TRAPPC13 Is a Novel Target of *Mesorhizobium amorphae* Type III Secretion System Effector NopP

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Similar to pathogenic bacteria, rhizobia can inject effector proteins into host cells directly to promote infection via the type III secretion system (T3SS). Nodulation outer protein P (NopP), a specific T3SS effector of rhizobia, plays different roles in the establishment of multiple rhizobia-legume symbiotic systems. *Mesorhizobium amorphae* CCNWGS0123 (GS0123), which infects *Robinia pseudoacacia* specifically, secretes several T3SS effectors, including NopP. Here, we demonstrate that NopP is secreted through T3SS-I of GS0123 during the early stages of infection, and its deficiency decreases nodule nitrogenase activity of *R. pseudoacacia* nodules. A trafficking protein particle complex subunit 13-like protein (TRAPPC13) has been identified as a NopP target protein in *R. pseudoacacia* roots by screening a yeast two-hybrid library. The physical interaction between NopP and TRAPPC13 is verified by bimolecular fluorescence complementation and coimmunoprecipitation assays. In addition, subcellular localization analysis reveals that both NopP and its target, TRAPPC13, are colocalized on the plasma membrane. Compared with GS0123-inoculated *R. pseudoacacia* roots, some genes associated with cell wall remodeling and plant innate immunity down-regulated in Δ nopP-inoculated roots at 36 h postinoculation. The results suggest that NopP in *M. amorphae* CCNWGS0123 acts in multiple processes in *R. pseudoacacia* during the early stages of infection, and TRAPPC13 could participate in the process as a NopP target.

Keywords: nodulation outer protein P, rhizobia, symbiosis, type III secretion system, trafficking protein particle complex subunit 13

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Symbiotic interactions established between rhizobia and legumes represent a major nitrogen (N₂)-fixing system that transforms atmospheric dinitrogen into ammonia. Since ammonia can be utilized directly by plants, the establishment of the symbiotic systems facilitates improvement in fertility and productivity of low-N soils and thereby, the enhancement of ecosystem functioning (Wagner 2011; Zahran 1999). In many cases, the formation of N₂-fixing nodules on legume roots requires the cooperation of type III secretion system (T3SS) and Nod factor signalings (Janczarek et al. 2015; López-Baena et al. 2016). T3SS is used to deliver effector proteins from pathogenic bacteria to the cytoplasm of the host cells of many plants and animals to facilitate bacterial survival and promote infection (Jiménez-Guerrero et al. 2017). Many rhizobia, similar to pathogenic bacteria, interfere with signal transduction pathways and impair plant defense systems to establish symbiotic interactions with legumes through a functional T3SS (Miwa and Okazaki 2017; Robert-Seilanianantz et al. 2011). Many rhizobial T3SS effectors (T3Es) have been identified based on genetic, genomic, and proteomic approaches (Jiménez-Guerrero et al. 2017; Miwa and Okazaki 2017). For example, 87 T3Es belonging to 47 families were identified from *Ensifer (Sinorhizobium) fredii* and *Bradyrhizobium japonicum* using a genomic approach (Kimbrel et al. 2013). Some of the rhizobial T3Es show similarities with pathogenic T3Es such as NopT and NopM. In contrast, others T3Es, such as NopP, NopC, NopL, and NopI, are rhizobia-specific and have no detectable homologs in other bacteria (Ausmees et al. 2004; Miwa and Okazaki 2017).

As a widespread rhizobia-specific T3E, NopP participates in the establishment of multiple rhizobia-legume symbiotic systems. The inactivation of *nopP* in *Sinorhizobium* sp. strain NGR234 dramatically reduces the number of nodules formed on *Flemingia congesta* and *Tephrosia vogelii* (Skorpil et al. 2005); however, it has no effect on other legumes responsive to the T3SS of NGR234 (Kambara et al. 2009). In addition, infection of *Vigna unguiculata* with the *nopP*_NGR234 knockout mutant strain induces the formation of 36% more nodules than infection with the wild-type strain (Ausmees et al. 2004). The inactivation of *nopP*_HH103 facilitates symbiosis between *E. fredii* HH103 and *Glycine max*; yet, it is highly detrimental when T3SS-dependent secretion is blocked (López-Baena et al. 2008, 2009). When the legume host is switched to *V. unguiculata*, mutation of *nopP*_HH103 has neutral effects on symbiosis, while mutation of *nopL*_HH103 increases nodule number and shoot biomass; however, mutation of both *nopP*_HH103 and *nopL*_HH103 impairs nodulation, causing decreases in nodule number and shoot biomass (Jiménez-Guerrero et al. 2017). In addition, NopL modulates signal transduction

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pathways, and *nopL* expression prevents full induction of pathogenesis-related defense genes in *Nicotiana tabacum* (Bartsev et al. 2004). Although it has recently been reported that NopP directly interacts with GmNNL1 from *Bradyrhizobium* USDA110 in soybean (Zhang et al. 2021), the biological functions and targets of NopP in other legumes has not been fully elucidated.

Robinia pseudoacacia is a pioneer tree species exploited in ecological and vegetation restoration programs. Due to its rapid growth rate, high drought tolerance, and robust seed germination and N fixation capacity, *R. pseudoacacia* has high ecological importance and is planted extensively in the Loess Plateau of China (Boring and Swank 1984; Qiu et al. 2010). *R. pseudoacacia* plantations play a vital role in the improvement of soil properties and ecological restoration in this plateau region associated with severe soil erosion (Li et al. 2004). Previously, a rhizobial strain designated as *Mesorhizobium amorphae* CCNWGS0123 (GS0123) was isolated from the root nodules of *R. pseudoacacia* grown in heavy metal tailings (Hao et al. 2012) and its specific infection of *R. pseudoacacia* was verified by plant cross-nodulation tests (Chen et al. 2013). In strain GS0123, there are two T3SS gene clusters, T3SS-I and T3SS-II. T3SS-I, which is located in the symbiotic plasmid, is an integrated cluster containing all the basal components, while T3SS-II, which is located in the chromosome, lacks some of the structural genes of T3SSs. Plant assays have shown that T3SS-I is necessary for legume nodulation by strain GS0123, and the expression of T3SS-I structure genes can be up-regulated in the presence of flavonoids, in contrast to that in T3SS-II (Wang et al. 2019). However, the role of specific T3Es in the establishment of symbiosis between *R. pseudoacacia* and strain GS0123 is still unclear.

To explore the role of NopP in symbiosis establishment, we generated a *nopP*_GS0123 knockout mutant ($\Delta nopP$) and screened a yeast two-hybrid (Y2H) library from GS0123-inoculated roots of *R. pseudoacacia* to identify the direct target protein of NopP in host plant cells. Transcriptome analysis was used to identify candidate genes related to NopP function. The data presented in this study could enhance our understanding of the molecular mechanisms underpinning the establishment of rhizobia-legume symbiotic interactions.

RESULTS

NopP is an effector of T3SS of GS0123.

According to GS0123 genome sequencing data, the *nopP* gene with a *tts* box conserved element in the promoter region was identified on symbiotic plasmid. When strain GS0123 was cultured in tryptone-yeast extract (TY) broth, *nopP*_GS0123 could be induced by flavonoids, as detected by quantitative (q)PCR (Fig. 1A). It was speculated that NopP was a T3E of strain GS0123.

To determine whether NopP was secreted through T3SS-I in GS0123, supernatant of the wild-type strain and the T3SS-I and T3SS-II mutant strains induced with flavonoids was extracted and detected by mass spectrometry, respectively. NopP was detected in extracellular medium of the wild-type strain and the T3SS-II mutant strain but not in extracellular medium of the T3SS-I mutant strain (Supplementary Table S1), indicating that NopP was a T3E in GS0123.

Expression profiles of *nopP*_GS0123 in *R. pseudoacacia*.

During infection of *R. pseudoacacia* plants by strain GS0123, root-hair curling and infection threads (ITs) were detected at 4 days postinoculation (dpi) and the number of ITs peaked at 5 dpi. Root nodules emerged at approximately 10 dpi. The nodules did not mature fully until 18 dpi, and senescent nodules emerged after 30 dpi (Chen et al. 2013; Wang et al. 2019). Based on these observations, the infection process can be divided into four phases: prophase of

early stage (0 to 3 dpi), anaphase of early stage (3 to 7 dpi), middle stage (7 to 18 dpi), and late stage (18 to 30 dpi).

To analyze the changes in *nopP*_GS0123 expression during symbiosis establishment, we determined the relative expression of *nopP*_GS0123 in *R. pseudoacacia* roots inoculated with the wild-type strain (GS0123), based on qPCR (Fig. 1B). During

Fig. 1. Changes in the expression of *nopP* in *Mesorhizobium amorphae* CCNWGS0123 (GS0123). **A**, Relative expression level of *nopP* at 48 h after flavonoid induction in liquid culture (GS0123: without flavonoid induction, GS0123*: with flavonoid induction). The flavonoids used for induction were apigenin, luteolin, and naringenin; **B** and **C**, Relative expression level of *nopP* during different stages of infection. Data shown are the mean $\pm$ standard deviation of the mean for three biological replicates, the comparisons of means were performed using the natural $\log_2$ -transformed data. Different letters above bars indicate significant differences (fold change value ≥ 2). hpi = hours postinoculation; dpi = days postinoculation.

different stages of infection, the highest relative expression of *nopP*_GS0123 was observed at 1 dpi, which decreased significantly with an increase in time of infection (4 to 30 dpi). Subsequently, gene expression over the first 120 h postinoculation (hpi) was measured, and the relative expression increased initially and peaked at 24 hpi (Fig. 1C). NopP could play a role in symbiosis establishment during the prophase of early infection according to these initial results.

The trafficking protein particle complex subunit 13-like (TRAPPC13) protein is a novel interaction partner of NopP.

To determine the intracellular interactors of NopP, we screened a cDNA library prepared from GS0123-inoculated roots of *R. pseudoacacia* by a Y2H assay. From 20,000 candidates screened, 159 clones were sequenced and 24 unique clones were identified, of which TRAPPC13 was the only one with stable and strong fluorescence in the bimolecular fluorescence complementation (BiFC) experiments among the annotated potential targets. The full-length sequences of *nopP* and *trappc13* were inserted into pGBKT7 and pGADT7 vectors, respectively. According to the Y2H assay results, NopP and TRAPPC13 expression had no toxic effects on yeast growth. Yeast carrying positive control plasmids pGADT7-T/pGBKT7-53 (encoding interacting gene products) turned blue on quadruple amino acid (–Leu/–Trp/–His/–Ade) dropout, aureobasidin A-positive (A), and X-α-Gal-positive (X) plates (QDO/X/A), while yeast carrying the negative control plasmids pGADT7-T/pGBKT7-Lam (encoding noninteracting gene products), pGBKT7-NopP, and pGADT7-TRAPPC13 did not grow. The results indicated that the bait NopP and the target TRAPPC13 could not autonomously activate the reporter genes; thus, they were suitable for screening the Y2H library. The positive result was verified by yeast growth on QDO/X/A plates only when both pGBKT7-NopP and pGADT7-TRAPPC13 plasmids were expressed, which indicated a direct interaction between

NopP and TRAPPC13 (Fig. 2; Supplementary Fig. S1). To confirm the protein-protein interaction between NopP and TRAPPC13, we conducted BiFC and coimmunoprecipitation (Co-IP) assays in *Nicotiana benthamiana* leaves. Observations under a laser confocal scanning microscope revealed that NopP fused with the C-terminal fragment of yellow fluorescent protein (YFP^C) and TRAPPC13 fused with the N-terminal fragment of yellow fluorescent protein (YFP^N), which jointly produced fluorescence on the plasma membrane (PM) in *N. benthamiana* leaves (Fig. 3). In addition, hemagglutinin (HA)-tagged TRAPPC13 was coexpressed in *N. benthamiana* leaves with Flag-tagged NopP, and the presence of NopP was detected by Western blotting with anti-Flag antibodies after incubation with anti-HA tag magnetic beads (Fig. 4). The results confirmed a strong interaction between NopP and TRAPPC13.

Subcellular localization of NopP and TRAPPC13 on the PM.

To examine the subcellular localization of NopP and TRAPPC13, *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* GV3101 containing fusion proteins NopP-eGFP (enhanced green fluorescent protein) and TRAPPC13-eGFP separately. Then, the fusion proteins were observed by laser confocal microscopy at 48 to 72 h postinfiltration. Green fluorescence was confined to the PM (Fig. 5), which indicated the accumulation of both NopP and TRAPPC13 on the PM. To confirm the result, NopP-eGFP and TRAPPC13-eGFP fusion proteins were coexpressed with the PM marker-*mCherry* fusion protein, which fluoresces red on the PM, in *N. benthamiana* leaves. An overlay of green fluorescence and red fluorescence in the infiltrated leaves indicated that NopP and TRAPPC13 were indeed localized on the PM (Fig. 5A and B). In view of the interaction between NopP and TRAPPC13, we constructed recombinant NopP-mCherry and TRAPPC13-eGFP vectors to determine whether TRAPPC13 was colocalized with NopP on the PM. The results showed that NopP

Fig. 2. Yeast two-hybrid assay of NopP-interacting targets in SD/DDO (synthetic defined/double amino-acid dropout) and SD/QDO/X/A (quadruple amino acid (–Leu/–Trp/–His/–Ade) dropout, aureobasidin A-positive and X-α-Gal-positive) media. Yeast cells coexpressing the indicated combinations of constructs were grown on nonselective (DDO) or selective (QDO/X/A) plates. Yeast growth on the selective plates indicated protein-protein interaction. Full-length NopP and TRAPPC13 were inserted into pGBKT7 and pGADT7 vectors, respectively. Cotransformation of NopP and TRAPPC13 with empty carrier, respectively, showed that there was no self-activation. Cotransformation of pGBKT7-p53 and pGADT7-T antigen was used as positive control and that of pGBKT7-Lam and pGADT7-T antigen was used as negative control. Ten-fold serially diluted cell cultures were inoculated on each spot as indicated by the triangle widths. A representative dataset of the experiment replicated three times on different biological samples was shown.

potentially interacted with TRAPPC13 on the PM based on their colocalization (Fig. 5C). Both the confocal image and correlation plot profile represented the fluorescence from only eGFP, and mCherry vectors were ubiquitous in the nucleus, cytoplasm, and PM and cannot be merged (Fig. 5D).

The absence of NopP slightly affects symbiosis between *M. amorphae* CCNWGS0123 and *R. pseudoacacia*.

To explore the role of NopP in symbiosis establishment, root nodule traits and plant growth performance were compared between GS0123- and $\Delta nopP$ -inoculated *R. pseudoacacia* plants. Inoculation with $\Delta nopP$ caused a slight but not significant increase in the nodule number and a minor decrease in the nodule biomass when compared with those in GS0123-inoculated plants (Fig. 6A and B). In addition, there was a significant decrease in nodule nitrogenase activity in $\Delta nopP$ -inoculated roots at 30 hpi, and the change was offset by *nopP* compensation (Fig. 6C). There were no significant differences in plant height, root length, or plant biomass between treatments, while leaf chlorophyll concentrations decreased considerably in $\Delta nopP$ -inoculated plants as compared with that in the GS0123-inoculated plants (Supplementary Fig. S2). In addition, the root nodule volumes were lower in the *nopP*-inoculated plants than in the GS0123-inoculated plants. However, microscopic observations of paraffin and ultrathin sections revealed no alterations in the internal structures of nodules or the morphological structures of bacteroids in the N_2 -fixation zone (Fig. 7).

Fig. 4. Confirmation of protein-protein interaction between NopP and TRAPPC13 by coimmunoprecipitation (Co-IP) assay in *Nicotiana benthamiana* leaves. NopP was tagged with Flag epitope, while TRAPPC13 was tagged with hemagglutinin (HA) epitope. Proteins in the input and Co-IP samples were detected with antibodies against Flag and HA by Western blotting. NopP was pulled out by anti-HA-tag magnetic beads, indicating that it was immunoprecipitated by TRAPPC13.

Fig. 3. Verification of protein-protein interaction between NopP and TRAPPC13 by bimolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana* leaves. **A**, BiFC assay between the N-terminal half of yellow fluorescent protein (YFP^N) and NopP fused to the C-terminal half of yellow fluorescent protein (YFP^C-NopP). **B**, BiFC assay between YFP^C and TRAPPC13 fused to YFP^N (YFP^N-TRAPPC13). **C**, BiFC assay between YFP^C-NopP and YFP^N-TRAPPC13. Reconstitution of yellow fluorescence indicated positive interaction on the plasma membrane (C) and the absence of fluorescence in negative controls (A and B). Scale bar = 50 μ m.

Gene expression responses of *R. pseudoacacia* to NopP during the prophase of early infection.

Two timepoints (36 and 72 hpi) were selected for transcriptome sequencing to study the gene expression responses of *R. pseudoacacia* to NopP during the prophase of early infection. Based on the RNA sequencing (RNA-seq) data, a relatively small number of genes were expressed in a specific manner, consistent with the substantial overlap between the genes expressed in GS0123- and $\Delta nopP$ -inoculated roots (Fig. 8A). Comparison of the genes expressed in $\Delta nopP$ -inoculated roots to those in GS0123-inoculated roots yielded 1,514 (321 upregulated and 1,193 downregulated) genes at 36 hpi and 165 (93 upregulated and 72 downregulated) genes at 72 hpi ($\log_2$ fold change ≥ 2 , $P < 0.05$) (Fig. 8C and D). Most of the differentially expressed genes (DEGs) showed significant differences in expression at one timepoint (Fig. 8B), suggesting a transcriptional reprogramming in response to $\Delta nopP$ infection; however, many genes were expressed only transiently during the prophase of early infection. The RNA-seq data were verified by qPCR analysis of the expression levels of 16 selected genes, and the mean values of $\log_2$ fold change for each gene were consistent with the RNA-seq data (Supplementary Fig. S3).

To annotate transcripts from gene products, we identified the functions of transcripts using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Based on GO classification, the DEGs were classified into three categories: biological processes, molecular functions, and cellular components. The top 30 enriched GO terms among the DEGs at 36 and 72 hpi are illustrated in Supplementary Figure S4. Under biological processes, “protein phosphorylation” followed by “phosphorylation” were the most enriched at 36 hpi, while “apoptotic process” and “programmed

cell death” were the top two enriched GO terms at 72 hpi. Under molecular functions, the DEGs were mostly enriched in “pepsin A activity” followed by “phosphotransferase activity, alcohol group as acceptor” and “protein kinase activity” at 36 hpi; “phospholipid binding” and “calcium-dependent phospholipid binding” were the most enriched GO terms at 72 hpi. In addition, under cellular components, a large number of DEGs were enriched in membrane followed by terms involved in vesicle (Supplementary Table S3).

By mapping the transcripts to the reference canonical pathways in the KEGG database, 747 of 1,514 DEGs at 36 hpi were assigned to 94 pathways, and 72 of 165 DEGs at 72 hpi were grouped into 36 pathways. At 36 hpi, the KEGG pathway with the highest enrichment of DEGs was plant-pathogen interaction, and most DEGs enriched in this pathway were associated with pathogen-associated molecular pattern-triggered immunity (PTI). At 72 hpi, only protein processing in the endoplasmic reticulum (ER), spliceosome, and 2-oxocarboxylic acid metabolism were enriched significantly (Supplementary Table S3).

DISCUSSION

Previously, two T3SS gene clusters have been found in *M. amorphae* CCNWGS0123 (GS0123), a rhizobia strain nodulating *R. pseudoacacia* (Wang et al. 2019). However, the role of specific T3Es, especially NopP, in the establishment of rhizobia-legume symbiosis has not been clarified. Here, we found that *nopP*_GS0123 is located in the symbiotic plasmid and is up-regulated under flavonoids induced in vitro, which is similar to genes encoding the structural components of T3SS-I. Thus, *nopP*_GS0123 can be induced by flavonoids secreted from *R. pseudoacacia* roots. A secretion assay showed that NopP was an

Fig. 5. Subcellular localization and colocalization of NopP and TRAPPC13 on the plasma membrane (PM) in *Nicotiana benthamiana* leaves. Correlation plot profiles between enhanced green fluorescent protein (eGFP) and mCherry fluorescence. **A**, Colocalization of NopP and PM marker, **B**, TRAPPC13 and PM marker, and **C**, NopP and TRAPPC13, and **D**, coexpressed of eGFP and mCherry as control. Fluorescence intensity profiles of eGFP (green) and mCherry (red) according to the distance (pixels, white dotted box in Merge images) were analyzed using Image J (National Institutes of Health, Bethesda, MD, U.S.A.). Scale bar = 50 μ m.

Fig. 6. Comparison of nodule traits in roots of *Robinia pseudoacacia* plants inoculated with the wild-type strain of *Mesorhizobium amorphae* CCNWSGS0123 (GS0123), the *nopP* mutant strain ($\Delta nopP$ -GS0123), its complementary strain (C $\Delta nopP$ -GS0123), and the wild-type strain containing the complementary vector carrying green fluorescent protein (GFP) (GS0123GFP) ($n = 100$ each). **A**, Nodule number per plant. **B**, Nodule biomass per group (each group had 15 plants, and six groups were measured). **C**, Nodule nitrogenase activity per group (each group had 15 plants, and six groups were measured). Different lowercase letters above the column indicate significant differences among the treatments ($P < 0.05$).

effector of T3SS-I in GS0123. Within the first 3 dpi, we observed an evident increase in the levels of expression of *nopP*_GS0123 in *R. pseudoacacia* roots, which indicates that *nopP*_GS0123 is involved in the molecular signaling between *M. amorphae* CCNWGS0123 and its legume host during the early stages of infection.

Using Y2H and subcellular localization assays, we observed that NopP interacts directly with TRAPPC13 on the PM. TRAPPC13 usually functions as a part of TRAPP complexes (Kim et al. 2016; Lipatova et al. 2016). These complexes share core TRAPPs, which are combined with complex-specific subunits (Lipatova et al. 2016). TRAPPC13 is a TRAPP II-specific subunit in yeast but a TRAPP III-specific subunit in metazoans (Kalde et al. 2019; Sacher et al. 2019). TRAPP II complex acts as a guanine nucleotide exchange factor (GEF) for Ypt31/32 to

regulate Golgi-to-PM transport in yeast, while TRAPP III complex activates Rab1 as a GEF and participates in autophagy and ER-to-Golgi transport and the coat protein II recruitment to ER in metazoans (Kim et al. 2016; Lipatova and Segev 2019; Yu and Liang 2012). Loss of TRAPPC13 in HeLa cells reduces apoptosis, impairs autophagic flux, and increases susceptibility to *Shigella flexneri* infection (Ramírez-Peinado et al. 2017). Trs65, the homolog of TRAPPC13 in yeast, contributes to GEF activity of TRAPP II in concert with two essential subunits, TRS120 and TRS130 (Liang et al. 2007). The deletion of Trs65 leads to lower Trs130 protein level (Liang et al. 2007). RNA-seq data shows significant increased expression levels of *trs120* and *trs130* (Gene IDs i5_LQ_ABWNT_c4911/f1p0/5138 and i4_LQ_ABWNT_c7102/f1p0/4434) in Δ *nopP*-inoculated roots at 36 hpi, suggesting that NopP may inhibit the expression of

Fig. 7. Morphological and physiological characteristics of *Robinia pseudoacacia* plants inoculated with *Mesorhizobium amorphae* CCNWGS0123. **A**, Growth status of plants inoculated with the wild-type strain (GS0123), the *nopP* knockout mutant strain (Δ *nopP*-GS0123), and sterile water (Control). **B**, Paraffin section of root nodules at 30 days postinoculation (dpi). **C**, Paraffin section of the nitrogen fixation zone in 30-dpi nodules. **D**, Transmission electron microscopic images of symbiosomes in 30-dpi nodules. **E**, A photograph of uninoculated plant roots (the absence of nodules in the control group demonstrated that the experiment was not contaminated by other rhizobia). **F**, Image of all nodules on a random plant at 30 dpi.

Trs120 and Trs130. Additionally, the mutation of Trs65/Kre11 reduces the 50% level of yeast cell wall (1→6)-glucan and increases sensitivity to oxidative-stress reagents (Brown et al. 1993; Liang et al. 2007). These results indicate that Trs65/Kre11 plays roles in cell-wall biogenesis (Brown et al. 1993) and oxidative-stress response (Fernandez-Ricaud et al. 2007). During the initiation of infection, rhizobia induce plant cell-wall remodeling enzymes, such as pectic enzymes, pectin methyl esterase, polygalacturonase, and pectate lyase (Fähhaeus and Ljunggren 1959; Muñoz et al. 1998; Xie et al. 2012), which are required for root-hair deformation and ITs formation (Brewin 2004). Although the mechanism has not been fully elucidated, some studies have reported that Nod factors can induce localized cell-wall degradation (Carol and Dolan 2002; van Spronsen et al. 1994). In this study, some genes associated with cell-wall remodeling

were down-regulated significantly in $\Delta nopP$ -inoculated roots at 36 hpi, including xyloglucan endotransglucosylase, pectinesterase, pectin methyltransferase, and glucan 1,3-beta-glucosidase. Conceivably, NopP may be related to the localized cell-wall degradation and remodeling via interacting with TRAPPC13 in the early infection.

In *Arabidopsis*, TRAPP II component mutants exhibit dwarfism, sterility, and partial photomorphogenesis in the dark and show obvious defects in cytokinesis, cross-wall formation, cell polarity, polarity of the auxin transporter PIN2, and seedling development (Garcia et al. 2020; Ravikumar et al. 2018; Rybak et al. 2014; Zhang et al. 2018), while the function of TRAPP III is still unknown in plants. The transformation of small GTPases from an inactive to active state is catalyzed by specific GEF (Goody et al. 2017; Rehman and Di Sansebastiano 2014). Trs65

Fig. 8. Overview of transcriptome analysis of differentially expressed genes (DEGs) in GS0123- and $\Delta nopP$ -inoculated *Robinia pseudoacacia* roots at 36 h postinoculation (hpi) (GS0123-36hpi and nopP-36hpi) and 72 hpi (GS0123-72hpi and nopP-72hpi). **A**, A heatmap depicting the expression changes in all the DEGs. **B**, A Venn diagram showing the comparative analysis of a set of genes changed at 36 hpi (yellow circle) and 72 hpi (purple circle). **C** and **D**, Volcano plots of DEGs between two different libraries at 36 and 72 hpi, respectively, with $\log_2(\text{fold-change})$ as the x axis and $-\log_{10}(\text{padj})$ as the y axis. Red dots indicate upregulated DEGs, green dots indicate downregulated DEGs, and blue dots indicate nonsignificant DEGs.

deletion leads to lower Ypt31 GEF activity in yeast, while TRAPPC13 deletion reduces Rab1a and Rab1b activity in Hela cells (Liang et al. 2007; Ramírez-Peinado et al. 2017). As molecular switches, Rab small GTPases are related to vesicle trafficking and signaling pathways involving hormones and biotic stresses (Rehman and Di Sansebastiano 2014). Among eight groups of plant Rab small GTPase, RabA (ortholog of Ypt31/32 in yeast and Rab11 in mammals) is extremely expanded and bears the unique characteristics in the plants (Minamino and Ueda 2019; Rehman and Di Sansebastiano 2014). Further, it has been identified that Rab small GTPases play important biological roles in nodule meristem activity and maintenance at an early stage of the infection process by alternating between GDP-bound (inactive) and GTP-bound (active) states (Flores et al. 2018; Wang et al. 2020; Yuksel and Memon 2008). Mutations of RabA2 result in bursting of ITs and a reduction in nodule number in *Phaseolus vulgaris* (Dalla Via et al. 2017). RabA2 is required for root-hair deformation and the progression and maintenance of membrane integrity of ITs by regulating vesicle trafficking of the materials involved in PM and cell-wall remodeling (Blanco et al. 2009; Dalla Via et al. 2017). In addition, Rab1p is essential for the development of the symbiosome membrane in effective symbiosis (Cheon et al. 1993). Recently, it has been demonstrated that SPIKE1 interacts with and activates the small GTPase LJROP6 as a GEF to guide the polar progression of ITs in *Lotus japonicus* (J. Liu et al. 2020). Thus, we infer that TRAPPC13 could be involved in the formation of ITs in *R. pseudoacacia* by activating the small GTPase.

In *Arabidopsis* and tomato, the T3E AvrPto from pathogenic bacteria *Pseudomonas syringae* targets FLAGELLIN SENSING 2 (FLS2) and RabE to block innate immunity (Bogdanove and Martin 2000; Speth et al. 2009; Xiang et al. 2008). Based on KEGG pathway analysis, 38 genes related to PTI, including calcium-dependent protein kinase, respiratory burst oxidase protein, calcium-binding protein CML, mitogen-activated protein kinase kinase 1 (MAPKK 1), MAPKK 1/2, and WRKY transcription factor, were down-regulated significantly in $\Delta nopP$ -inoculated roots at 36 hpi, suggesting that NopP can activate PTI downstream of FLS2. But this activation effect cannot be sustained, because the expression levels of related genes, except genes encoding respiratory burst oxidase isogeny protein A, are no different from those in the GS0123-inoculated roots at 72 hpi. Subcellular localization of FLS2 is strictly regulated by membrane trafficking. RabA subgroup members not only act in flg22 peptide-induced endocytosis of FLS2 from PM to compartments but also act in transport of newly synthesized FLS2 to the PM (Choi et al. 2013). For example, RabA4b collaborates with PLANT U-BOX13 E3 ubiquitin ligases and PI(4)P to attenuate FLS2-dependent immune responses by mediating ligand-induced degradation of the FLS2 (Lu et al. 2011; Minamino and Ueda 2019). RabA6a and RabA4c also regulate different steps in the endocytic trafficking of FLS2, while Rab1b facilitates the secretion of de novo-synthesized FLS2 to the PM (Choi et al. 2013). These findings suggest that the interaction between NopP and TRAPPC13 reduces the activity of RabA, leading to the transient stronger expression of genes related to PTI, which was inhibited subsequently during the infection.

Although evidence of the importance of Rab small GTPases of rhizobial infection have been obtained in a variety of ways, we did not observe significant changes in phenotypes of *R. pseudoacacia* inoculated with $\Delta nopP$ mutant strains except for a slight decrease in both nodule volume and nitrogenase activity. There are many possibilities for this result. First, multiple effectors work together to facilitate the establishment of symbiosis with legume hosts, while a single effector can play a limited role during rhizobial infection. Second, small GTPase is a large protein family with many functional redundancies, and Trs65

has been identified to be a nonessential subunit due to its function overlap with Trs33 in the TRAPP II assembly in yeast (Liang et al. 2007; Tokarev et al. 2009). As a homolog of Trs65, TRAPPC13 may also be replaced by Trs33 in some cases. In addition, most of the differences in the expression of related genes are only present at 36 hpi and could not be sustained, which weakened the influence of *nopP* deficiency on the establishment of the symbiotic system. In order to understand how TRAPPCs work in plants and how membrane trafficking has been adapted in the rhizobia-legume symbiotic system, much more research is needed. In conclusion, NopP is an effector of T3SS-I in *M. amorphae* CCNWGS0123, playing a role at the early stage of infection. We have demonstrated the interaction of NopP with a novel target protein (TRAPCC13) in the PM. The results of this study could enhance our understanding of the role of NopP in the establishment of rhizobia-legume symbiotic systems.

MATERIALS AND METHODS

Construction of mutant and complementary strains.

In this study, *nopP* was knocked out from the wild-type strain GS0123 of *M. amorphae* CCNWGS0123 by homologous recombination to construct a mutant strain, named $\Delta nopP$ _GS0123. The homologous arm was constructed by overlapping PCR and was linked to the suicide plasmid pK18mobsacB, and was then transferred into *M. amorphae* CCNWGS0123 by electroporation. Double-exchange mutants were selected on TY agar plates containing 5% sucrose (Ausmees et al. 2004) and were validated by sequencing. The amplification primers of the upstream homologous arms were 5'-ATCATGGGAAAGACGAGACG-3' and 5'-TGAACCAAGGATAAGGCACCA-3', and the primers of the downstream homologous arms were 5'-TCACTTTTCTGGCGAATGAAGACC-3' and 5'-CGGGCTCTGTTAGACGATTG-3'. Both pairs of primers were designed using Primer Premier v5 (Premier Biosoft International). To construct a complementary strain, *nopP* was inserted into pBBR1-mcs5; afterward, the recombinant vector was transferred into the $\Delta nopP$ _GS0123 mutant strain.

Plant growth and rhizobial infection.

R. pseudoacacia seeds were soaked in H₂SO₄ for approximately 8 min to remove the surface wax layer; thereafter, the seeds were treated with 95% (vol/vol) ethanol and 50% (vol/vol) NaClO to sterilize the surface (Chen et al. 2013). The seeds were then washed with sterile water and were germinated in sterile Petri dishes in the dark. After seed germination, the seedlings were transplanted into plastic pouches (two seedlings per pouch) containing 800 ml of a sterilized mixture of vermiculite-perlite (2:1, vol/vol) and 400 ml of Fahraeus N-free nutrient solution (Chou et al. 2016; Liang et al. 2016). Seven days later, each pouch was inoculated with a 5-ml suspension (optical density at 600 nm = 0.1) of an *M. amorphae* CCNWGS0123 culture in TY broth. The inoculated plants were incubated in a controlled greenhouse (humidity, 70%; day condition, 22°C, 16 h; night condition, 16°C, 8 h) (Wang et al. 2019) and were harvested at different timepoints (2, 4, 8, 12, 24, 48, 72, and 120 hpi and 12 and 30 dpi). Approximately 30 pouches of uninoculated seedlings were used as negative controls to ensure that the plants were not contaminated by other rhizobia.

RNA extraction and qPCR analysis.

Total bacterial RNA was extracted from purified rhizobial cultures of *M. amorphae* CCNWGS0123 grown in TY broth with or without flavonoid induction using the hot phenol method (Hosoda et al. 2003). A mixture of flavonoids (apigenin, luteolin, and naringenin) was added to the cultures, with final

concentrations of 1 μ M (Scheidemann and Wetzel 1997; Veitch et al. 2010). Total plant RNA was extracted from infected roots and shoots of *R. pseudoacacia* plants using the MiniBEST plant RNA extraction kit (TaKaRa). First-strand cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa). Bacterial cDNA was synthesized using random primers and plant cDNA was synthesized with primer Oligo (dT).

qPCRs were performed using the EvaGreen qPCR MasterMix (Abm, Canada). All primers used for qPCR (Supplementary Table S2) were designed using Primer premier v5 (Premier Biosoft International). The qPCR was performed on a QuantStudio 6 Flex real-time PCR system (Life Technologies). The obtained data were normalized to 16S rRNA and 18S rRNA expression and were then analyzed using the Quantstudio 6 real-time PCR software. Quantification of gene expression was performed using the relative quantification ($2^{-\Delta\Delta CT}$) method, and comparisons of the means were performed by using the $\log_2$ -transformed data. The DEGs were considered induced or repressed if the normalized fold change was >2 .

Secretion assay.

M. amorphae CCNWGS0123 wild-type strain and T3SS-I and T3SS-II deficient mutant strains, whose *rhcN1/rhcN2* encoding ATPases were disrupted, were grown at 28°C with flavonoid induction for 48 h, respectively. Supernatant proteins were collected from GS0123 wild-type strain, T3SS-I mutant strain, and T3SS-II mutant strain culture medium by three consecutive centrifugations (using a Sigma 3K15), one at 5,500 rpm and the others at 7,500 rpm. Then, ammonium sulfate was added into bacteria-free culture medium at a final saturation of 80%. Samples were desalted overnight by dialysis bag and were concentrated by ultrafiltration (Merck Millipore) (Marie et al. 2003; Sánchez et al. 2009). Extracellular proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were stained with Coomassie blue. As the molecular mass of NopP is about 31 kDa, the bands between 25 and 35 kDa were cut and detected by mass spectrometry.

Plant symbiosis analysis.

At 30 dpi, *R. pseudoacacia* plants inoculated with the wild-type strain of *M. amorphae* CCNWGS0123 (GS0123), the Δ nopP_GS0123 mutant strain (Δ nopP-GS0123), its complementary strain (C Δ nopP-GS0123), and the wild-type strain containing the complementary vector carrying GFP (GS0123GFP) were harvested. To examine symbiosis establishment, we measured plant height, plant biomass, root length, nodule number, and nodule biomass. In addition, the nitrogenase activity of root nodules was monitored using acetylene reduction assay (Das and De 2018). Briefly, fresh nodules from every 15 plants were placed in 10-ml sealed glass bottles, and 400 μ m of acetylene gas was injected into each bottle with a sample injector. The nodule samples were allowed to react in darkness at 28°C for 2 h. Afterward, the ethylene concentrations were analyzed by gas chromatography (Trace GC Ultra, Thermo Fisher Scientific). Chlorophyll concentrations in leaf samples at similar positions (two leaves per plant) were measured using a portable chlorophyll meter (SPAD-502 plus, Minolta Camera Co. Ltd.). Each sample had three biological replicates.

To observe the structures of root nodules, their morphology was examined microscopically using paraffin sections. Fresh nodules were wrapped in paraffin and cut into 5- μ m slices, stained with 0.05% toluidine blue for approximately 5 min, to visualize bacteroids inside the nodule cells. After staining, the samples were washed using distilled water and were then observed under an optical microscope (BX53; Olympus). In addition, ultrathin sections were prepared to observe the internal

morphology of the N₂-fixing cells in the root nodules. The N fixation zones of fresh nodules were cut into 2 $\times$ 2 mm² blocks, and the blocks were immersed in glutaraldehyde for 24 h, followed by 1% (vol/vol) osmium tetroxide for 2 h. Subsequently, the blocks were subjected to gradient dehydration with 30 to 100% ethanol, and then, were embedded in 812 resin. Resin polymerization was performed at 55°C for more than 48 h, and the ultrathin sections were observed using a transmission electron microscope (HT7700, Hitachi) (Zou et al. 2020).

Y2H screening of NopP interacting proteins.

To screen for the targets of NopP in *R. pseudoacacia* root cells, a cDNA library was prepared from GS0123-inoculated roots using a SMART cDNA library construction kit (Clontech). The roots were sampled at different timepoints, mainly during the early stages of infection. Total RNA was extracted, mixed, reverse-transcribed, and then, was used to construct a three-reading-frame normalized root cDNA library, in which each gene could express the same proteins expressed in live plants, and each protein had the similar chances of binding to the bait.

nopP_GS0123 was cloned into the bait vector pGBKT7 and cotransformed into Y2HGold yeast strain with cDNA (pGADT7-SfilB) from the constructed root cDNA library. Subsequently, the transformed yeast was spread onto double amino acid (–Leu/–Trp) dropout (DDO) plates. The DDO colonies were streaked onto QDO/X/A plates. The pGADT7-T (encoding SV40 large T-antigen [84-708]) and pGBKT7-53 (encoding murine p53 protein [72-390]) are known to interact and served as the positive control, while pGADT7-T and pGBKT7-Lam (encoding Human lamin C [66-230]) were used as negative controls because lamin C does not interact with most other proteins (Wong et al. 2002). The vector blue colonies from QDO/X/A plates were isolated, and the purified colonies were screened by colony PCR using Matchmaker Insert Check PCR Mix 2 (Clontech). The colonies with only one electrophoretic band were sequenced, and all unigenes were searched using BlastX (Altschul et al. 1997).

Verification of protein-protein interaction by BiFC and Co-IP assays.

First, a BiFC assay was carried out with binary vectors pSPYNE-35S and pSPYCE-35S. NopP was fused with YFP^C into pSPYCE-35S and its candidate targets were fused with YFP^N into pSPYNE-35S. Afterward, the constructed vectors were transferred into *A. tumefaciens* GV3101 separately and were expressed transiently in 3- to 4-week-old *N. benthamiana* leaves. BiFC-induced yellow fluorescence was observed 3 days after agroinfiltration under a confocal laser-scanning microscope (Andor Revolution WD) (Bhat et al. 2006; Verhoef and Wade 2017).

Next, a Co-IP assay was used to confirm the interaction between NopP and its candidate targets. NopP fused with Flag-tag was cloned into the pEarleyGate202-YC vector and its candidate targets fused with HA-tag were cloned into the pEarleyGate201-YN vector. The constructs were transformed into *A. tumefaciens* GV3101 and then infiltrated into 3- to 4-week-old *N. benthamiana* leaves. Three days after agroinfiltration, the fusion proteins were extracted from leaf samples expressing Co-IP constructs, using a lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% NP-40) supplemented with 1 M dithiothreitol. The samples were incubated on ice for 15 min and were then centrifuged at 12,000 $\times$ g at 4°C for 15 min. The supernatant was filtered with a 45- μ m filter membrane, and a small amount of the filtered proteins was used as the input material for SDS-PAGE. The remainder of the proteins was incubated at 4°C with anti-HA-tag magnetic beads for 3 h. After removing the supernatant, beads were washed with a wash buffer (150 mM NaCl, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.5) for 5 min. Both the input and resuspended Co-IP samples

were boiled for 5 min to dissociate the immunocomplexes (Li et al. 2019). Following a 2-min ice bath, centrifugation and electrophoresis were carried out. Western blotting was used to detect the immunoprecipitation of the two proteins.

Subcellular localization of NopP and its target TRAPPC13.

To locate NopP and its target protein TRAPPC13 in plant cells, the vectors *p35S::nopP-eGFP*, *p35S::nopP-mCherry* and *p35S::trappc13-eGFP* were generated. NopP and TRAPPC13 were digested with *Xma*I and *Spe*I restriction enzymes and were inserted into a binary expression vector called pYJGFP, to generate NopP-eGFP and TRAPPC13-eGFP fusion protein, respectively, under the control of the cauliflower mosaic virus 35S promoter [66]. Subsequently, the modified vectors were transformed into *A. tumefaciens* GV3101. Simultaneously, mCherry-labeled PM marker was used in the colocalization experiment, and the empty vectors (*p35S::mCherry* and *p35S::eGFP*) were used as control, providing information about infiltration-induced leaf fluorescence. Leaves of 3- to 4-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* GV3101 in an agroinfiltration medium consisting of 10 mM MgCl₂, 10 mM morpholineethane sulfonic acid, and 150 mM acetosyringone (S. Liu et al. 2020). After 48 to 72 h of incubation, signals were detected under a confocal laser-scanning microscope (Andor Revolution WD).

mRNA transcriptome analysis.

For RNA-seq, GS0123- and Δ *nopP*-inoculated roots of *R. pseudoacacia* were sampled at 36 and 72 hpi. The mRNA was purified from total RNA and cDNA was synthesized. The cDNA was amplified to generate cDNA libraries, and second-generation high-throughput sequencing was performed on an Illumina HiSeq 2000 platform (Illumina Inc.) by Allwegene, using a paired-end sequencing strategy (150 bp). Meanwhile, total RNA from GS0123-infected roots were extracted to construct a cDNA combined SMRTbell library and third-generation sequencing was performed on a PacBio RS II platform (Pacific Biosciences). Three biological replicates for each sample were subjected to RNA extraction. Since no *R. pseudoacacia* reference genome is available, the full-length transcriptome (Iso-Seq) obtained by third-generation sequencing was used as the reference sequence (Supplementary Table S2), and clean reads of each sample were mapped to the reference sequence using the RSEM software (Li and Dewey 2011). The RNA-seq data are available at National Center for Biotechnology Information Short Read Archive under Bioproject PRJNA644405.

The DEG input data were read count data obtained from gene expression analyses. *R. pseudoacacia* genes of Δ *nopP*-inoculated roots with signal intensities more than twofold (or 0.5-fold) that of GS0123-inoculated roots were considered upregulated (or downregulated). DESeq was used for data analysis (Anders and Huber 2010), and the screening threshold was *padj* < 0.05. GO enrichment analysis was performed using the Goseq software based on Wallenius noncentral hypergeometric distribution (Young et al. 2010), and KEGG enrichment analysis was performed using KOBAS v2.0.

Statistical analyses.

Data were presented as the means $\pm$ standard deviation of three biological replicates. Different capital letters indicated significant differences (*P* < 0.05) of the different strains inoculated. Significant differences between means of samples were checked by analysis of variance and the Student's *t* tests (α = 0.05). Statistical analyses were performed by SPSS 19.0 (SPSS Inc.), and figures were performed with Origin 2019 (OriginLab Corp) and Microsoft Excel 2010.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BlastX tool: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>

KOBAS v2.0: <http://kobas.cbi.pku.edu.cn>

National Center for Biotechnology Information Short Read Archive: <https://www.ncbi.nlm.nih.gov/bioproject/644405>

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