genes**

Mutations of CLAVATA1, RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), and some other shoot apical meristem (SAM)-related genes confer resistance against cyst nematode infection (Replogle et al. 2013). Therefore, we observed spatial promoter activity using GUS marker lines to examine the expression of SAM-related genes. In the absence of nematodes, GUS activity of CUP-SHAPED COTYLEDON 2 (CUC2::GUS), KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1::GUS), BARELY ANY MERISTEM 1 (BAM1::GUS), and SHOOT MERISTEMLESS (STM::GUS) was observed at shoot apical meristems. Weak promoter activity of CUC2, KNAT1, and BAM1 was also detected around lateral root primordium. Following inoculation with nematodes, GUS signals of CUC2::GUS and BAM1::GUS were detected in both the vascular and central cylinders at 7 dpi. The KNAT1::GUS signal spread in the vascular and central cylinder regions during gall development. Promoter activity of the KNAT1 gene seemed to be restricted to the giant cell region. A strong GUS signal of STM::GUS was observed in the vascular regions (Figure 4). In addition to CLAVATA1 and RPK2, CUC2, KNAT1, BAM1, and STM might also be involved in gall development.

**Vascular-related genes**

We have previously reported the crucial function of vascular-related genes during gall formation (Yamaguchi et al. 2017). Here, we examined some other vascular-related genes. We confirmed GUS reporter gene activity of the Arabidopsis H+-ATPase3 (AHA3::GUS), ALTERED PHLOEM DEVELOPMENT (APL::GUS), SUCROSE-PROTON SYMPORTER 2 (SUC2::GUS), and VASCULAR HIGHWAY 1 (VH1::GUS) in vascular cells, as previously reported (Bonke et al. 2003; Clay and Nelson 2002; DeWitt et al. 1991; Truerenit and Sauer 1995). Following inoculation with nematodes, all of the marker lines showed GUS signals along with the vascular cells. The signals were restricted to areas around vascular cells (Figure 5). Given that AHA3, SUC2, and APL are expressed in the phloem, number of phloem strand increased during gall development, probably because of vascular re-arrangement that occurred during gall development. Further, the promoter activity of the vascular gene, VH1, was observed both in the vascular regions and in the central cylinder (Figure 5). These results suggest that AHA3, APL, SUC2, and VH1 are also responsible for normal gall development.
Discussion

In this study, we showed that the number of galls was reduced in myb3r4, and that there was obvious up-regulation of MYB3R4 gene expression in galls. These results indicated that the transcription activator MYB3R4 is required for successful gall formation. Previous studies have demonstrated that MYB3R activators (Act-MYBs) from tobacco and Arabidopsis are phosphorylated and activated by CDK (Araki et al. 2004; Haga et al. 2007). Mitotic cyclins, one of the targets of MYB3Rs, form active CDK–cyclin complexes, so Act-MYBs are further phosphorylated and activated through a positive feedback loop, thus causing a burst of G2/M-specific gene expression. Interestingly, it has been reported that KRP6 functions as a CYCLIN-DEPENDENT KINASE/CYCLIN (CDK/CYC) inhibitor and that overexpression of KRP6 suppresses normal gall development induced by nematode infection (Vieira et al. 2014). These results suggest that CYC/CDK-ActMYB is responsible for gall formation.

In a recent study, ago1 or ago2 mutation reduced egg mass number after nematode inoculation (Medina et al. 2017), suggesting the importance of micro RNA (miRNA) signaling pathways for successful nematode infection. In this study, we did not observe any significant differences in the gall numbers of ago1 and ago2, which is the previous step of egg mass development, suggesting that Ago1 and Ago2 may be responsible only for the late stages of gall development and/or nematode maturation steps to produce egg masses. On the other hand, miR390 is reported to be involved in the early stages of gall development (Cabrera et al. 2016). Here, we showed a reduced gall formation rate in ago7. Together with previous reports that miR390-AGO7 complexes function in distinct cleavage or non-cleavage modes at two target sites in ta-siRNA gene (TAS3a) transcripts (Montgomery et al. 2008), TAS3a transcripts may also be responsible for normal gall development.AGO4 (or its homologs, AGO6 and AGO9) dependent siRNAs are secondary siRNAs dependent on the prior activity of the RNA-directed DNA methylation (RdDM) pathway (Wang and Axtell 2017). It has been suggested that the RdDM pathway is involved in the maintenance of genome integrity during the reprogramming process of galls/GCs from their vascular precursor cells, and/or in ensuring faithful DNA replication during the repeated mitosis/endoreduplication that coincides with feeding site formation (Ruiz-Ferrer et al. 2018). Consistent with this previous report, our assays also supported the importance of AGO4 and AGO6 for gall development, because the ago4 and ago6 mutants showed a reduced number of galls.AGO10 functions as a decoy for miR166/165 to maintain SAM activity, preventing their incorporation into AGO1 complexes and the subsequent repression of Homeodomain–Leucine zipper (HD-Zip) III gene expression (Zhu et al. 2011). Here we showed a reduced infection rate in ago10 mutants, suggesting that the mechanism of AGO10-HD-ZIPIII may be important, even during gall formation.

In this study, ara6, raba, and vps9a-2 showed resistance to gall formation. ARA6, which is activated by VPS9A, and VAMP727 are plant-specific machinery components of membrane trafficking (Ebine et al. 2011; Goh et al. 2007). It is also known that the RABA group is extremely expanded during land plant evolution (Minamino et al. 2018), implying a plant-specific regulatory system for membrane trafficking. These results suggest that plant-specific membrane trafficking mechanisms may be involved in gall formation.

Interestingly, GUS signals controlled by SAM-related genes promoters was observed in galls.

On the other hand, it was recently reported that the expression of class-I KNOX genes including KNAT1 and STM was increased in the early development stage of gall by insects (Hirano et al. 2020). Taking these findings into consideration, RKN and insect might share some of the SAM maintenance mechanisms for gall formation.

In this study, we screened candidate genes that may be responsible for nematode infection, using Arabidopsis mutants and GUS reporter transgenic plants. Our findings suggest that many genes are involved in normal gall development following nematode inoculation. It would be interesting to examine these gene functions against different types of nematodes, such as cyst nematodes. Cyst nematodes have a CLE peptide gene in their genome; it has been suggested that injection of the CLE peptide into plant cells regulates successful infection against different types of nematodes, such as cyst nematodes. In this study we screened candidate genes that may be responsible for nematode infection, using Arabidopsis mutants and GUS reporter transgenic plants. Our findings suggest that many genes are involved in gall development following nematode inoculation. It would be interesting to examine these gene functions against different types of nematodes, such as cyst nematodes. Cyst nematodes have a CLE peptide gene in their genome; it has been suggested that injection of the CLE peptide into plant cells regulates successful infection (Guo et al. 2017). However, the CLE peptide gene has still not been reported in RKNs. So, the infection mechanisms appear to be different. Here, we examined many genes that may commonly be used in the infection mechanisms used by many types of pests. However, they might be used differently for their respective successful infections. The information we have presented here will provide useful candidates for comparing the infection mechanisms of many pests; this may assist in understanding how these pest–host relationships evolved.

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