

Subunit 3 of the SUPERKILLER complex mediates microRNA172-directed cleavage of Nodule Number Control 1 in *Medicago truncatula*

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Abstract

Legumes and rhizobia establish a nitrogen-fixing symbiosis that involves the formation of a lateral root organ, the nodule, and the infection process that allows intracellular accommodation of rhizobia within nodule cells. This process involves substantial gene expression changes regulated at the transcriptional and post-transcriptional levels. We have previously shown that a transcript encoding subunit 3 of the SUPERKILLER Complex (SKI), which guides mRNAs to the exosome for 3'-to-5' degradation, is required for nodule formation and bacterial persistence within the nodule, as well as the induction of early nodulation genes, including early nodulin40 (MtENOD40), during the *Medicago truncatula*–*Sinorhizobium meliloti* symbiosis. Here, we reveal through transcript degradome and small RNA sequencing analysis that knockdown of MtSKI3 impairs the miR172-directed endonucleolytic cleavage of the mRNA encoding Nodule Number Control 1 (MtNNC1), an APETALA2 transcription factor that negatively modulates nodulation. Knockdown of MtNNC1 enhances nodule number, bacterial infection, and the induction of MtENOD40 upon inoculation with *S. meliloti*, whereas overexpression of an miR172-resistant form of MtNNC1 significantly reduces nodule formation. This work identifies miR172 cleavage of MtNNC1 and its control by MtSKI3, a component of the 3'-to-5' mRNA degradation pathway, as a regulatory hub controlling indeterminate nodulation.

Introduction

mRNA decay is a key regulatory process that modulates gene expression by affecting the stability and, thus, the steady-state levels of cellular mRNAs for protein synthesis. Decay of mRNAs occurs either through the action of exoribonucleases or the endonucleolytic cleavage guided by microRNAs (miRNAs) and small interference RNAs (siRNAs). Bulk degradation of cytoplasmic mRNAs by exonucleases is accomplished by distinct deadenylation-dependent and deadenylation-independent pathways (Chantarachot and Bailey-Serres 2018). In the deadenylation-dependent pathways, the protecting polyA tail at the 3' end of translationally repressed mRNAs is removed by one of the different types of deadenylases. Subsequently, the deadenylated mRNAs can undergo decapping and 5'-to-3' decay, which in plants is catalyzed mainly by the 5'-to-3' EXORIBONUCLEASE 4 (XRN4) (Potuschak et al. 2006; Rymarquis et al. 2011) or be guided by the SUPERKILLER (SKI) complex to the RNA exosome, where degradation is in the 3'-to-5' direction (Liu and Chen 2016; Lange and Gagliardi 2021). The deadenylation-independent pathway occurs co-translationally in the 5'-to-3' direction on ribosome-associated mRNAs, whose translation has been paused or stacked at upstream open reading frames, the stop codon of main open reading frames, or in the

proximity of noncleavable miRNA target sites (Yu et al. 2015, 2016; Hou et al. 2016). This pathway, which requires the removal of the 5' cap and the function of XRN4, exhibits a decay with a 3-nucleotide periodicity that reflects the codon-by-codon translocation of elongating ribosomes along the mRNA (Pelechano et al. 2015; Yu et al. 2015, 2016; Carpentier et al. 2020). Degradation of mRNAs by endonucleolytic cleavage is guided by miRNAs or siRNAs bound to a member of the ARGONAUTE (AGO) protein family in a process known as post-transcriptional gene silencing (PTGS) (Bartel 2004; Vaucheret 2006). mRNA endonucleolytic cleavage results in the production of a deadenylated 5' cleavage fragment and a decapped 3' cleavage fragment, which can subsequently be degraded by the SKI/RNA exosome complex and XRN4, respectively. Also, both 5' and 3' fragments can be substrates of RNA-dependent RNA polymerase 6 (RDR6), triggering the production of secondary siRNAs (Allen et al. 2005; Yoshikawa et al. 2005; Manavella et al. 2012).

SKI is an exosome-associated complex, evolutionarily conserved in eukaryotes with key functions in the deadenylation-dependent 3'-to-5' mRNA degradation, the surveillance mechanisms of nonsense-mediated decay, and nonstop decay (Arribere and Fire 2018; Szádeczky-Kardoss et al. 2018), as well as in small RNA-mediated endonucleolytic cleavage of mRNAs in metazoa

Received June 7, 2025. Accepted July 22, 2025.

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and plants (Orban and Izaurralde 2005; Zhang et al. 2015). The SKI complex is a tetramer composed of SKI2, SKI3, and 2 SKI8 subunits. The SKI2 subunit functions as a helicase that unwinds the RNA. The SKI3 subunit is a scaffold protein with tetratricopeptide repeats (TPR), whereas SKI8 is a WD40 repeat-containing protein. Both TPR and WD40 repeats have been involved in protein–protein interactions (Halbach et al. 2013). In animals, different lines of evidence suggest that the SKI complex can act co-translationally to channel translationally stalled mRNAs to the RNA exosome for degradation through its association with 80S ribosome-bound mRNAs (Schmidt et al. 2016; Zinoviev et al. 2020; Kögel et al. 2022). In yeast, biochemical and cryo-electron microscopy structural analyses have shown that the SKI complex binds the 80S ribosome via SKI2, the N-terminal of SKI3, and one of the SKI8 subunits (Schmidt et al. 2016), whereas in mammals the interaction of the SKI complex with the 40S ribosomal subunit is exclusively mediated by SKI2 encompassing a gatekeeping mechanism that switches from a closed conformational state to an ATP induced open conformational state that facilitates the extraction of 80S ribosome-bound mRNAs and their delivery to the RNA exosome for degradation (Kögel et al. 2022). In plants, the interaction of the SKI complex with 80S ribosomes and its potential role in the co-translational RNA surveillance pathway remain unexplored. Instead, genetic and molecular evidence points to the role of the SKI complex in preventing the ectopic production of secondary siRNA from endogenous miRNA targets and transgenes in Arabidopsis (Branscheid et al. 2015; Yu et al. 2015; Zhang et al. 2015; Vigh et al. 2022). Simultaneous disruption of bidirectional RNA decay (i.e. 5′-to-3′ and 3′-to-5′) by mutations in genes encoding subunits of the SKI complex and the cytoplasmic 5′-to-3′ exoribonuclease Ethylene Insensitive 5 (EIN5)/XRN4, enhanced the production of 21 and 22 siRNAs in Arabidopsis, indicating that 3′-5′ and 5′-3′ RNA decay pathways mediated by the SKI complex and EIN5/XRN4, respectively, suppress PTGS (Zhang et al. 2015). Concomitantly, Yu et al. (2015) reported that mutation of SKI3 restores sense PTGS in a hypermorphic *ago-1* mutant background, whereas Branscheid et al. (2015) reported that mutation of Arabidopsis SKI2 enhanced the production of secondary siRNAs generated from either the 5′ or the 3′ cleavage fragments of miRNA targets, and these siRNAs mapped close to the miRNA cleavage site. Also, the authors found that mutation in any of the SKI genes, SKI2, SKI3, or SKI8, compromised the degradation of 5′ cleavage fragments but not 3′ cleavage fragments derived from miRNA endonucleolytic cleavage (Branscheid et al. 2015). A subsequent study revealed that in the absence of a functional SKI complex, most of the transcripts that accumulate in the cytoplasm are redirected to the 5′-to-3′ XRN4 pathway for degradation (Zhao and Kunst 2016). More recently, Vigh et al. (2022) showed that inactivation of the exosome subunit Ribonuclease PH45B (RRP45B) results in the accumulation of 5′ cleavage fragments and enhanced production of secondary siRNAs, similar to that observed in *ski2* mutants. Interestingly, mutation of the Release Factor PELOTA1, which is required for ribosomal subunit dissociation of stalled ribosomes, led to the production of siRNAs from miRNA targets that overlap but are distinct from those produced in the *ski* and *rpb45b* mutants, suggesting that PELOTA limits siRNA amplification by reducing ribosome stalling (Vigh et al. 2022).

In a previous study, we identified a transcript encoding the SKI3 subunit in the model legume *Medicago truncatula* (MtSKI3), which is subjected to translational regulation during the nitrogen-fixing symbiosis with *Sinorhizobium meliloti* (Traubenik et al. 2020). Knockdown of MtSKI3 results in a reduction in the number of

nitrogen-fixing specialized organs, i.e. the nodules, but also prevents the persistence of the bacteria within these nodules, compromising nodule viability and nitrogen fixation (Traubenik et al. 2020). Considering that disruption of one mRNA degradation pathway might alter the functioning of other degradation pathways, in this study, we investigated whether knockdown of MtSKI3 altered the 5'-to-3' degradation and/or miRNA-guided endonucleolytic cleavage of mRNAs in *M. truncatula* roots under symbiotic and nonsymbiotic conditions by the use of the genome-wide mapping of uncapped and cleaved transcripts (GMUCT) (Willmann et al. 2014). GMUCT identifies the 3' cleavage products of miRNA or siRNA guided-endonucleolytic cleavage and other uncapped mRNAs by selecting those molecules with a free 5' monophosphate (5'P). Hundreds of genes with free 5'P reads enriched or depleted in MtSKI3-silenced roots were identified. One of the 3' cleavage fragments with lower abundance in MtSKI3-silenced roots was the product of endonucleolytic cleavage mediated by miR172 on its target mRNA, which encodes an APETALA2/Ethylene Response Factor (AP2/ERF) transcription factor referred to as Nodule Number Control 1 (NNC1). In soybean (*Glycine max*), GmNNC1 acts as a negative modulator of the formation of determinate nodules and a transcriptional repressor of the early *nodulin40* (*GmENOD40*) gene (Wang et al. 2014, 2019). Here, we show that whereas MtNNC1 mRNA levels decrease upon inoculation with *S. meliloti* in control plants, they remain high in MtSKI3-silenced roots. The silencing of MtSKI3 does not alter the abundance of miR172a or miR172/b/c; rather, it leads to impaired endonucleolytic cleavage of the MtNNC1 transcript. Knockdown of MtNNC1 leads to more numerous nodules and infection events, as well as higher levels of MtENOD40, whereas overexpression of a miR172-resistant form of MtNNC1 recapitulates the reduced nodulation phenotype observed in MtSKI3-silenced roots (Traubenik et al. 2020). These results uncover a role of the SKI complex in the cleavage of MtNNC1 mediated by miR172 during the establishment of the root nodule symbiosis.

Results

SKI3-dependent RNA fragments with a free 5'P accumulate in mock and symbiotic conditions

The underlying mechanisms by which MtSKI3 mediates nodule formation and bacterial viability are unknown. To evaluate whether RNA interference (RNAi) of MtSKI3 in *M. truncatula* roots is altering the 5' to 3' RNA decay and/or sRNA-guided endonucleolytic cleavage in response to rhizobia, we performed a degradome analysis by applying the GMUCT 2.0 method (Willmann et al. 2014) on GUS RNAi and SKI3 RNAi roots inoculated with *S. meliloti* or with water (mock). This approach specifically identifies products of miRNA or siRNA-guided endonucleolytic cleavage and decapped mRNAs by selecting those molecules with a free 5'P (Fig. 1A). Hundreds of 5'P end peaks differentially accumulate in SKI3 RNAi roots as compared to GUS RNAi roots under symbiotic (94 up and 154 down) and mock conditions (118 up and 59 down) with $2 < \text{FC} < 0.5$ and $P\text{-value} < 0.05$ (Fig. 1B, Supplementary Data Set 1). Under mock conditions, transcripts with increased 5'P end peaks in SKI3 RNAi roots versus GUS RNAi roots are enriched in the functional categories of Perception/Signaling and Metabolism, whereas transcripts with decreased 5'P peaks are enriched in the categories of Transcriptional Regulation and Perception/Signaling (Supplementary Fig. S1A, Supplementary Data Set 2). Similarly, under symbiotic conditions, the prevalent functional categories in SKI3 RNAi roots versus GUS RNAi are Transcriptional Regulation, Metabolism, Perception/Signaling, and

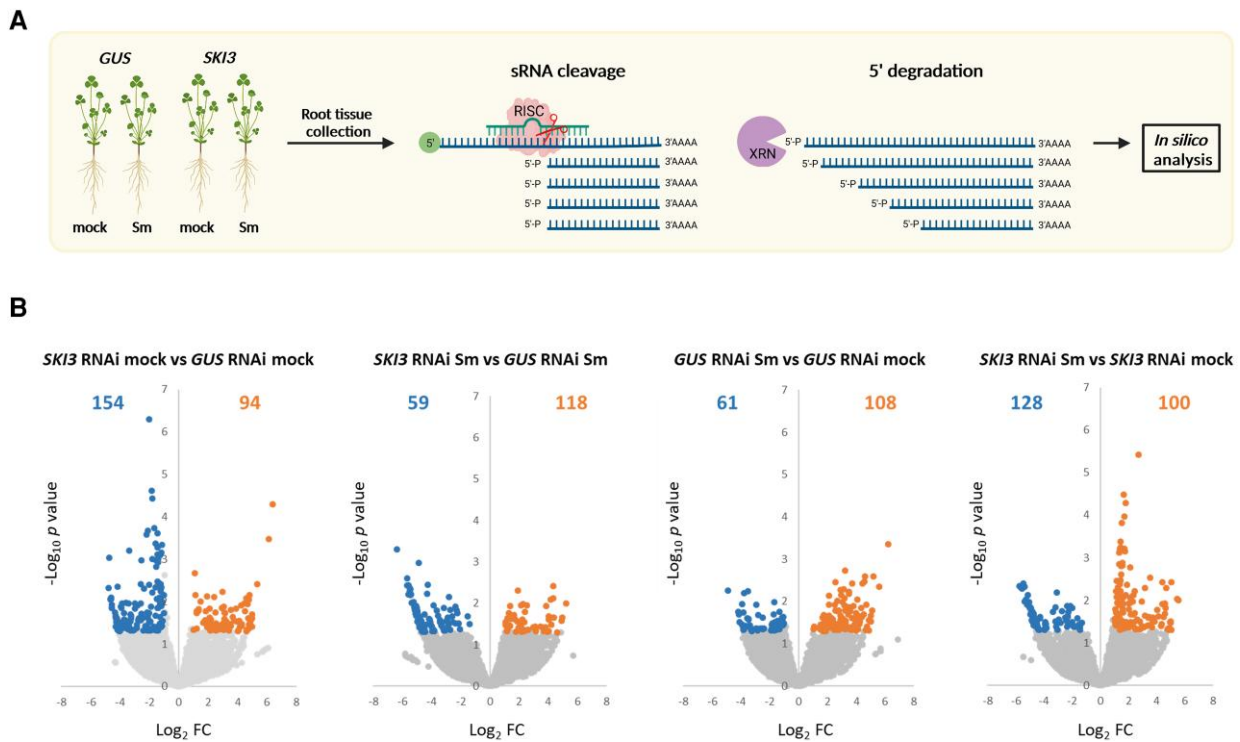


Figure 1. Transcripts with differential free 5' P peaks in mock and *S. meliloti*-inoculated *GUS* RNAi and *SKI3* RNAi roots. **A)** Schematic overview of the experimental design using GMUCT to identify transcripts with 5' P. Roots of *M. truncatula* were inoculated with water (mock) or with *S. meliloti* (Sm). Total RNA was used to generate degradome libraries of RNA molecules with a free 5' P caused by decapping and 5'-to-3' degradation by XRN4 or by sRNA-mediated endonucleolytic cleavage, created via [Biorender.com](https://www.biorender.com). **B)** Volcano plots representing changes in the abundance of transcripts with a free 5' P peak. Each dot represents one transcript. Gray dots represent transcripts with no significantly different 5' P peaks in each comparison, blue dots represent transcripts with significantly downregulated 5' P peaks ($FC < 0.05$, $P\text{-value} < 0.05$), and orange dots represent transcripts with significantly upregulated 5' P peaks ($FC > 2$, $P\text{-value} < 0.05$). Numbers in each plot indicate the number of upregulated and downregulated transcripts with 5' P peaks.

Chromatin remodeling (Supplementary Fig. S1B). Interestingly, when comparing genes with differential 5' end peaks in *S. meliloti*-inoculated versus mock roots, functional categories are more diverse in *SKI3* RNAi roots than in *GUS* RNAi, including Transcriptional Regulation and Perception/Signaling as well as Cell Wall Remodeling and DNA/RNA Metabolism (Supplementary Fig. S1, C and D, Supplementary Data Set 2). In the category of Transcriptional Regulation, transcription factors encoded by transcripts with differential 5' P peaks belong to diverse gene families, including the Auxin Response Factor (ARFs), APETALA2/Ethylene Response Factor (AP2/ERF), the basic Helix-Loop-Helix (bHLH), the C2C2-Dof, the zinc finger C2H2 (Cys₂His₂), the Homeobox WOX, MYB, and the WD40 repeats families (Supplementary Fig. S2). Particularly, we have found that upon inoculation with rhizobia, *MtSKI3* RNAi roots exhibited enhanced degradation (i.e. increased 5' peaks) of the mRNA MtrunA17Chr8g0385791 that encodes the Auxin Response Factor 2 (MtARF2). This enhanced degradation in response to rhizobia was not observed in *GUS* RNAi roots (Supplementary Fig. S2, Supplementary Data Set 2). MtARF2 is part of the miR390/TAS3/ARF module that functions in nodulation (Hobecker et al. 2017) and directly targets the *Lateral Organ Boundaries Domain 17/29a* (MtLBD17/29a) gene, which is required for rhizobia infection and nodule organogenesis (Kirolinko et al. 2024). We also found that the mRNA encoding the AP/ERF transcription factor ERF required for Nodulation 3 (MtERN3, MtrunA17Chr8g0378811), which was proposed to be a transcriptional repressor of the early nodulation gene *early nodulin11* (MtENOD11) (Andriankaja et al. 2007), exhibited reduced degradation (i.e. decreased 5' peaks) in *MtSKI3*-silenced roots as compared with

GUS RNAi under mock conditions (Supplementary Fig. S2, Supplementary Data Set 2). In addition, it was found that the mRNA MtrunA17Chr2g0328391 encoding a SCARECROW-like transcription factor within the GRAS family exhibited reduced degradation in *MtSKI3* RNAi roots as compared with *GUS* RNAi roots under symbiotic conditions (Supplementary Data Set 2). Since MtARF2, MtERN3, and SCARECROW-like transcription factors have been shown to play a role in the root nodule symbiosis (Andriankaja et al. 2007; Battaglia et al. 2014; Hobecker et al. 2017; Dong et al. 2021; Kirolinko et al. 2024), it is possible to speculate that the misregulation of these transcription factors caused by the silencing of *MtSKI3* might contribute to the impaired nodulation phenotype previously observed in *MtSKI3* RNAi roots (Traubenik et al. 2020).

SKI3-dependent miRNA-directed target cleavage of specific transcripts

We then investigated whether miRNA-mediated cleavage is affected by the silencing of *MtSKI3*. We predicted the targets and their cleavage sites for the conserved canonical miRNAs of *M. truncatula* annotated in miRBase (Kozomara and Griffiths-Jones 2014) using the miRanda algorithm (Betel et al. 2010) and then determined whether there was evidence of cleavage at these targets using degradome data, allowing a window of ± 2 nucleotides from the predicted cleavage site. Our analysis confirmed 615 miRNA targets containing 5' P ends cleavage reads at the predicted miRNA cleavage site (Supplementary Data Set 3), indicating that our degradome data can robustly quantify 5' P end reads produced by miRNA-mediated cleavage. Degradome data were analyzed to

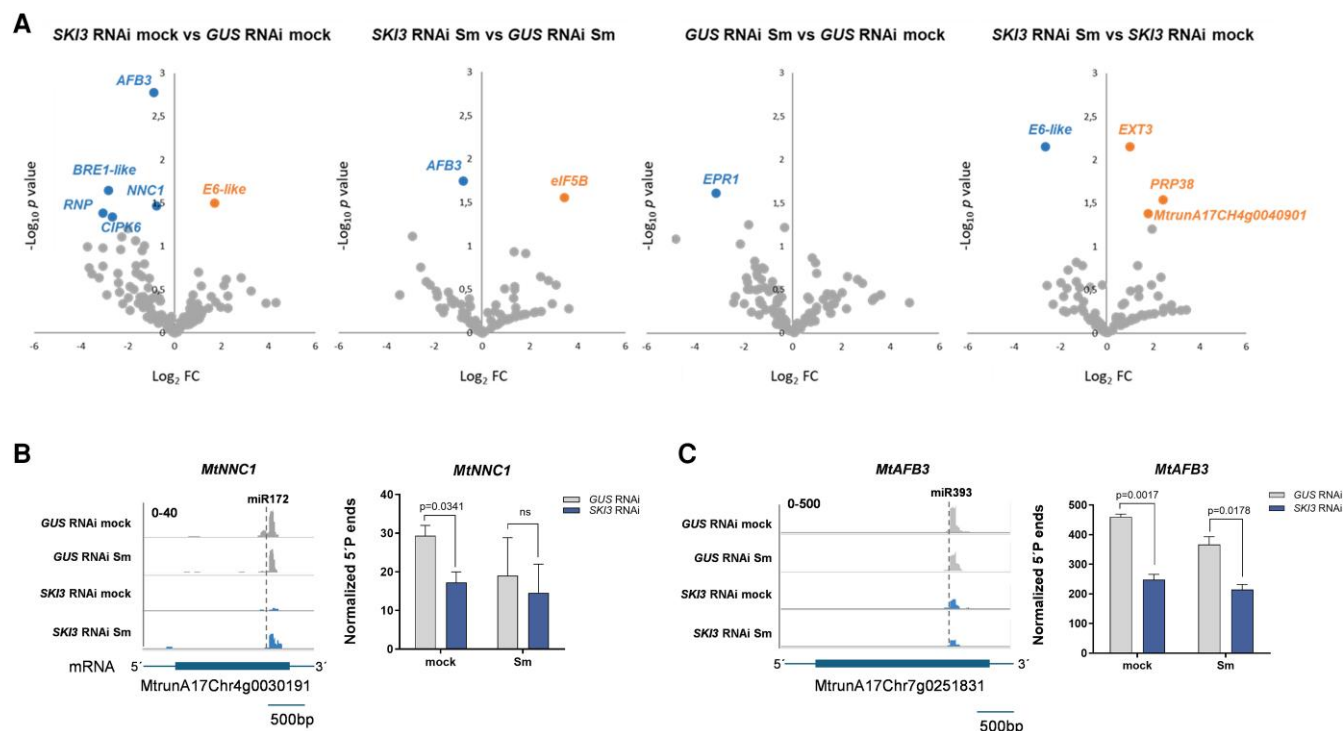


Figure 2. miRNA target transcripts with differential free 5'P peaks in mock and *S. meliloti*-inoculated GUS RNAi and SKI3 RNAi roots. **A)** Volcano plots representing miRNA target transcripts that contain a free 5'P. Each dot represents one transcript. Gray dots represent transcripts with no significantly different 5'P peaks in each comparison, blue dots represent transcripts with significantly downregulated 5'P peaks ($FC < 0.58$, $P\text{-value} < 0.05$), and orange dots represent transcripts with significantly upregulated 5'P peaks ($1.7 < FC$, $P\text{-value} < 0.05$). **B, C)** 5'P read coverage of *MtNNC1* (B) and *MtAFB3* (C). GUS RNAi samples are shown in gray, and SKI3 RNAi samples in blue. Left panels: The dashed line indicates the cleavage sites of miR172 (B) and miR393 (C). Transcript models are displayed at the bottom. The numbers on the top left indicate the maximum read value of the scale, which was the same for all samples. Right panels: Normalized counts of 5'P reads ends obtained from GMUCT experiments in SKI3 RNAi and GUS RNAi roots inoculated with *S. meliloti* (Sm) or water (mock). Error bars represent standard deviation of the mean (SD). P-values obtained by the Wald test using DESeq2 for each comparison are indicated. P-values < 0.05 were considered significant. ns, no significant difference.

quantify products of miRNA-guided endonucleolytic cleavage in both GUS RNAi and SKI3 RNAi roots under mock and symbiotic conditions, and differential 5'P peaks in all pairwise comparisons were identified using DESeq2 using a $P\text{-value} < 0.05$ (Love et al. 2014) (Fig. 2A). Under mock conditions, we identified 5 transcripts with significant downregulated 5'P peaks in SKI3 RNAi roots as compared to GUS RNAi encoding the Auxin Signaling F-Box 3 receptor (*MtAFB3*), the E3 ubiquitin-protein ligase BRE1 (*MtBRE1*), a heterogeneous nuclear ribonucleoprotein (*MtRNP*), a CBL interacting protein kinase (*MtCIPK6*), and an ethylene-responsive transcription factor of the AP2/ERF family, which was designated as *MtNNC1* (see below). Only a single transcript showed upregulated 5'P peaks in SKI3 RNAi roots as compared to GUS RNAi, encoding an E6-like protein. Under symbiotic conditions, 2 transcripts showed differential accumulation of 5'P peaks in SKI3 RNAi as compared with GUS RNAi roots: one upregulated transcript encoding the eukaryotic translation initiation factor 5B (*MtEIF5B*), and one downregulated transcript encoding *MtAFB3*. These results suggest that silencing of *MtSKI3* does not globally enhance miRNA endonucleolytic cleavage of *M. truncatula* targets, but rather affects the miRNA-mediated cleavage of specific miRNA targets, mainly reducing the amount of 3' cleavage products with a free 5'P derived from miRNA cleavage. In GUS RNAi roots, inoculation with *S. meliloti* diminished the degradation of only one transcript encoding a proline-rich extensin-like protein 1 (*EPR1*). Conversely, in SKI3 RNAi roots, the transcript encoding the E6-like protein exhibited 5'P fragments downregulated in response to *S. meliloti*, whereas 3 transcripts showed the

opposite behavior. These transcripts encode an extensin domain-containing protein 3 (*MtEXT3*), the pre-mRNA-splicing factor precursor RNA processing 38 (*MtPRP38*), and a hypothetical protein (*MttrunA17Chr4g0040901*). Two of the transcripts with downregulated 5'P peaks in SKI3 RNAi versus GUS RNAi roots were targets of evolutionarily conserved miRNAs, i.e. the transcripts encoding the AP2 transcription factor *MtNNC1* and *MtAFB3* are targeted by miR172 and miR393, respectively (Fig. 2, B and C, respectively). Both miRNAs and their target mRNAs have been shown to play a role during nodulation in soybean plants (Wang et al. 2014, 2019; Cai et al. 2017).

Downregulation of *MtNNC1* mRNA levels in response to rhizobia is impaired by silencing of *MtSKI3*

The gene encoding the AP2 transcription factor identified here (*MttrunA17Chr4g0030191*) is homolog and syntenic to *Glycine max* Nodule Number Control 1 (*NNC1*) (Fig. 3A), which has been described as a negative regulator of nodule initiation and a transcriptional repressor of *GmENOD40* (Wang et al. 2014) and later, involved in the autoregulation of nodule number (Wang et al. 2019); thus, we named it as *MtNNC1*. To further link the action of *MtSKI3* to symbiotic nodulation, we focused our analysis on this miRNA target, since its regulation by *MtSKI3* may help explain the impaired nodulation phenotype previously observed in SKI3 RNAi roots (Traubenik et al. 2020). The reduced number of 5'P end reads found in the *MtNNC1* transcript in SKI3 RNAi roots, as compared

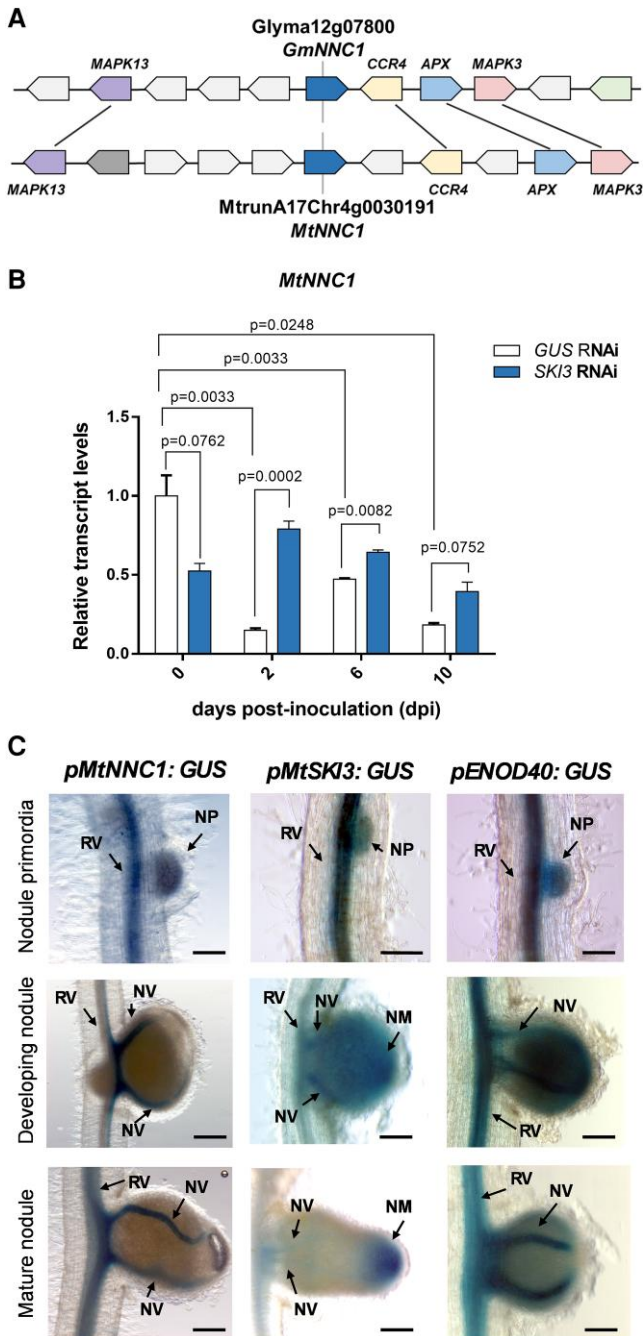


Figure 3. Synteny and expression analysis of MtNNC1 in GUS RNAi and SKI3 RNAi roots during symbiosis. **A)** Syntenic regions of *M. truncatula* and *G. max* NNC1. Anchor genes flanking MtNNC1 and GmNNC1 are connected with colored lines. **B)** Relative transcript levels of MtNNC1 in GUS RNAi and SKI3 RNAi roots inoculated with water (0 dpi) or with *S. meliloti* at 2, 6, and 10 dpi. Expression values were determined by RT-qPCR, normalized to MtHIS3L, and plotted relative to the mock sample at time 0. Each bar represents the mean $\pm$ SEM of 3 biological replicates. P-values in an unpaired 2-tailed Student's t-test are indicated for each comparison. P-values < 0.05 were considered significant. **C)** GUS staining of pMtNNC1: GUS, pMtSKI3: GUS, and pMtENOD40: GUS in *S. meliloti* inoculated roots (upper panels), developing (middle panels), and mature nodules (lower panels). RV, root vasculature; NP, nodule primordia; NV, nodule vasculature; NM, nodule meristem. Bars: 100 μ m in upper panels and 200 μ m in middle and lower panels.

to GUS RNAi roots, suggested that cleavage by miR172 might be compromised in SKI3-silenced roots. Thus, we analyzed whether

MtNNC1 mRNA levels were modified by silencing of MtSKI3 under mock and symbiotic conditions using RT-qPCR and primers that spanned at each side of the miR172a/b/c cleavage site. We found that levels of noncleaved MtNNC1 were significantly downregulated at 2 and 10 dpi with *S. meliloti* in GUS RNAi roots, in agreement with the previously reported repression in soybean roots under symbiotic conditions (Wang et al. 2014). However, this repression in response to rhizobia was abolished in SKI3 RNAi roots at all the time points analyzed, indicating that inactivation of the SKI complex compromises rhizobia-induced miR172-mediated repression of MtNNC1 (Fig. 3B).

Silencing of MtSKI3 modulates the expression of MtNNC1, and MtNNC1 has been shown to act as a negative regulator ENOD40; therefore, we investigated whether these genes were expressed in the same tissue types in roots and nodules at different stages of nodule development. Promoter:GUS analysis revealed that MtNNC1 promoter is active in the root vasculature, as well as in the vascular tissue of developing and mature nodules (Fig. 3C). MtSKI3 promoter activity was also detected in the vasculature of the root, as well as in the nodule primordia, as well as in the vasculature and the meristematic zone of developing and mature nodules (Fig. 3C). MtENOD40 promoter activity was found in the root vasculature, the nodule primordia, and the vasculature of the developing and mature nodules (Fig. 3C). This analysis indicates that the expression of MtNNC1 coincides with that of MtSKI3 and MtENOD40 in the root and nodule vasculature. On the other hand, examination of a publicly available *M. truncatula* single nuclei RNA-seq dataset reported (Cervantes-Perez et al. 2022) allowed us to quantitatively assess steady-state mRNA levels of these genes in different root cell types at an early time point after inoculation with rhizobia. This analysis revealed that MtNNC1 levels are downregulated in nonroot hair-forming epidermal cells, cortical cells, as well as in endodermal and stele cells at 48 hpi with *S. meliloti*, whereas MtSKI3 transcript levels do not show significant changes in most cell types upon inoculation with *S. meliloti* except for mild increases in endodermal and stele cells (Supplementary Fig. S3), which also agrees with our previous RNA-seq data of whole root tissue (Traubenik et al. 2020). Normalized expression levels of the MtENOD40 were too low in the nuclei of most cell types at this point after rhizobia inoculation to extract robust conclusions of its steady-state mRNA levels.

MtNNC1 showed translational downregulation during symbiosis

MtSKI3 was previously found to be upregulated at the translational level at early stages (2 dpi with *S. meliloti*) of the root nodule symbiosis (Traubenik et al. 2020). Since miR172 was shown to act at the level of translational repression in Arabidopsis plants (Aukerman and Sakai 2003; Chen 2004) and we previously found that miR172 was bound to translating ribosomes in *M. truncatula* roots (Reynoso et al. 2013), we tested whether the association of MtNNC1 with actively translating ribosomes was affected during the root nodule symbiosis using TRAP (Translating Ribosomes Affinity Purification) in *M. truncatula* roots. TRAP is based on the expression of a FLAG-tagged version of the Ribosomal Protein of the Large Subunit 18 (FLAG-RPL18) and the affinity purification of mRNAs bound to one or more ribosomes containing FLAG-RPL18 using anti-FLAG antibodies coupled to magnetic beads, followed by the isolation of the ribosome-bound RNA referred to as TRAP RNA (Zanetti et al. 2005). As a control, we also determined the association of MtSKI3 mRNA with the ribosomes at different stages of the root nodule symbiosis. Using RT-qPCR

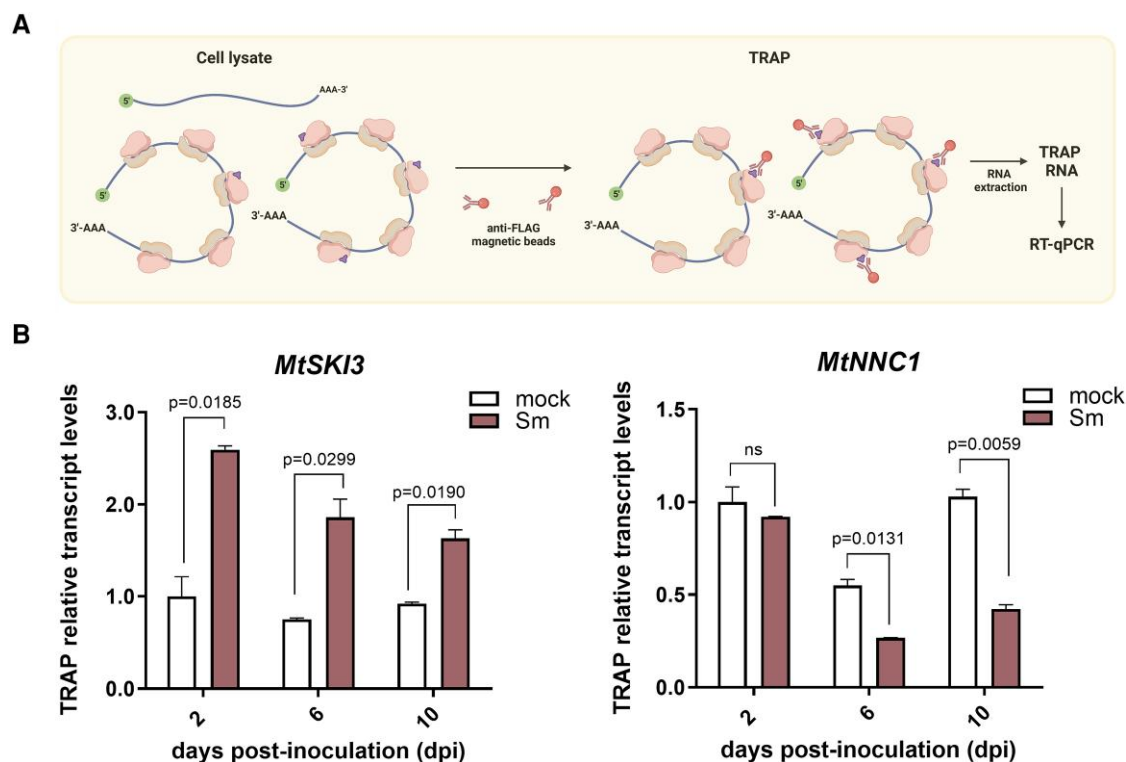


Figure 4. Association of *MtSKI3* and *MtNNC1* with translating ribosomes during symbiosis. **A)** Schematic representation of the TRAP methodology. Cell lysates were prepared from root tissue expressing the FLAG-tagged MtRPL18 protein (FLAG-RPL18) under the control of the CaMV 35S promoter. mRNA bound to one or multiple ribosomes containing FLAG-tagged MtRPL18 was immunopurified using anti-FLAG coupled to magnetic beads to obtain the TRAP samples. RNA was extracted from the TRAP samples and subjected to RT-qPCR. Created with [BioRender.com](#). **B)** Relative transcript levels of *MtSKI3* or *MtNNC1* in TRAP samples from roots inoculated with *S. meliloti* (Sm) or water (mock) at the indicated time points. Expression values were determined by RT-qPCR, normalized to *MtHIS3L*, and plotted relative to the mock sample at 2 dpi. Each bar represents the mean $\pm$ SEM of 3 biological replicates. P-values in an unpaired 2-tailed Student's t-test are indicated for each comparison. P-values < 0.05 were considered significant. ns, no significant differences.

on TRAP RNA samples, we found that the association of MtSKI3 transcripts was upregulated not only at 2 dpi with *S. meliloti*, but also at later stages of the symbiosis, i.e. 6 and 10 dpi (Fig. 4). By contrast, the association of MtNNC1 mRNA with translating ribosomes was unaffected at 2 dpi with rhizobia but reduced at later stages, i.e. roots at 6 and 10 dpi (Fig. 4), indicating that in addition to its regulation at the level of miRNA endonucleolytic cleavage, MtNNC1 may be subjected to translational regulation, which could be mediated by miR172 associated with translating ribosomes.

MtSKI3 knockdown has no impact on miR172a/b/c and miR393 levels but is required for endonucleolytic cleavage

To evaluate whether the silencing of MtSKI3 in *M. truncatula* roots affects small RNA levels and distribution, notably the miR172 and miR393 that emerged from the degradome analysis, we performed small RNA sequencing (sRNA-seq) on GUS RNAi and SKI3 RNAi roots under mock conditions. No differences in the accumulation levels of miR172a, miR172b/c, and miR393a/b are evident in SKI3 RNAi roots as compared with GUS RNAi roots in mock conditions (Fig. 5A, Supplementary Data Set 4). Stem-loop RT-qPCR confirmed sRNA-seq data, indicating that silencing of MtSKI3 does not modify the abundance of mature miR172a, miR172b/c or miR393a/b levels in *M. truncatula* roots, and further revealed that both miR172a/b/c and miR393a/b levels increase upon inoculation with *S. meliloti* in both GUS RNAi and SKI3 RNAi roots

(Fig. 5B and Supplementary Fig. S4). Silencing of MtSKI3 does not lead to significantly enhanced production of 21 and 22 nt secondary siRNA derived from the MtNNC1 or MtAFB3 transcripts (Supplementary Fig. S5) but enhances the production of 21 and 22 nt siRNAs from other loci, mainly repetitive sequences, and retrotransposon (Supplementary Fig. S6, Supplementary Data Set 5). To evaluate whether there is a differential stabilization of specific miR172-directed 5' and 3' cleavage fragments derived from the MtNNC1 mRNA due to the silencing of MtSKI3, we conducted RT using random primers followed by qPCR with primer pairs designed to target the 5' or the 3' region of MtNNC1 mRNA, which will detect both the full-length transcript as well the 5' or the 3' cleavage fragments, respectively. In addition, we used primers that anneal at regions flanking the miR172 cleavage site to detect exclusively the full-length MtNNC1 mRNA (Fig. 5C). This analysis revealed that the abundance of the full-length MtNNC1 and/or the cleavage fragments does not show significant differences between SKI3 RNAi and GUS RNAi under mock conditions. In addition, the abundance of the full-length MtNNC1 and/or the cleavage fragments decreases in GUS RNAi roots upon inoculation with *S. meliloti*, but not in SKI3 RNAi roots (Fig. 5C). These findings reveal that miR172-directed cleavage of MtNNC1 in response to rhizobia is lost in SKI3 RNAi roots. This effect might not be attributable to the differential stabilization of specific regions within the MtNNC1 transcript but to other mechanisms, such as alterations in RISC activity and/or mRNA degradation kinetics that underlie the observed accumulation of siRNAs derived from MtNNC1 in MtSKI3-silenced roots.

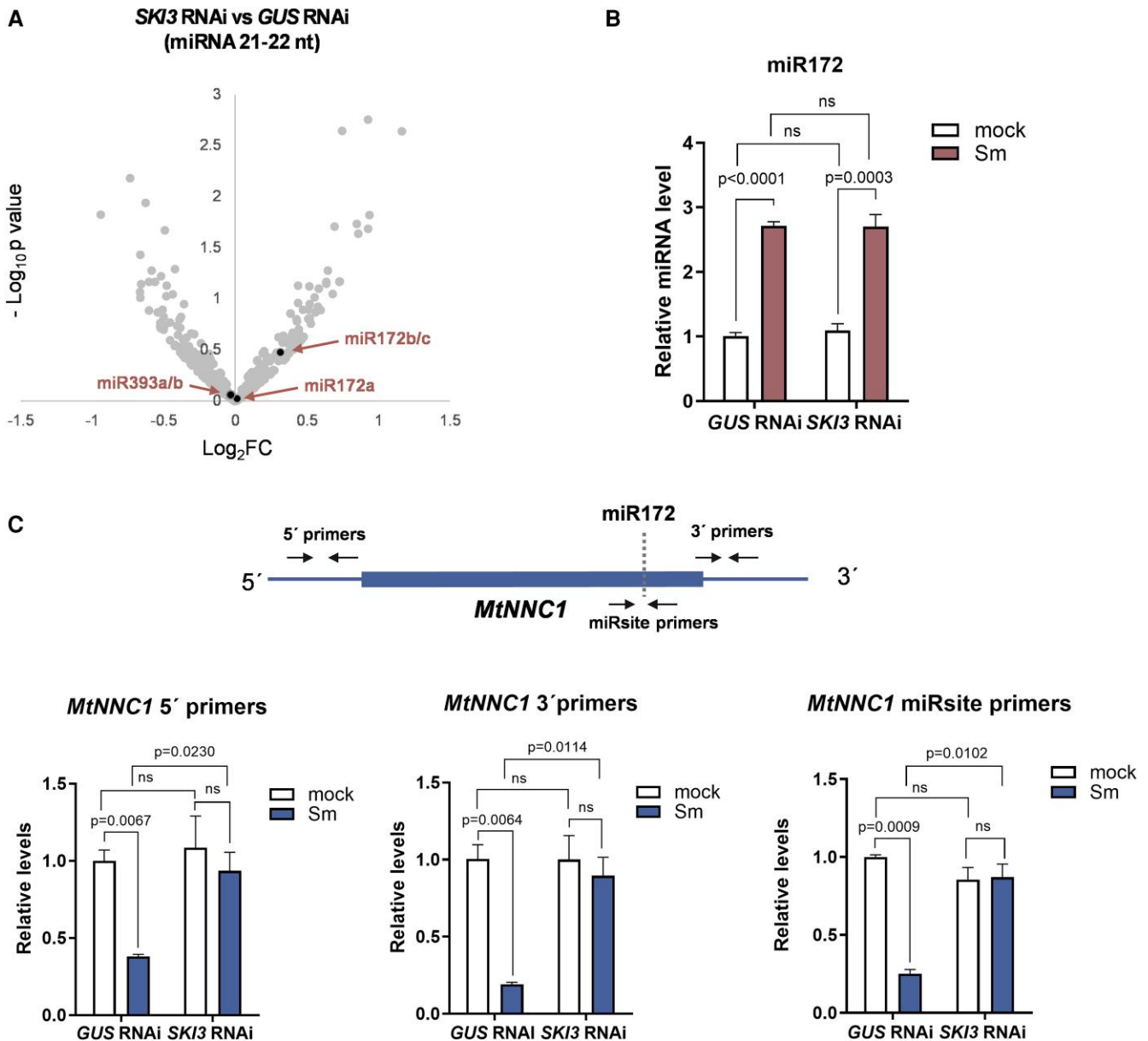


Figure 5. Analysis of miR172 levels and MtNNC1 in SKI3 RNAi and GUS RNAi roots. **A)** Volcano plots showing the Log₂FC as the mean expression level for each small RNA between SKI3 RNAi mock and GUS RNAi mock samples. Each dot represents one small RNA. miR172a, miR172b/c, and miR393a/b are colored black. **B)** Stem-loop RT-qPCR of miR172 levels in GUS RNAi and SKI3 RNAi roots at 2 dpi with *S. meliloti* (Sm) or water (mock). Levels were normalized to the level of miR162 and plotted relative to the mock GUS RNAi sample. **C)** Relative transcript levels of MtNNC1 in GUS RNAi and SKI3 RNAi roots at 2 dpi with *S. meliloti* (Sm) or water (mock) using the primers indicated in the top panel: 5' primers, 3' primers, and miRsite primers. Expression values were determined by RT-qPCR, normalized to MtHIS3L, and plotted relative to the GUS RNAi. In (B) and (C), each bar represents the mean $\pm$ SEM of 3 biological replicates. P-values of an unpaired 2-tailed Student's t-test are indicated. P-values < 0.05 were considered significant. ns, not significant differences.

MtNNC1 negatively regulates nodule number and infection by *S. meliloti*

NNC1 function has been described in determinate nodule-forming legumes, i.e. soybean and common bean (Wang et al. 2014; Nova-Franco et al. 2015). To gain insight into the function of MtNNC1 during the formation and infection of indeterminate nodules, we used RNAi to knock down MtNNC1 transcripts in *M. truncatula* hairy roots. This strategy decreases levels of MtNNC1 by more than 75%, but not the levels of its homolog MtERF101 (Fig. 6A). The knockdown of MtNNC1 results in a significant increase in the number of nodules formed by *S. meliloti* at all time points analyzed (7, 10, 14, and 21 dpi) compared with GUS RNAi

roots (Fig. 6B). The density of infection events also increases at 7 dpi by silencing MtNNC1 (Fig. 6C), but not their progression since the density is significantly higher only for those infection events at the microcolony stage or for infection threads (ITs) that elongate within the root hair, but not for ITs that reach the epidermal cells or the root cortex (Fig. 6D). The nodulation and infection phenotypes were confirmed by a second independent RNAi construct (MtNNC1 RNAi2), which produced similar results (Supplementary Fig. S7). To better understand the molecular events affected by the knockdown of MtNNC1 and considering that in our previous study, we observed that the induction of MtENOD40 in response to rhizobia was impaired in

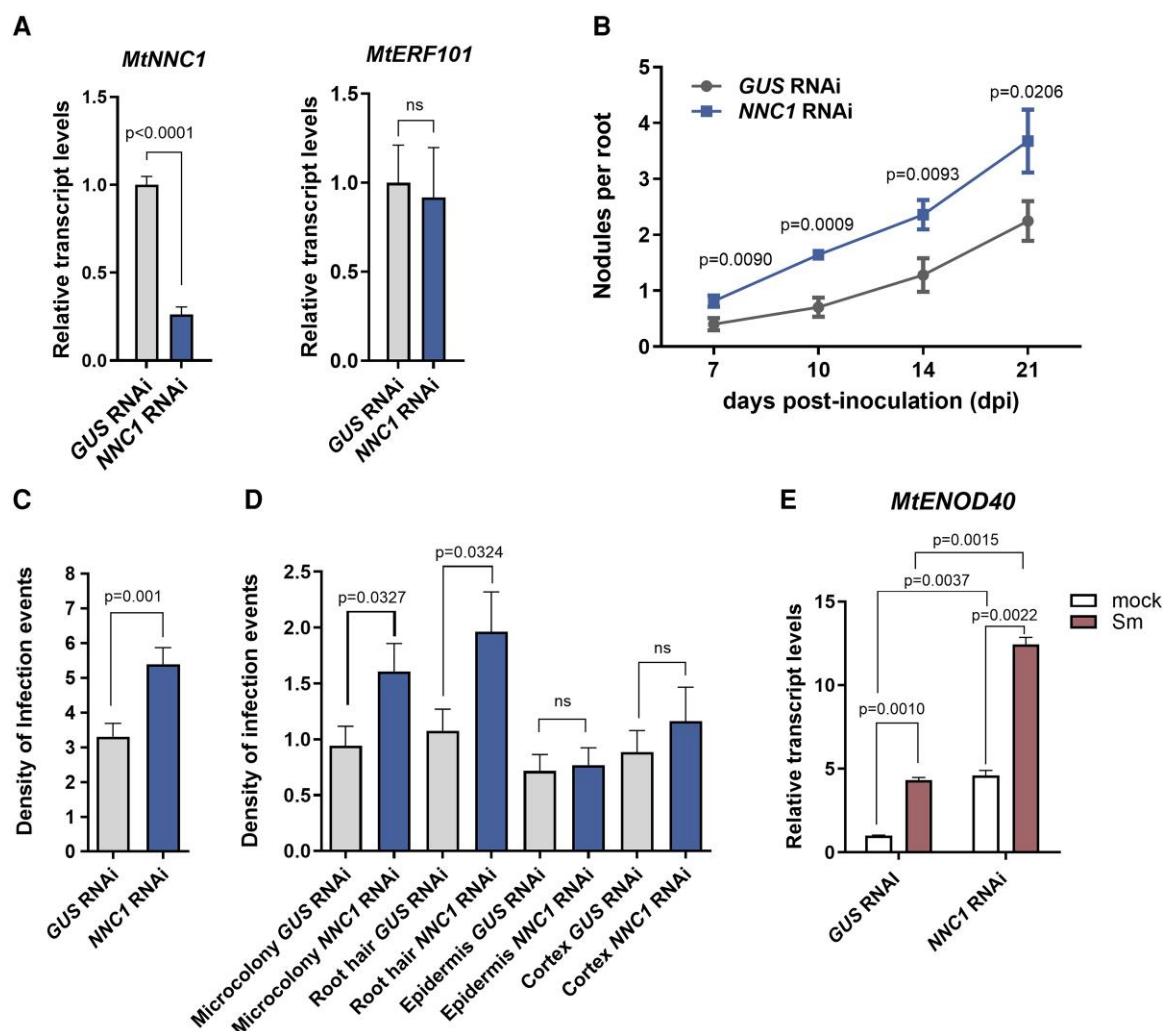


Figure 6. Silencing of *MtN1C1* enhanced nodulation, bacterial infection, and expression of *MtENOD40*. **A)** Relative transcript levels of *MtN1C1* and its homolog *MtERF101* in GUS RNAi and NNC1 RNAi roots. Expression values were determined by RT-qPCR, normalized to *MtHIS3L*, and plotted relative to the level in GUS RNAi roots. Each bar represents the mean $\pm$ SEM of 3 biological replicates. *P*-values in an unpaired 2-tailed Student's *t*-test are indicated. *P*-values < 0.05 were considered significant. **B)** Nodules per root formed in GUS RNAi and NNC1 RNAi roots at 7, 10, 15, and 21 dpi with *S. meliloti*. Error bars represent the mean $\pm$ SEM of 3 independent biological replicates with at least 50 roots in each biological replicate. *P*-values in an unpaired 2-tailed Student's *t*-test between GUS RNAi and *MtN1C1* RNAi roots at each time point are indicated. *P*-values < 0.05 were considered significant. **C)** Infection threads (ITs) per centimeter of root developed at 7 dpi in GUS RNAi and NNC1 RNAi roots. **D)** Progression of infection events in GUS RNAi and NNC1 RNAi roots. Infection events were classified as ITs that end in microcolony, in the root hair, in the epidermal cell layer, or reach the cortex at 7 dpi. In (C) and (D), each bar represents the mean $\pm$ SEM of 3 independent biological replicates with at least 50 roots in each biological replicate. *P*-values in an unpaired 2-tailed Student's *t*-test are indicated. *P*-values < 0.05 were considered significant. **E)** Relative transcript levels of *MtENOD40* in GUS RNAi and NNC1 RNAi roots. Expression values were determined by RT-qPCR, normalized to *MtHIS3L*, and plotted relative to the GUS RNAi. Each bar represents the mean $\pm$ SEM of 3 biological replicates. *P*-values in an unpaired 2-tailed Student's *t*-test in each comparison are indicated. *P*-values < 0.05 were considered significant.

MtSKI3-silenced roots compared to control roots, we tested whether the expression of *MtENOD40* was altered by silencing of *MtN1C1*. RT-qPCR experiments revealed that *MtENOD40* transcripts accumulated to significantly higher levels in *MtN1C1* RNAi roots as compared with GUS RNAi roots under both mock and *S. meliloti*-inoculated conditions (Fig. 6E), suggesting that *MtN1C1* might act as a repressor of *MtENOD40* during the formation of indeterminate nodules.

To investigate the functional impact of miR172 regulation on *MtN1C1*, plants ectopically overexpressing a miR172-resistant version of *MtN1C1* (*rN1C1*) were generated. RT-qPCR experiments verified that *rN1C1*-expressing roots accumulated higher levels of *MtN1C1* as compared with roots transformed with the empty vector (EV) (Fig. 7A). *rN1C1* roots exhibited a considerable reduction

in the number of nodules formed by *S. meliloti* at all time points (7, 10, 14, and 21 dpi) analyzed as compared to the EV-expressing roots (Fig. 7B). These results indicate that the overexpression of a miR172-resistant variant of *MtN1C1* negatively impacts nodule formation, suggesting that tight regulation of *MtN1C1* is crucial for the nodulation process. Indeed, *MtN1C1* knockdown shows an opposite phenotype to *SKI3* RNAi. To further dissect the regulatory dynamics between miR172 and *MtN1C1*, we analyzed the levels of miR172 transcripts in roots overexpressing *rN1C1* and EV roots at 2 dpi with *S. meliloti*. The results revealed a significant increase in miR172 accumulation in *rN1C1* roots as compared to EV roots (Fig. 7C). The higher accumulation of miR172 in *rN1C1* roots exposes a regulatory feedback loop between miR172 and its target *MtN1C1*. On the other hand, neither

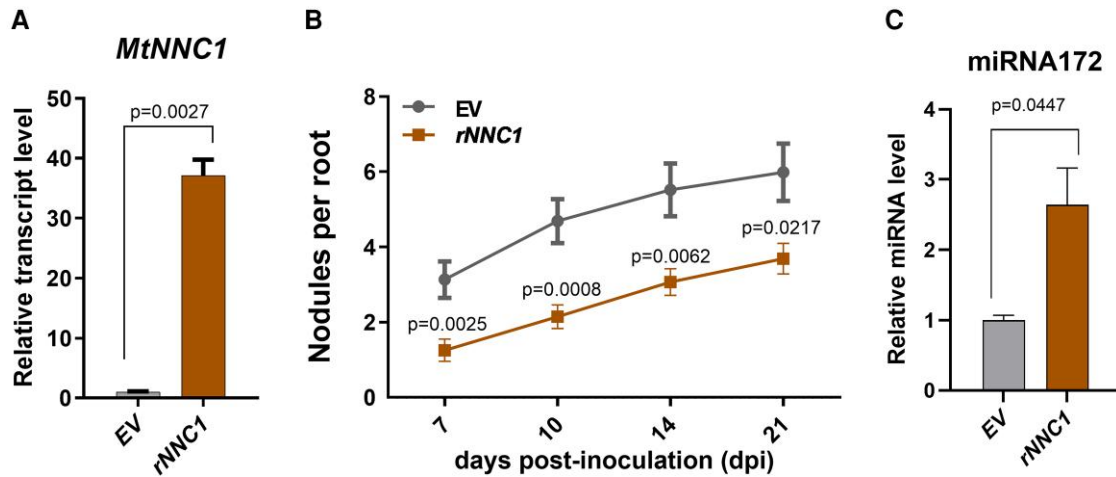


Figure 7. Expression of a miR172-resistant form of MtNNC1 (rNNC1) reduced nodulation and increased miR172 levels. **A)** Relative transcript levels of MtNNC1 in EV (empty vector) and rNNC1 roots. Expression values were determined by RT-qPCR, normalized to *MtHIS3L*, and plotted relative to the EV. **B)** Nodules per root formed in EV and rNNC1 roots at 7, 10, 15, and 21 dpi with *S. meliloti*. Error bars represent the mean $\pm$ SEM of 3 independent biological replicates, each with at least 60 roots. P-values in an unpaired 2-tailed Student's t-test at each time point are indicated. P-values < 0.05 were considered significant. **C)** Relative transcript levels of miR172 in EV, and rNNC1 roots. Expression values were determined by stem-loop RT-qPCR, normalized to miR162a, and plotted relative to the EV. In **(A)** and **(C)**, each bar represents the mean $\pm$ SEM of 3 biological replicates. P-values in an unpaired 2-tailed Student's t-test are indicated. P-values < 0.05 were considered significant.

silencing of MtNNC1 nor overexpression of rNNC1 altered levels of MtSKI3 mRNA under mock or *S. meliloti*-inoculated conditions, suggesting that MtSKI3 might not be part of this feedback loop (Supplementary Fig. S8).

Discussion

mRNA degradation is executed by multiple players, including 5'-to-3' exonucleases, the SKI/exosome complex that degrades deadenylated mRNAs in the 3'-to-5' direction, and miRNAs that guide AGO proteins by base pair complementary to their targets for endonucleolytic cleavage. Here, we uncover an interconnection between the SKI/exosome degradation pathway and the endonucleolytic cleavage mediated by miRNAs during the root nodule symbiosis. Using both 5' P transcript (degradome) and sRNA-seq approaches, we found that inactivation of MtSKI3 function impairs miR172-mediated cleavage of the MtNNC1 transcript with no alteration of miR172 levels. This suggests that the action rather than the biogenesis of miR172 is altered by the knockdown of MtSKI3. Upon rhizobia inoculation, miR172a and miR172b/c levels increase in both GUS RNAi and SKI3 RNAi roots, in agreement with previous reports in other leguminous plants (Wang et al. 2014; Nova-Franco et al. 2015). However, whereas MtNNC1 levels decrease upon inoculation with rhizobia in GUS RNAi control roots, they remain high in SKI3 RNAi roots. This suggests that the repression of MtNNC1 by miR172-mediated cleavage in response to rhizobia may involve MtSKI3. Reduced endonucleolytic cleavage was also observed for the miR393 target transcript MtAFB3 in SKI3 RNAi roots in mock- and rhizobia-inoculated conditions, despite an increase in miR393a/b levels upon rhizobia inoculation in both GUS RNAi and SKI3 RNAi roots. Soybean TIR/AFB homologs have been shown to mediate auxin signaling to modulate nodule number and the formation of infection foci (Cai et al. 2019). Thus, our results suggest that a functional SKI complex might be a requirement for cleavage of specific miRNA target transcripts during the root nodule symbiosis. Consistent with these results, it has been described that simultaneous mutation in genes encoding the exoribonuclease EIN5/XRN4 and SKI2 in

Arabidopsis leads to overaccumulation of miRNA target transcripts, many of which encode transcription factors of the ARF, HD-ZIP, and LBD families (Zhang et al. 2015). Besides the reduced miRNA-guided cleavage of MtNNC1 and MtAFB3, we observed that neither the miR172a and miR172b/c nor the miR393a/b levels changed in MtSKI3-silenced roots as compared with control roots under mock-inoculated conditions, reinforcing the idea that defects in RISC cleavage activity or the kinetics of cleavage rather than changes in miRNA levels are affected in MtSKI3-silenced roots.

In soybean, GmNNC1 was described as a negative regulator of nodule formation and a transcriptional repressor that directly binds to the *GmENOD40* promoter (Wang et al. 2014). Here, our phenotypic characterization has shown that knockdown of MtNNC1 results in the formation of numerous nodules in *M. truncatula* roots, which agrees with that previously observed in soybean and common bean (Wang et al. 2014; Nova-Franco et al. 2015). Thus, NNC1 seems to be a negative regulator of nodulation in both determinate and indeterminate nodule-forming legumes. Moreover, levels of MtENOD40, which is required for nodule formation, were higher in MtNNC1 RNAi roots as compared with GUS RNAi roots under both symbiotic and nonsymbiotic conditions. This is also consistent with the fact that GmNNC1 functions as a transcriptional repressor of *GmENODs* in soybean (Wang et al. 2014) as well as with increased or reduced levels of PvENOD40 in roots overexpressing miR172c or a miR172-resistant form of the NNC1 homolog in *P. vulgaris*, respectively (Nova-Franco et al. 2015). Moreover, the common expression pattern observed here for MtNNC1, MtSKI3, and MtENOD40 in the root and nodule vasculature, which partially agrees with that previously reported for the expression of GmNNC1 in soybean (Wang et al. 2014), favors the idea that the MtSKI3, MtNNC1, and MtENOD40 function in the same tissue types to control nodule development, where MtNNC1 is downregulated at post-transcriptional level by miR172, presumably releasing the transcriptional repression of MtENOD40. Remarkably, MtNNC1 also participates in the control of rhizobia infection since silencing of MtNNC1 results in a higher frequency of infection events, a feature

that was not previously investigated in other legumes, although these extra infection events do not seem to progress to the root cortex. Based on single nuclei RNA-seq analysis reported by Cervantez-Perez et al. (2022), MtNNC1 mRNA levels were downregulated in epidermal, cortical, and vascular tissues of *M. truncatula* roots at 48 hpi with *S. meliloti*, sustaining the notion that MtNNC1 must be repressed at early stages of the root nodule symbiosis to sustain successful infection. On the other hand, overexpression of a miR172-resistant variant (*rNNC1*) results in a severe reduction in nodule number in *M. truncatula*. This agrees with that previously observed in soybean and common bean (Wang et al. 2014; Nova-Franco et al. 2015), supporting the negative role of NNC1 in nodulation as a conserved feature in determinate and indeterminate nodule-forming legumes. In addition, overexpression of *rNNC1* recapitulates the phenotype observed in MtSKI3-silenced roots (Traubenik et al. 2020), reinforcing the idea that MtSKI3 is required for proper downregulation of MtNNC1 during symbiosis. Interestingly, the expression of a miR172-resistant version of MtNNC1 leads to elevated miR172a/b/c levels, suggesting a potential compensatory mechanism that counteracts the overexpression of *rNNC1* to restore the balance in the miR172-MtNNC1 regulatory axis. This differs from that previously described in soybean, where miR172c levels were reduced in roots overexpressing a miR172-resistant variant of *GmNNC1*. Moreover, in soybean, miR172c is transcriptionally repressed by *GmNNC1* (Wang et al. 2019). The opposite behavior of *M. truncatula* and soybean might reflect differences in the regulatory mechanisms for miR172 transcriptional activation between the 2 legume species, i.e. *M. truncatula* and soybean, but also differences in the time points after rhizobia inoculation used to evaluate miR172 levels.

Endonucleolytic cleavage of mRNAs mediated by miRNAs and/or siRNAs, as well as the production of siRNAs, was proposed to occur in polysomes (Brodersen et al. 2008; Liu et al. 2012; Li et al. 2016; Traubenik et al. 2020). Moreover, miRNA-mediated translational inhibition of target mRNAs seems to be a widespread mechanism present in Arabidopsis and maize plants (Brodersen et al. 2008; Yang and Thompson 2024). The miR172 is one of the miRNAs that, in addition to acting at the endonucleolytic cleavage level (Schwab et al. 2005), has been shown to repress its target, the homeotic gene AP2, through translation inhibition in Arabidopsis (Aukerman and Sakai 2003; Chen 2004). We previously demonstrated that miR172 is highly associated with polysomes in *M. truncatula* roots (Reynoso et al. 2013). Here, we found that MtNNC1 was associated with polysomes and that its association with polysomes decreased in roots in response to inoculation with *S. meliloti* at 6 and 10 dpi, whereas MtSKI3 increased its association with polysomes as early as 2 dpi and remained high up to 10 dpi. This indicates that regulation of MtSKI3 at the translational level could impact the miR172-mediated endonucleolytic cleavage, which most likely occurs while MtNNC1 is bound to the polysomes, although translational inhibition and stalling of MtNNC1 during symbiosis cannot be excluded. The SKI complex has been found bound to ribosomes in mammals and yeast, likely facilitating the delivery of 80S ribosome-bound translationally stalled mRNAs to the exosome for mRNA decay (Kögel et al. 2022). The requirement of MtSKI3 observed here for miR172-mediated repression of MtNNC1, as well as for other miRNA targets (e.g. MtAFB3), might reflect a function of the plant SKI complex to extract translational repressed mRNAs and/or the 5' products of miRNA cleavage and their delivery to the exosome for 3' to 5' degradation. Based on this, it could be hypothesized that SKI3-mediated extraction of the MtNNC1 5' product of miR172 endonucleolytic cleavage from polysomes and its delivery to the

exosome might be required for subsequent cleavage of additional MtNNC1 molecules upon symbiotic conditions. This hypothesis would explain not only the downregulation of MtNNC1 at the total mRNA abundance and polysome association upon rhizobia infection but also the higher levels of MtNNC1 mRNA found in MtSKI3-silenced roots as compared with GUS RNAi roots at different stages of the root nodule symbiosis. Altogether, this study highlights the fine-tuning of gene regulation involving miRNAs, translation, and mRNA decay processes required for the establishment of a successful symbiosis between legume plants and nitrogen-fixing bacteria.

Materials and methods

Biological material and vector construction

Medicago truncatula Jemalong A17 seeds were obtained from INRA Montpellier, France (<http://www.montpellier.inra.fr>). SKI3 RNAi and GUS RNAi constructs were previously generated (Traubenik et al. 2020). The MtNNC1 RNAi 1 or MtNNC1 RNAi 2 constructs were generated by amplification of the MtNNC1 fragment using *M. truncatula* cDNA as a template and the MtNNC1RNAi 1 F and MtNNC1RNAi 1R or MtNNC1RNAi 2 F and MtNNC1RNAi 2 R primers, respectively, which are listed in Supplementary Data Set 6. The MtNNC1 amplified fragments were cloned into the pTOPO/ENTR vector (Thermo Fisher Scientific) and recombined into the destination vector pK7GW1WG2DII (Karimi et al. 2007). The miR172-resistant variant of MtNNC1 (mirResMtNNC1) was generated by PCR-directed mutagenesis, introducing specific nucleotide changes (CAT to GCA) at the 9th, 10th, and 11th positions of the miR172 binding site while preserving the encoded serine residues. These modifications were made using the mirResMtNNC1 F and mirResMtNNC1 R primers listed in Supplementary Data Set 6. The amplified fragment was cloned into the pENTR/D-TOPO vector and then recombined into the Gateway-compatible binary vector pK7WG2D,1 (Karimi et al. 2007), in which the expression of MtNNC1 is driven by the cauliflower mosaic virus 35S promoter. All constructs were verified by sequencing. Binary vectors were introduced into *Agrobacterium rhizogenes* Arqua1 (Quandt et al. 1993) by electroporation. *S. meliloti* strain 1021 (Meade and Signer 1977) or the same strain expressing RFP (Tian et al. 2012) was used for root inoculation as previously described (Hobecker et al. 2017).

Plant growth conditions, hairy root transformation, and rhizobia inoculation

Seeds were sterilized and germinated as previously described (Traubenik et al. 2020). Germinated seedlings were transferred to Petri dishes containing agar Fahraeus media (Fahraeus 1957) covered with sterile filter paper. Transgenic roots were generated by *A. rhizogenes*-mediated transformation as previously described (Boisson-Dernier et al. 2001). Plants that developed hairy roots were transferred to slanted boxes containing Fahraeus media free of nitrogen. Seedlings were grown at 25 °C with a long day period (16-h day/8-h night cycle) and 50% humidity. Roots were inoculated with 10 ml of a 1:1,000 dilution of *S. meliloti* 1021 (Meade and Signer 1977) culture grown in liquid TY media until OD₆₀₀ reached 0.8 or with 10 ml of water as a control (mock treatment). One hour later, the excess liquid was discarded, and the seedlings were incubated vertically under the temperature and light

conditions for growth described above. For RNA isolation, root tissue was harvested, frozen in liquid N₂, and stored at -80 °C.

GMUCT 2.0 and Small RNA libraries preparation

GMUCT 2.0 and Small RNA libraries were prepared from RNA samples isolated from GUS RNAi or SKI3 RNAi roots inoculated with water (mock) or *S. meliloti* for 48 h. Whole root tissue was collected from more than 300 plants and pooled. Three biological replicates were conducted, consisting of independent experiments performed on different days. GMUCT 2.0 libraries were constructed following Willmann et al. (2014), replacing kit components with commercially available supplies. Briefly, RNA was extracted using Trizol (Thermo Scientific), and 30 µg of each sample was used to obtain PolyA RNA using oligo dT conjugated to magnetic beads. The 5' adapter was ligated to the RNA using T4RNA ligase 1, and a second round of polyA selection was performed in order to purify unligated 5' adapters. The selected RNA was ligated to a 3' adapter, and after reverse transcription, the library was amplified with PCR primers from BrAD-seq (Townsend et al. 2015). KAPA HiFi DNA Polymerase (Kapa Biosystems) was used for amplification. Libraries were characterized using gel electrophoresis, and the amplified libraries were isolated and sequenced using the Illumina NextSeq500 platform at the IIGB Genomics Core facility at UC Riverside (<https://genomics.iigb.ucr.edu/>).

Small RNA libraries were constructed using the NEBNext Multiplex Small RNA Library Prep Set for Illumina, following the manufacturer's instructions, starting with 500 ng of Total RNA extracted with Trizol. Briefly, 3' and 5' adaptors were ligated to the ends of the single-stranded RNA. After ligation of the adaptors, the small RNAs were used as a template for the synthesis of one complementary strand of DNA. Reverse transcribed molecules that contain both adaptor sequences were then amplified by PCR. Libraries were characterized using gel electrophoresis, and the amplified libraries were isolated. Small RNA-seq libraries were sequenced using the Illumina HiSeq 2500 RP platform at BGI Korea (<https://www.bgi.com/global>). The size of DNA fragments in the GMUCT 2.0 and Small RNA libraries was verified on an Agilent 2100 Bioanalyzer using the DNA-HS kit (Agilent).

GMUCT2-seq analysis

GMUCT2.0 reads were aligned to the *M. truncatula* A17 Mt5.0 mRNAs (Pecrix et al. 2018) in the CyVerse Discovery Environment using STAR v2.4.0.1, reporting the best alignment with ≤2 nt mismatches. Count abundances were calculated using eXpress v1.5.1, with default parameters. Peaks were detected with the "Findpeaks" function of the HOMER package (Heinz et al. 2010) with the parameters "-regionRes 1," "-minDist 150," and "-region." Peaks overlapping in at least 2 replicates were conserved for further analysis using the function "findOverlapsOfPeaks" of the ChipPeakAnno R package (Zhu et al. 2010). Reads in the peaks were quantified in each replicate using the "summarizeOverlaps" function of the GenomicRanges R package (Lawrence et al.). miRNA sequences were obtained from miRbase (v22) (Kozomara and Griffiths-Jones 2014) and target sites were predicted using Miranda software (Enright et al. 2003) over cDNA sequences of the Mt5.0 genome with parameters of gap-opening penalty set to -2 and energy threshold of -22 kcal/mol. Genomic locations predicted for 3' fragments resulting from miRNA cut-sites were deducted. Reads were quantified strictly over those genomic coordinates. The counts for peaks and cut sites were statistically evaluated using DESeq2 (Love

et al. 2014). Genotype differences over features that had a log fold change value of 1 or more, or -1 or less, and a *P*-value < 0.05 were identified as differential GMUCT regions or cut-sites.

Small RNA-seq analysis

Following sequencing, small RNA sequencing adapters were removed using cutadapt (v2.10). Sequences matching ribosomal or transfer RNA were filtered out using bowtie (v1.3.1). Reads of 21 and 22 nucleotides were mapped on the Mt5.0 genome (Pecrix et al. 2018) with ShortStack (v3.8.5) under strict no-mismatch conditions (-mismatches 0), keeping all primary multimapping (-bowtie_m all) and correcting multimapped reads based on uniquely mapped reads (-mmap u). The read accumulation of 21/22nt for each gene annotation was quantified using ShortStack (Axtell 2013). To assess miRNA abundance, the count of sequences corresponding to each mature miRNA in *Medicago* miRBase (v22) was used (Kozomara and Griffiths-Jones 2014). Differential 21/22nt sRNA accumulation and log2 fold changes between conditions were computed using DESeq2 (v1.34.0) (Love et al. 2014). FDR correction of the *P*-value was used.

Isolation of polysomes by TRAP

Isolation of polysomes by TRAP was accomplished as previously described (Traubenik et al. 2020). TRAP material was subjected to RNA extraction using Trizol, following the manufacturer's recommendations (Thermo Scientific).

RT-qPCR

Total RNA was extracted using Trizol (Thermo Fisher Scientific), following the manufacturer's recommendations, and digested with RNase-free DNase (Promega). Total and TRAP RNA samples were subjected to first-strand cDNA synthesis using Moloney Murine Leukemia Virus reverse transcriptase (Promega). Expression analysis by RT-qPCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad) and the CFX96 qPCR system (Bio-Rad) as described previously (Blanco et al. 2009). For each pair of primers, the presence of a unique PCR product of the expected size was verified in agarose gels. The *M. truncatula* HISTONE LIKE 3 (*HIS3L-MtrunA17_Ch4g0054151*) was selected as a reference transcript for the normalization of RT-qPCR data based on previously reported geNORM analysis (Reynoso et al. 2013). Primers used are listed in Supplementary Data Set 6. microRNA quantification was performed by stem-loop RT-qPCR as described previously (Hobecker et al. 2017) using the primers listed in Supplementary Data Set 6. miR162 was used as a reference transcript for normalization. The statistical significance of the differences between samples was determined by unpaired 2-tailed Student's *t*-tests using 3 biological replicates and 3 technical replicates.

Phenotypic analysis

Nodule number was recorded at different time points after inoculation with *S. meliloti* as described previously by Hobecker et al. (2017). Infection events were quantified at 7 dpi as described by Traubenik et al. (2020). Three biological replicates with more than 50 roots were analyzed. The statistical significance of the differences for each parameter was determined by unpaired 2-tailed Student's *t*-tests for each construct.

GUS staining

The *pMtSKI3::GUS-GFP*, *pMtNNC1::GUS-GFP*, and *pMtENOD40::GUS-GFP* constructs were generated by amplifying the region upstream

of the translational initiation codon of MtSKI3, MtNNC1, and MtENOD40 using the primers listed in [Supplementary Table S6](#). The resulting DNA fragment was cloned into the pENTR/D-TOPO vector (Thermo Scientific) and then recombined into the Gateway-compatible binary vector pKGWFS7.0 ([Karimi et al. 2002](#)) using LR Clonase according to the manufacturer's instructions (Thermo Scientific). *M. truncatula* plants were grown in pots containing perlite:sand (3:1) mixed substrate, irrigated with Fahraeus medium free of nitrogen, and inoculated with *S. meliloti*. Histochemical GUS staining was performed in roots and nodules according to [Jefferson et al. \(1987\)](#). GUS-stained tissue was visualized and photographed under white light using an inverted microscope (Olympus IX51).

Accession numbers

Raw sequence files (fastq files) supporting the conclusions of this article were deposited at Gene Expression Omnibus under series entry numbers GEO GSE135920 and GSE285134. All processed data were included in [Supplementary Datasets S1 to S5](#).

Sequence data from this article can be found in MtrunA17r5.0-ANR, (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>) or in miRbase (v22) (<http://www.mirbase.org>) under the following accession numbers: MtSKI3 (MtrunA17_Ch5g0393431), MtNNC1 (MtrunA17_Ch4g0030191), MtENOD40 (MtrunA17_Ch8g0368441), MtHISL3 (MtrunA17_Ch4g0054151), MtERF1 (MtrunA17_Ch2g0325911), miR172a (MI0005600), miR172b (MI0018368), miR393a (MI0001745), miR393b (MI0005601), and miR162 (MI0001738).

Author contributions

S.T., M.E.Z., F.B., M.C., and J.B.-S. designed the research. S.T., M.A.R., F.S.-R., M.Y., M.H., A.C., and T.B. performed the research. S.T. and M.E.Z. wrote the original draft of the article. S.T., M.A.R., F.S.-R., M.Y., T.B., and M.E.Z. analyzed the data. S.T., M.A.R., A.C., T.B., M.C., J.B.-S., F.B., and M.E.Z. reviewed and edited the article. Funding was acquired by M.E.Z., F.B., M.C., J.B.-S., T.B., and S.T. M.E.Z., F.B., M.C., T.B., and J.B.-S. supervised the study.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Gene ontology functional categories found among transcripts with differential 5'P peaks in GMUCT2.0.

Supplementary Figure S2. Transcription factors with differential 5'P peaks.

Supplementary Figure S3. Expression of MtSKI3 and MtNNC1 in root cells.

Supplementary Figure S4. miR393 levels in mock and *S. meliloti*-inoculated SKI3 RNAi and GUS RNAi roots.

Supplementary Figure S5. sRNA-seq read coverage of MtNNC1 and MtAFB3.

Supplementary Figure S6. Differentially accumulated 21-22 nt sRNA levels in SKI3 RNAi and GUS RNAi roots.

Supplementary Figure S7. Silencing of MtNNC1 using NNC1 RNAi 2-enhanced nodulation, and bacterial infection.

Supplementary Figure S8. Silencing of MtNNC1 or overexpression of rNNC1 does not affect MtSKI3 mRNA levels under mock or *S. meliloti*-inoculated conditions.

Supplementary Data Set 1. 5'P peaks detected by GMUCT2.0 in SKI3 RNAi and GUS RNAi mock and *S. meliloti* (Sm)-inoculated roots.

Supplementary Data Set 2. GO Functional classification of genes with differential 5'P peaks in GMUCT2.0 in all comparisons.

Supplementary Data Set 3. miRNA target transcripts with GMUCT2.0 5'P peaks at the miRNA cleavage sites.

Supplementary Data Set 4. Log₂ fold change (FC) of microRNA levels identified by sRNA-seq using DeSeq2.

Supplementary Data Set 5. Log₂ fold change (FC) of 21-22 nt sRNA levels identified by sRNA-seq using DeSeq2 in SKI3 RNAi versus GUS RNAi roots under mock conditions.

Supplementary Data Set 6. Primers used in this study.

Funding

This work was supported by grants of the Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación (Agencia I+D+I) of Argentina, FONCYT (PICT2019-00554, PICT2019-0029, PICT2020-00053, and PICT-2021-I-A-00170), the Ministerio de Ciencia, Tecnología e Innovación (MINCYT) of Argentina (RIBOLEG, CONVE-2023-100766842), the Centre National de la Recherche Scientifique (CNRS) through the International Research Project LOCOSYM, and Saclay Plant Sciences-SPS (ANR-17-EUR-0007). S.T. is supported by the LUMIROOT (101110703) project funded by the Marie Skłodowska-Curie Actions and the MICROLUP project (ANR MICROLUP 19-CE13-0029-02NA) funded by the Agence Nationale de la Recherche ANR, and was funded by a CONICET fellowship and a Fulbright-Williams Foundation fellowship. S.T., F.S.-R., and T.B. benefit from the ECOS-SUD Exchange Program (no. A20N05). M.A.R., F.A.B., and M.E.Z. are members of CONICET, Argentina. F.S.-R. is funded by a Saclay Plant Sciences-SPS fellowship. M.Y. is funded by a CONICET fellowship. A.C., T.B., and M.C. are members of CNRS, France. J.B.-S. is a member of NSF, USA.

Conflict of interest statement. None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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