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Abstract

MicroRNAs (miRNAs) are a major class of small non-coding RNAs with emerging functions in biotic and abiotic interactions. Here, we report on a new functional role of Arabidopsis miR827 and its NITROGEN LIMITATION ADAPTATION (NLA) target gene in mediating plant susceptibility to the beet cyst nematode Heterodera schachtii. Cyst nematodes are sedentary endoparasites that induce the formation of multinucleated feeding structures termed syncytia in the roots of host plants. Using promoter:GUS fusion assays we established that miR827 was activated in the initial feeding cells and this activation was maintained in the syncytium during all sedentary stages of nematode development. Meanwhile, the NLA target gene, which encodes an ubiquitin E3 ligase enzyme, was post-transcriptionally silenced in the syncytium, to permanently suppress its activity during all nematode parasitic stages. Overexpression of miR827 in Arabidopsis resulted in hyper-susceptibility to H. schachtii. In contrast, inactivation of miR827 activity through target mimicry or by overexpression a miR827-resistant cDNA of NLA produced the opposite phenotype of reduced plant susceptibility to H. schachtii. Gene expression analysis of several pathogenesis-related genes together with Agrobacterium–mediated transient expression in Nicotiana benthamiana provided strong evidence that miR827-mediated downregulation of NLA to suppress basal defense pathways. In addition, using yeast two-hybrid screens we identified several candidates of NLA-interacting proteins that are involved in a wide range of biological processes and molecular functions, including three pathogenesis-related proteins. Taken together, we conclude that nematode-activated miR827 in the syncytium is necessary to suppress immune responses in order to establish infection and cause disease.

Keywords

miR827, Arabidopsis, cyst nematodes, Heterodera schachtii, post-transcriptional gene silencing, syncytium, immune responses, ubiquitin E3 ligases, yeast two-hybrid

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Arabidopsis miR827 mediates post-transcriptional gene silencing of its ubiquitin E3 ligase target gene in the syncytium of the cyst nematode *Heterodera schachtii* to enhance susceptibility

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Key words: miR827, Arabidopsis, cyst nematodes, *Heterodera schachtii*, post-transcriptional gene silencing, syncytium, immune responses, ubiquitin E3 ligases, yeast two-hybrid.

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Summary

MicroRNAs (miRNAs) are a major class of small non-coding RNAs with emerging functions in biotic and abiotic interactions. Here, we report on a new functional role of Arabidopsis miR827 and its *NITROGEN LIMITATION ADAPTATION* (*NLA*) target gene in mediating plant susceptibility to the beet cyst nematode *Heterodera schachtii*. Cyst nematodes are sedentary endoparasites that induce the formation of multinucleated feeding structures termed syncytia in the roots of host plants. Using promoter:GUS fusion assays we established that miR827 was activated in the initial feeding cells and this activation was maintained in the syncytium during all sedentary stages of nematode development. Meanwhile, the *NLA* target gene, which encodes an ubiquitin E3 ligase enzyme, was post-transcriptionally silenced in the syncytium, to permanently suppress its activity during all nematode parasitic stages. Overexpression of miR827 in Arabidopsis resulted in hyper-susceptibility to *H. schachtii*. In contrast, inactivation of miR827 activity through target mimicry or by overexpression a miR827-resistant cDNA of *NLA* produced the opposite phenotype of reduced plant susceptibility to *H. schachtii*. Gene expression analysis of several pathogenesis-related genes together with Agrobacterium–mediated transient expression in *Nicotiana benthamiana* provided strong evidence that miR827-mediated downregulation of *NLA* to suppress basal defense pathways. In addition, using yeast two-hybrid screens we identified several candidates of NLA-interacting proteins that are involved in a wide range of biological processes and molecular functions, including three pathogenesis-related proteins. Taken together, we conclude that nematode-activated miR827 in the syncytium is necessary to suppress immune responses in order to establish infection and cause disease.

Introduction

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Plant endogenous small RNAs, including microRNAs (miRNAs), play key roles in regulating gene expression and host immune responses against pathogen infection (reviewed by Weiberg *et al.,* 2014). Mature miRNAs are small non-coding RNA molecules, about 20-22 nucleotides in length, that mediate gene repression through cleavage of the target transcripts containing complementary sequences, causing post-transcriptional gene silencing. Several studies of miRNA expression analysis in various plant species have linked miRNA expression changes with pathogenesis. Nevertheless, the molecular mechanisms through which miRNA gene expression changes impact host immune responses are limited to a few cases. miR393 is the first miRNA shown to activate innate immune responses following bacterial infection (Navarro *et al.,* 2006). The expression of miR393 was induced by bacterial flagellin, and this induction resulted in downregulation of three auxin receptors, leading to the suppression of auxin signaling pathways that are associated with disease susceptibility (Navarro *et al.,* 2006).

Subsequent analyses indicated that miRNA genes can function as negative or positive regulators of plant innate immunity. For example, overexpression of miR160a, miR398b and miR773 in Arabidopsis established a role of these miRNAs in modulation of pathogen-associated molecular pattern–induced callose accumulation, which impacted plant responses to both virulent and nonpathogenic strains of *Pseudomonas syringae* (Li *et al.,* 2010). Barley miR398 was shown to be regulated by the disease resistance proteins Mla (Mildew resistance locus a) and ROM1 (Restoration of Mla resistance1), and this regulation impacts the chloroplast copper/zinc superoxide dismutase 1 (SOD1) protein, which in turn influences barley cell death in response to infection by the powdery mildew fungus (Xu *et al.,* 2014). Transient overexpression of both barley and Arabidopsis miR398 in barely repressed the accumulation of SOD1, which impeded Mla-triggered hydrogen peroxide and hypersensitive reaction (Xu *et al.,* 2014). Similarly, Arabidopsis miR400 was found to negatively affect the defense response by targeting peptatricopeptide repeat (*PPR*) genes. miR400–directed cleavage of *PPR* genes rendered Arabidopsis plants more susceptible to the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 and the fungus *Botrytis cinerea* (Park *et al.,* 2014b). By contrast, differential gene expression analysis pointed to the implication of several rice miRNAs in immunity against the blast fungus *Magnaporthe oryzae* (Li et *al.,* 2014). Recently, a role of miRNA genes in regulating the nucleotide binding site-leucine-rich repeat (NBS-LRR) innate immune receptors has been elucidated (Zhai *et al.,* 2011; Li *et al.,* 2012a; Shivaprasad *et al.,* 2012, Liu *et al.,* 2014a). It has

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been demonstrated that cleavage of *NBS-LRR* transcripts by miRNAs triggers the production of phased, secondary siRNAs (phasiRNAs). Because phasiRNAs can function both in *cis* and in *trans*, targeting homologous transcripts, miRNAs can extend their suppression activity through the production of secondary phasiRNAs, thereby regulating many other *NBS-LRR* genes.

Cyst nematodes are sedentary, biotrophic plant parasites of root systems in many crop plants. These parasites penetrate the roots and migrate intracellularly to the central cylinder where they induce the formation of multicellular feeding sites called syncytia. The nematode first initiates contact with a single competent cell known as initial feeding cell. This contact also results in dramatic cellular modifications in the neighboring cells that include endoreduplication, cell wall modification and dissolution, disappearance of large vacuoles, and increased number of organelles (Golinowski *et al.,* 1996). The successive cell-to-cell fusion of several hundred cells surrounding the initial feeding cells results in the formation of one large multinucleated syncytium in the roots (Sobczak and Golinowski, 2009). The initiation and formation of these permanent feeding structures, as the sole source for nematode nourishment, are associated with active gene expression changes in which small RNAs-mediated gene regulation are believed to play key roles (reviewed by Hewezi and Baum, 2015). Biogenesis of small RNAs in plants requires the activity of various dicer-like (DCL) proteins and RNA-dependent RNA polymerases (RDRs) (Bologna and Voinnet, 2014). The reduced susceptibility of mutant lines defective in DCL or RDR activity to the beet cyst nematode *Heterodera schachtii* provided the initial indication for the involvement of small RNA pathways in cyst nematode parasitism of host plants (Hewezi *et al.,* 2008a). This indication was further supported by the findings that several Arabidopsis and soybean miRNAs profoundly changed expression in response to nematode infection (Hewezi *et al.,* 2008a; Li *et al.,* 2012b).

Detailed genetic and molecular analyses of one of these miRNAs, miR396 and its Growth-Regulating Factor 1 (*GRF1*) and *GRF3* target genes, during the compatible interaction between Arabidopsis and *H. schachtii* pointed to a key role of this regulatory system in reprogramming root cells into syncytial cell-type (Hewezi *et al.,* 2012; Hewezi and Baum, 2012). The miR396/GRF regulatory loop was found to directly or indirectly control the expression of a significant portion of syncytium differentially expressed genes, particularly those associated with transcriptional activity, hormone signaling, defense responses and disease resistance (Hewezi *et*

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al., 2012; Liu *et al.,* 2014b). Recently, analyzing small RNA components isolated from early developing galls formed by the root knot-nematode *Meloidogyne javanica* in Arabidopsis revealed significant differences in the abundance and distribution of small RNA components between galls and control roots (Cabrera *et al.,* 2016). In addition, a role of the miR390/TAS3 regulatory module in gall formation, presumably through the regulation of auxin-response factors, was demonstrated (Cabrera *et al.,* 2016). In the current study, we describe a new functional role for Arabidopsis miR827 and its *NITROGEN LIMITATION ADAPTATION* (*NLA*) target gene in suppressing the plant immune response to mediate plant susceptibility to the beet cyst nematode *H. schachtii.*

Results

miR827 is strongly upregulated in the initial feeding cells of *Heterodera schachtii*

In Arabidopsis, miR827 is encoded by one gene (AT3G59884) and negatively regulates the expression of *NITROGEN LIMITATION ADAPTATION* (*NLA*), an ubiquitin E3 ligase gene (AT1G02860), which contains a perfect complementary binding site to the mature miR827 located between nucleotides 257 and 278 of the mRNA (Fahlgren *et al.,* 2007). miR827–directed cleavage of *NLA* was confirmed using RNA ligase-mediated 5' rapid amplification of cDNA ends (RLM 5'-RACE) (Fahlgren *et al.,* 2007, Hsieh *et al.,* 2009) and by degradome sequencing (Addo-Quaye *et al.,* 2008). To examine the temporal and spatial expression patterns of miR827 in roots following infection with *Heterodera schachtii*, we generated several independent transgenic lines expressing the GUS reporter gene under the control of the miR827 promoter. Under Pi-sufficient conditions, the GUS activity resulting from the miR827 promoter in non-infected plants was observed in the cotyledons (Figure S1a). However, GUS activity was not detected in root tissues at various developmental stages (Figure S1b-d). Following nematode infection, GUS activity was assayed every 24 h for the first 4 days. Very interestingly, specific GUS activity was detected in the initial feeding cells as early as 1 day post infection (dpi) (Figure 1a). At 2, 3 and 4 dpi, GUS activity was observed in the developing feeding sites formed by the second-stage juvenile (J2) (Figure 1b–d). In the well-developed syncytia of the early and late third-stage juveniles (J3) at 7 and 10 dpi, respectively, the miR827 promoter showed substantial increase in activity (Figure 1e, f). At 14 dpi, the miR827 promoter became less active

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in the syncytium of the fourth-stage juvenile (J4), and GUS staining can only be seen in the feeding site around nematode head (Figure 1g). In contrast, strong GUS activity was observed in the syncytium induced by male nematodes (Figure 1h). These expression patterns point to a functional role of miR827 not only during the very early stage of syncytium initiation but also during syncytium formation and later development stages.

NLA gene is post-transcriptionally regulated by miR827 in the syncytium

Because miRNAs function through the negative regulation of their target genes, it was of interest to test whether the *NLA* gene is post-transcriptionally regulated by miR827 in the syncytium. We tested several transgenic lines expressing two different *NLA* promoter:GUS constructs designed to allow assessment of transcriptional and post-transcriptional regulation of *NLA* transcripts by miR827 in response to *H. schachtii* infection under Pi-sufficient conditions. Because the miR827 target site is located in the 5'-UTR, the first construct was generated to include the *NLA* promoter upstream of the 5'-UTR fused to GUS (*pNLA:GUS*), which should reflect the transcriptional activity of *NLA*. The second construct contained the *NLA* promoter upstream of the translation start codon (ATG) fused to GUS ($pNLA_{miR827}$*:GUS*), which should reflect the post-transcriptional regulation of *NLA* transcripts by miR827. The expression patterns of these two promoter constructs in independent transgenic lines under non-infected conditions are provided in Figure S2. Under infected conditions, GUS activity of three independent transgenic lines expressing the *pNLA:GUS* construct was tested in syncytia induced by *H. schachtii* at various nematode developmental stages. Strong GUS activity was observed in the developing syncytia induced by the J2 nematodes at 3 dpi (Figure 2a). GUS activity was maintained at high levels in the syncytia at 7 and 10 dpi during early and late J3 stages (Figure 2b, c). At 14 dpi, the GUS activity persisted in the syncytia of J4 nematodes (Figure 2d). Similarly, the histochemical staining of GUS activity of three transgenic lines expressing $pNLA_{miR827}$*:GUS* construct was examined in the roots during *H. schachtii* infection. Interestingly, GUS staining indicated clear downregulation of *NLA* in the syncytia throughout all stages of nematode infection (Figure 2e–h). These spatiotemporal expression patterns suggest post-transcriptional regulation of *NLA* by miR827 in the syncytium during the course of *H. schachtii* parasitic stages.

To provide additional evidence for miR827-mediated *NLA* cleavage during nematode infection, we used qPCR to quantify the levels of both cleaved and total *NLA* transcripts in the roots of

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wild-type (Col-0) plants at 4 and 8 d after infection with *H. schachtii* relative to non-infected plants. Two pairs of primers were designed, as previously described by Gutierrez *et al.* (2009), to estimate the extent of miR827-directed *NLA* cleavage. One primer pair was flanking the miR827 cleavage site, which would reflect the relative abundance of uncleaved *NLA* transcripts, and the other pair was designed downstream of the miR827 cleavage site, which would reflect the relative abundance of total (cleaved and uncleaved) *NLA* transcripts. As shown in Figure S3, the levels of total and uncleaved *NLA* transcripts were significantly increased in the infected roots relative to non-infected control roots at 4 and 8 dpi. However, the level of uncleaved transcripts was about 2 fold lower than the level of total transcripts at both time points (Figure S3), suggesting that *H. schachtii* infection activates posttranscriptional degradation of *NLA*.

Overexpression of miR827 enhanced plant susceptibility to *H. schachtii*

To test the potential involvement of miR827 in modulating the interaction between Arabidopsis and *H. schachtii*, we constitutively overexpressed the hairpin–shaped precursor of miR827 in Arabidopsis using the 35S promoter. Three independent non-segregating T2 lines were selected for phenotypic analyses. The overexpression lines displayed noticeable developmental defects in leaves and reduced plant size (Figure 3a), whereas root system architecture and development were indistinguishable from that of the wild-type plants (Figure 3b). Consistent with a post-transcriptional regulation of *NLA* by miR827, these miR827 overexpression lines showed between 2- and 3-fold downregulation of the transcript levels of *NLA* relative to wild-type Col-0 plants (Figure 3c). These lines were used in nematode inoculation assays to determine susceptibility levels to *H. schachtii*. Ten-day-old plants were inoculated with infective J2 nematodes, and the number of J4 females per root system was determined 3 weeks after inoculation and used to approximate plant susceptibility. Notably, the miR827 overexpression lines showed hyper-susceptibility phenotypes in response to *H. schachtii* with an increase in susceptibility between 90 and 125% relative to the wild-type Col-0 (Figure 3d). This strong hyper-susceptibility phenotype indicates a key role of increased miR827 activity in mediating plant susceptibility to *H. schachtii* infection.

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Inactivation of miR827 expression decreased plant susceptibility to *H. schachtii*

We next examined whether inactivation of miR827 expression will produce the opposite phenotype of miR827 overexpression, giving rise to reduced plant susceptibility to *H. schachtii*. The target mimicry approach (Franco-Zorrilla et al., 2007) was used to suppress miR827 expression. In this approach we replaced the miR399 binding site in the non–protein coding gene *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) from Arabidopsis by mimic sequences for miR827 (MIM827) containing a mismatched loop to prevent miR827-guided cleavage (Figure 4a). The modified *IPS1* gene was overexpressed in Col-0 plants under control of the 35S promoter and several MIM827-overexpressing lines were generated and quantified for miR827 expression levels. The transgenic lines showed a phenotype comparable to that of the miR827 overexpression lines, including developmental defects in leaves and reduced plant size (Figure 4b). However, the root system was largely unaltered (Figure S4a). Three MIM827-overexpressing lines showing between 14- and 25-fold downregulation of the mature miR827 levels (Figure 4c) were selected and subjected to nematode susceptibility assays. Interestingly, the MIM827-overexpression lines showed a statistically significant decrease in susceptibility to *H. schachtii* relative to the wild-type control (Figure 4d). Again, these data indicate that miR827 functions as a positive regulator of plant susceptibility to *H. schachtii* infection.

Inactivation of *NLA* expression phenocopied miR827 overexpression phenotype of increased nematode susceptibility

Our nematode susceptibility assays described above suggest that increased nematode susceptibility in miR827 overexpression lines is mediated through downregulation of its *NLA* target gene. To test this hypothesis, the *nla-3* (FLAG_352A03) knockout mutant in the Wassilewskija (Ws) background (Lin *et al.*, 2013) was phenotypically analyzed. Under normal growth conditions, the *nla-3* mutant showed no noticeable morphological changes compared to the wild-type Ws (Figure 5a, and Figure S4b), confirming the results of Lin *et al.* (2013). The *nla-3* mutant and the wild-type Ws were assayed for nematode susceptibility in two independent experiments. Interestingly, *nla-3* exhibited a statistically significant increase in susceptibility to *H. schachtii* relative to the wild-type control (Figure 5d), thereby phenocopying the miR827 overexpression lines. These data indicate that NLA functions as a negative regulator of plant susceptibility to *H. schachtii* infection

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Overexpression of a miR827-resistant variant of the *NLA* gene reduced nematode susceptibility

To provide additional support for the negative impact of NLA on nematode susceptibility, we generated transgenic lines overexpressing the wild-type mRNA of *NLA* (*P35S:wtNLA*) or a miR827-resistant non-cleavable mRNA variant (*P35S:rNLA*) under the control of the 35S promoter (Figure 5b, c and Figure S5). The non-cleavable variant was generated by introducing mutations in the miR827 target site. No effect of overexpression of *wtNLA* or *rNLA* on the root system morphology was observed (Figure S4c, d). The transgenic lines overexpressing *wtNLA* mRNA showed little or no significant effects on plant susceptibility to *H. schachtii* compared with the wild-type control (Figure 5e). In contrast, the transgenic lines overexpressing the miR827-resistant variant of *NLA* gene showed statistically significant reduction in nematode susceptibility compared to the wild-type plants (Figure 5f). Overexpressed *wtNLA* did not show a phenotype because the intrinsic stability of these transcripts and/or protein translation likely were decreased as a result of transcript cleavage by the nematode-induced miR827. However, expression of *NLA* variant resistant to miR827 exhibited a function in the syncytia and impeded nematode infection success. These results again demonstrate that post-transcriptional silencing of *NLA* expression is the mechanism through which miR827 brings about plant susceptibility to *H. schachtii*.

NLA activates basal defense responses

Previous reports point to a role of NLA in regulating the accumulation of salicylic acid (SA) and immune response against *Pseudomonas syringae* pv *tomato* (Pst DC3000) (Yaeno and Iba, 2008). Therefore, we used qPCR to quantify the mRNA abundance of selected pathogenesis-related (PR) genes, whose activation is associated with the SA (*PR1, PR5, PAD4* and *EDS1*), jasmonic acid (JA, *PR3* and *PR4*) or JA/ethylene (*PDF 1.2*) signaling pathways in the roots of transgenic plants overexpressing a miR827-resistant version of *NLA* (line 6-4) as well as in the *nla-3* mutant line relative to the wild-type plants. Interestingly, all seven *PR* genes showed significant upregulation in transgenic plants overexpressing *rNLA* relative to the wild-type control (Col-0) with *PR1, PR4* and *PDF 1.2* showing the largest levels of upregulation (Figure 6a). In contrast, the expression of these genes showed little or no change in the *nla-3* mutant

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plants when compared with the wild-type (Ws) (Figure 6a). These results suggest that NLA functions in activating basal defense responses.

To further investigate the involvement of the miR827/*NLA* regulatory module in modulating plant basal defense responses, we measured the expression of the seven *PR* genes in the roots of transgenic plants overexpressing miR827 (line 18-9) as well as in the *P35S:wtNLA* plants (line 13-2). Interestingly, *PR4*, *PR5*, *PDF1.2*, *PAD4*, and *EDS1*, were downregulated in the miR827 overexpression line, whereas *PR1* and *PR3* were unaffected compared with the wild-type Col-0 plants (Figure 6b). In contrast to the high expression levels of PR genes detected in the *P35S:wtNLA* plants, the *P35S:wtNLA* plants showed slight increases in the expression of *PR1*, *PR3,* and *PR4*, but the remaining four *PR* genes were comparable to the wild-type plants (Figure 6b).

In additional experiments we assayed the impact of the miR827/*NLA* regulatory system on growth of the non-pathogenic bacteria *Pseudomonas fluorescens* strain EtHAn using Agro-infiltration assays. Binary plasmids for overexpressing miR827, MIM827 and *NLA in planta* were introduced into *Agrobacterium tumefaciens* strain GV3101. The empty binary plasmid was also introduced into the GV3101 strain and used as a negative control. These constructs were expressed in *Nicotiana benthamiana* leaves by Agro-infiltration one day prior to the inoculation with *P. fluorescens* strain EtHAn. Remarkably, the survival of EtHAn was profoundly increased after overexpression of miR827 (Figure 6c). On the contrary, expression of MIM827 and *NLA* showed the opposite effects of reduced bacterial survival (Figure 6c). Taken together, these results provide strong evidence that miR827-mediated downregulation of *NLA* results in suppression of basal defense pathways.

Identification of putative NLA interacting protein substrates

Ubiquitin E3 ligases catalyze the transfer of ubiquitin from the E2 to the targeted protein substrates through direct interaction. To search for Arabidopsis proteins that physically associate with the E3 ligase NLA we performed yeast two-hybrid (Y2H) screens using the full-length NLA fused to GAL4 DNA-binding domain as bait. Three prey libraries prepared from Arabidopsis roots inoculated with *H. schachtii* and containing more than 15 million yeast

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colonies (Hewezi *et al.*, 2008b) were screened. A total number of 42 clones were identified in the initial screen. Forty clones were found to code for ubiquitin-conjugating enzyme 8 (UBC8, AT5G41700), confirming the results previously reported by Peng *et al.* (2007). The remaining two clones were found to code for ubiquitin-conjugating enzyme 10 (UBC10, AT5G53300) (Figure 7a).

Frequently, identifying substrates for ubiquitin E3 ligases is challenging because of the transient nature of the interactions. In addition, all RING motif-containing proteins form dimers and this dimerization is required for their function (Xie *et al.*, 2002; Seo *et al.*, 2003). Recently, NLA was found to homodimer via its C-terminal RING domain (Park *et al.*, 2014a). Thus, we generated a bait construct (Figure 7b) in which we fused the C terminal region containing the RING domain (amino acid 220-335) to the N terminus of the full-length *NLA* and mutated the conserved cysteine residue at position 279 to adenine (NLA^{C279A}), which disrupts the RING domain activity (Lin *et al.*, 2013) and would increase the NLA/substrate binding by inhibiting the events that trigger substrate release. Screening the prey libraries using this modified bait construct ($^{RING}NLA^{C279A}$) yielded a high number of positive colonies on the selective synthetic dropout (SD/-Leu/-Trp/-Ade/-His) medium. These colonies were re-streaked on the selective medium containing X-α-Gal to assay expression of *MEL1* reporter gene encoding α-galactosidase. This process resulted in the identification of 59 colonies that maintained their ability to grow on the selective medium and strongly activated the *MEL1* reporter gene. Prey plasmid isolation and sequencing revealed that these prey clones code for 51 independent Arabidopsis proteins (Table S1). From these preys, nine were selected to test interaction specificity, including possible interactions with two negative controls, including the empty bait vector and bait vector containing the human Lamin C gene, using yeast co-transformation assay. In this assay, yeast AH109 cells were co-transformed with each of these 9 preys together with the modified NLA bait vector, or with the negative control vectors. Interestingly, these partial clones were found to interact specifically with the modified NLA bait and grow on the stringent SD/-Leu/-Trp/-Ade/-His selective medium (Figure 7c). These nine clones code for LOG2-like ubiquitin ligase1 (AT5G03200), INDOLE-3-ACETIC ACID INDUCIBLE 27 (IAA27, AT4G29080), MAP kinase 17 (MPK17, AT2G01450), MAP kinase kinase 2 (MKK2, AT4G29810), pathogenesis-related thaumatin superfamily protein (AT5G24620), pathogenesis-related 4, (PR4, AT3G04720), papain family cysteine protease (AT4G16190), beta-1,3-endoglucanase

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(AT4G16260), and cytochrome P450 (CYP72A14, AT3G14680). Finally, we examined whether the 51 NLA-interacting clones are among the 7,725 genes whose expression was significantly altered in Arabidopsis syncytium (Szakasits *et al.,* 2009). Importantly, 31 of these clones are syncytium differentially expressed (Table S1). Together these data highlight the potential of NLA for regulating a significant number of cellular processes including plant-nematode interactions.

Discussion

Plants have evolved an array of basal defense responses to resist nematode parasitism. However, plant factors and signaling pathways that regulate basal host defenses have not yet been identified, and hence the role of basal defenses in shaping the compatibility of plant-nematode interactions remains largely unknown (Goverse and Smant, 2015; Holbein *et al.,* 2016). Genome-wide gene expression studies of nematode-infected roots and isolated feeding sites revealed that basal defense responses are triggered during nematode migration phase both in susceptible and resistant plants, but they are actively repressed inside the feeding sites formed on susceptible plants (reviewed in Kyndt *et al.,* 2013; Goverse and Smant, 2015; Holbein *et al.,* 2016). Thus, the ability of plant-parasitic cyst nematodes to suppress host defense responses specifically in the initial feeding cells is critical for dictating the fate of the interaction between the host plants and the nematodes during nutrient acquisition and sedentary stage development. In this study, we report new functional roles of the miR827/*NLA* regulatory system in suppressing basal defense responses in the nematode feeding site to promote parasitism. The spatiotemporal expression patterns of miR827 and its *NLA* target in the syncytium revealed that upregulation of miR827 is required to stably maintain repression of NLA activity during all stages of syncytium initiation, formation, and maintenance. In response to *H. schachtii,* miR827 was strongly up-regulated in the initial feeding cells as early as 1 dpi and this upregulation was maintained in the syncytium during the parasitic J2 and J3 stages and to lesser extent during the J4 stage. Meanwhile, the miR827 target gene *NLA* was post-transcriptionally silenced in the syncytium during all stages of nematode development. The visualization of post-transcriptional silencing of *NLA* by miR827 in the syncytium was facilitated by including the miR827 target sequences upstream of the *GUS* reporter gene directed by the *NLA* promoter. *NLA* was also

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reported to be significantly downregulated in the *H. schachtii*-induced syncytium in Arabidopsis roots (Szakasits *et al.*, 2009). qPCR quantification of total and uncleaved levels of *NLA* transcripts in wild-type plants after *H. schachtii* infection further confirmed the posttranscriptional cleavage of *NLA* transcripts. It may be important to mention that differences in the spatial expression patterns between the *pNLAmiR827:GUS* and *pNLA:GUS* plants were observed under non-infected conditions. These differences could be due to various factors, including GUS detection sensitivity, integration site, expression levels, and Pi availability. In addition, a role of the 5'UTR in transcriptional activity and/or transcript stability is not eliminated.

A recent study demonstrated a role of miR827 in inhibiting plant immunity in response to infection by the blast fungus *Magnaporthe oryzae* in rice (Li *et al.*, 2014). In line with this finding, our data indicate that miR827 functions as a negative regulator of plant immunity in response to *H. schachtii* through suppressing the activity of *NLA* in the syncytium. Several lines of experimental evidence support this claim. First, overexpression of miR827 in Arabidopsis produced hyper-susceptibility phenotype to *H. schachtii* infection. Second, suppression of miR827 activity through target mimicry or through overexpression of a non-cleavable variant of its *NLA* target gene produced the opposite phenotypes of reduced nematode susceptibility. Third, post-transcriptional silencing of *NLA* in the syncytium contributed to successful parasitism. The importance of the post-transcriptional silencing of *NLA* by miR827 in facilitating plant susceptibility to nematode infection was established by showing that overexpression of a wild-type of *NLA* did not significantly affect plant susceptibility to *H. schachtii,* while overexpression of a miR827-resistant version of *NLA* exhibited significant reduction in susceptibility. It may be worth mentioning that overexpression levels of *rNLA* were not linearly correlated with the magnitude of plant resistance to *H. schachtii*. It seems most likely that abundance of *rNLA* transcripts beyond a certain threshold had no additional impacts on plant resistance to *H. schachtii.*

The Arabidopsis genome codes for 499 RING-type E3 ubiquitin ligases (Craig *et al.,* 2009). Despite this large number, only few have been shown to regulate plant defense signaling. For instance, a number of the ARABIDOPSIS TóXICOS EN LEVADURA (ATL) family has been

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demonstrated to be involved in defense responses (Marino *et al.*, 2012). Overexpression of *ATL2* in Arabidopsis resulted in activation of defense-related genes (Serrano and Guzmán, 2004), whereas a loss-of-function mutant of *ATL9* exhibited enhanced susceptibility to *Golovinomyces cichoracearum* (Ramonell *et al.*, 2005). Functional analysis of Arabidopsis RING proteins RIN2 and RIN3 revealed their involvement in the RPM1- and RPS2-mediated hypersensitive response (Kawasaki *et al.*, 2005). Also, Arabidopsis Botrytis Susceptible1 Interactor (BOI) was found to interact with and ubiquitinate Arabidopsis BOS1 (Luo *et al.*, 2010), an R2R3-MYB family transcription factor, which is required for disease resistance (Mengiste *et al.*, 2003). RNAi-mediated downregulation of BOI transcripts led to increased plant susceptibility to the necrotrophic fungus *Botrytis cinerea* (Luo *et al.*, 2010). The Arabidopsis RING1 was also found to function as a positive regulator of plant programmed cell death (Lin *et al.*, 2008). Our study indicates that NLA contributes to the activation of basal defenses because overexpression of the *NLA* gene induced expression of several *PR* genes under non-infected conditions. In addition, Agrobacterium-mediated transient expression of *NLA* in *N. benthamiana* resulted in suppression of bacterial growth. It is worth mentioning that all tested *PR* genes were upregulated in the *NLA* overexpressing plants with *PR1* showing the highest level of induction of about 20-fold increase. Consistent with this finding, ectopic expression of a poplar RING-type E3 ubiquitin ligase gene in tobacco resulted in strong activation of numerous defense related genes with *PR1* being the most strongly upregulated gene (Bopopi *et al.*, 2010). Similarly, activation of *PR* and SA-responsive genes following overexpression of various *ATL* genes also has been documented (Serrano and Guzmán, 2004; Hondo *et al.*, 2007; Liu *et al.*, 2008). Though the mechanisms through which RING-type E3 ubiquitin ligases regulate defense responses are largely unknown, these findings suggest that the mode-of-action may involve stimulation of cellular responses that might be functionally equivalent to that of SA treatment.

It is worth noting that the set of results reported here is inconsistent with the finding of Yaeno and Iba (2008), who suggested that NLA functions as a negative regulator of immune response based on the analysis of the *benzoic acid hypersensitive1-Dominant* (*bah1-D*) mutant, an allele to the *nla* mutant. One possible explanation is that ubiquitination of target proteins for degradation by the ubiquitin-26S proteasome is a fundamental cellular process required to maintain optimal cellular functions and that interrupting NLA-mediating ubiquitination of target proteins by repressing *NLA* expression may also activate immune responses but to a much lesser extent

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compared to *NLA* overexpression lines. In agreement with this explanation we found that under non-infected conditions the expression of *PR1* and *PR5* was induced about 2.0- and 1.7-fold, respectively in the *nla-3* plants, compared to 20 and 4.5 fold in the *NLA* overexpressing plants (Figure 6a).

NLA encodes a protein containing an N-terminal SPX domain and a C-terminal RING domain and its ubiquitin E3 ligase activity has been recently confirmed both *in vitro* and *in planta* (Park *et al.,* 2014a). The RING domain of NLA was found to be involved in homodimerization, which is necessary for its ligase activity (Park *et al.,* 2014a). Though the functional role of the SPX domain in plants is unknown, it may act as a binding site for substrate proteins (Lin *et al.* 2013). Using the wild-type variant of NLA as bait in Y2H screens, we identified UBC8 and UBC10 as the only interactors. NLA was previously reported to interact with UBC8 (Peng *et al.,* 2007) and UBC24 (Park *et al.,* 2014a), suggesting that NLA associates with different E2 conjugases to perform cell- or tissue-specific functions. However, none of these E2 conjugases were found among the syncytium differentially expressed genes. Considering the fact that the Arabidopsis genome contains 37 ubiquitin conjugating genes (Kraft et al., 2005), many of which are regulated by nematode infection (Szakasits *et al.,* 2009), it will be of interest to determine which of these enzymes partner with NLA in the syncytium to modify target proteins.

Efforts to identify substrates for ubiquitin E3 ligases are frequently unsuccessful. This is likely due to the transient nature of the interactions of the ubiquitin E3 ligases with their substrates and their requirement to form dimers. We overcame these limitations by generating a modified NLA bait construct in which the C- terminal region containing the RING domain was fused to the N-terminus allowing for dimerization. In addition, the RING domain activity in this construct was disrupted by mutating the conserved cysteine residue at position 279, as previously described by Lin *et al.* (2013). As a result, a high number of interacting proteins were identified in the Y2H screens. The putative NLA-interacting proteins are involved in a wide range of biological processes and molecular functions, consistent with the role of RING domain-containing E3 ligase proteins in plant growth and development as well as biotic and abiotic interactions (Craig *et al.,* 2009; Dreher and Callis 2007; Schwechheimer *et al.,* 2009; Marino *et al.,* 2012; Stone 2014). Taking into consideration that *NLA* is regulated by miR827 in the syncytium, we reasoned that putative NLA-interacting clones with roles in nematode parasitism should change expression

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in the syncytium. Interestingly, out of the 51 NLA-interacting clones, 31 were found among the syncytium differentially expressed genes reported by Szakasits *et al.* (2009). Of particular note are those involved in defense responses, including the PR proteins thaumatin, PR4, and beta-1,3-endoglucanase. NLA-mediated activation of defense response may be directly linked to its physical association with these PR proteins. Nevertheless, it is unlikely that the interaction of NLA with these PR proteins mediates their degradation in the syncytium. NLA may impact their stability, protein interactions, or cellular localization. Among these putative interactors, beta-1,3-endoglucanase was recently found to be targeted by the cyst nematode 30C02 effector (Hamamouch *et al.*, 2012). Also, we found several transcription factors among the NLA-interacting clones that are differentially expressed in the syncytium. NLA may inhibit the transcription activity of these transcription factors by targeting them for degradation in the syncytium. Also, NLA may target negative regulators or suppressors of defense responses to activate immune responses. In this context, MKK2 may represent a bona fide target of NLA. In Arabidopsis, the MEKK1-MKK1/MKK2-MPK4 mitogen-activated protein (MAP) signaling cascade negatively regulates immune responses (Pitzschke *et al.*, 2009). Thus, targeting and disrupting the activity of MKK2, a central component of this cascade, is expected to trigger immune responses. Nevertheless, no experimental evidence implicates this MAP signaling cascade in plant-nematode interactions. While we have not established the functions of these putative interactors in plant–nematode interactions, the stringency and specificity of the interactions position these interactors as candidate downstream components for miR827-NLA regulatory system in the nematode feeding sites. Further functional analyses including *in plant* interaction, localization and ubiquitination assays, among others, will be required to confirm the biological significance of these interactions.

Increasing evidence indicates that miRNA genes have broad functions in regulating various developmental and physiological processes and that the same set of miRNA genes can respond to a wide range of biotic and abiotic stresses, demonstrating the complexity of miRNA-mediated gene regulation (Khraiwesh *et al.*, 2012). In this context, miR827 seems to play key roles in various signaling pathways. Detailed genetic and molecular analysis demonstrated the implication of the miR827-NLA regulatory loop in nitrate starvation responses (Peng *et al.*, 2007), maintaining phosphate homoeostasis (Kant *et al.*, 2011; Lin *et al.*, 2013, Park *et al.*, 2014a), and immune responses (this study). In addition, a role of this regulatory system in the

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modulation of nitrogen and phosphorus status in the syncytium can be postulated. Further identification and functional characterization of NLA targeted substrates will provide comprehensive understandings of the biochemical and molecular basis of NLA-mediated biotic and abiotic stress responses.

Experimental procedures

Plant materials and growth conditions

All transgenic lines were generated using Arabidopsis ecotype Col-0 except for the *nla-3* (FLAG_253H09) mutants, which is in the Ws background. Transgenic Arabidopsis lines expressing the *pNLA:GUS* construct (Lin et al., 2013) were kindly provided by Dr. Tzyy-Jen Chiou. Plants were grown in a growth chamber at 24°C under long-day conditions with 16 hours light and 8 hours dark.

Construction of binary vectors and generation of transgenic Arabidopsis plants

The miR827 overexpression construct was generated by cloning a 160-bp genomic fragment containing the precursor of miR827 under the control of 35S promoter in the binary vector pBI121 using the BamHI and SacI sites. The overexpression construct of the wild-type variant of *NLA* was generated by placing the cDNA sequence containing the miR827 binding site under the control of 35S promoter in the same binary vector using BamHI and SalI sites. The miR827-resistant variant of *NLA* was generated by introducing 10 mismatches in the miR827-binding sites. For overexpression of a target mimicry of miR827 (MIM827), the binding site of miR399 in the non–protein coding gene *IPS1* (INDUCED BY PHOSPHATE STARVATION1) from Arabidopsis was replaced by mimic sequences for miR827 (MIM827) using PCR. The mimic sequences are complementary to miR827 sequences and contained a mismatched loop (AGG) between the nucleotide number 12 and 13, in addition to two mismatches in nucleotide number 1 and 13 in the pairing region. The modified *IPS1* gene was cloned in the binary vector pBI121 using BamHI and SacI sites. All PCR amplification was carried out using the high-fidelity primeSTAR GXL DNA polymerase (Takara) following the manufacturer's instructions

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For the miR827 promoter construct, a 1342-bp genomic fragment upstream of the precursor sequence of miR827 was amplified from Arabidopsis genomic DNA using oligonucleotide primers containing SalI and BamHI restriction sites in the forward and reverse primers, respectively. For the *NLA* promoter construct (*pNLA*$_{miR827}$*:GUS*) a 2214-bp genomic fragment upstream of the translation start codon (ATG) was amplified using oligonucleotide primers containing SalI and BamHI restriction sites in the forward and reverse primers, respectively. The PCR products were cloned into the corresponding restriction sites of binary vector pBI101. All constructs were verified by sequencing and then introduced into *Agrobacterium tumefaciens* strain C58 and used for genetic transformation of Arabidopsis ecotype Col-0 using floral dip method (Clough and Bent, 1998). Transgenic lines were identified by screening on Murashige and Skoog medium containing kanamycin (50 mg/L). The primer sequences used for plasmid construction are provided in Table S2.

Histochemical staining of GUS activity

The histochemical analysis of GUS activity was performed as previously described by Jefferson *et al.* (1987). The specific promoter activity of the transgenic lines was observed both in non-infected and in *Heterodera schachtii*–infected plants at different time points.

Nematode Infection Assay

Nematode infection assays were conducted as described by Hewezi *et al.* (2015) with 20 replicates per line. The number of J4 females was used as a measurement to assess plant susceptibility. Statistically significant differences between the transgenic lines and wild-type controls were computed using a modified t-test with a *P* value less than 0.05.

Root length measurements

Arabidopsis seeds were planted in 4-well tissue culture plates (BD Biosciences) containing modified Knop's medium and grown vertically. Ten days after planting, the root length of at least 30 plants per line was measured as the distance between the crown and the tip of the main root in three independent experiments. Statistically significant differences between mutant lines and the corresponding wild-type were determined by unadjusted paired *t* tests ($P < 0.01$).

RNA isolation and qPCR analysis

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For assaying the expression level of miR827, total RNA was isolated from the roots of two-week-old plants with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNase-treated total RNA (10 µg) was polyadenylated and reverse transcribed using the Mir-X miRNA First-Strand Synthesis Kit (Clontech) according the manufacturer's instructions. The synthesized cDNAs then were diluted to a concentration equal to 50 ng total RNA/µl and used as a template in qPCR reactions to quantify the expression levels of mature miR827. PCR was performed using a universal reverse primer (mRQ; supplied with the Mir-X miRNA First-Strand Synthesis Kit), complementary to the poly(T) and the mature miR827 sequences as specific forward primer. The miR827-specific forward primer was extended by two adenine residues on the 3' end to ensure the binding to the poly(T) region of the mature miRNA cDNA and to prevent its hybridization to the miRNA precursor cDNA, as previously described by Hewezi *et al.* (2012). The PCR was run in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using the following program: 95°C for 3 min, and 40 cycles of 95°C for 30 s, and 60°C for 30 s. The U6 small nuclear RNA was used as an internal control to normalize the expression levels of mature miR827. For quantification of the expression levels of *NLA* and *PR* genes in overexpression and mutant lines, total RNA was isolated from root tissues of these lines and wild-type plants. For quantification of the expression levels of cleaved and total *NLA* transcripts in wild-type (Col-0) plants post *H. schachtii* infection, RNA was isolated form the root tissues at 4 and 8 dpi from both infected and non-infected plants. One primer pair flanking the miR827 cleavage site was used to quantify the abundance of uncleaved *NLA* transcripts. Another primer pair designed downstream of the cleavage site was used to quantify the abundance of total (cleaved and uncleaved) *NLA* transcripts. Total RNA was treated with DNase I (Invitrogen) and 10 ng of DNase-treated RNA were used for cDNA synthesis and PCR amplification using the Verso SYBR Green One-Step qRT-PCR Kit (Thermo Scientific) according to the manufacturer's instructions. *Actin8* (AT1G49240) was used as an internal control to normalize the level of mRNA. The specificity of each amplification reaction was verified using a heat dissociation curve (from 55 to 95°C). Normalization and quantification of relative gene expression values were computed as described in Hewezi *et al.* (2015). Primers used in qPCR assays are listed in Table S2.

Agrobacterium- mediated transient expression in *N. benthamiana*

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The binary plasmids for overexpressing miR827, MIM827 and *NLA* were introduced into *A. tumefaciens* strain GV3101. Agrobacteria were grown overnight on Luria agar medium containing the appropriate antibiotics at 28°C. Bacteria were collected, resuspended in inoculation medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone; Acros Organics), and then incubated for 3 h at room temperature before inoculation. Leaves of 5-week-old *N. benthamiana* plant were hand-inoculated with bacterial suspension at a concentration of 2×10^8 cells/mL using a needless syringe. After 24 h, inoculated patches were challenged by non-phytopathogen bacteria *Pseudomonas fluorescens* strain EtHAn with a concentration of 2×10^6 cfu/mL in 10 mM $MgCl_2$. Leaves of the same age on the same branch were used for each experimental treatment. Plants were kept under 16-h light/day at 22°C. For each treatment, four leaf discs (0.5 cm^2) were collected from one leaf at 0 and 3 dpi, pooled, ground in 10 mM $MgCl_2$, and then spotted on LB plates containing the appropriate antibiotics for EtHAn in triplicate to determine the bacterial load in each inoculated leaf. Three biological replicates (i.e., three plants) were used, and the experiment was repeated three times.

Yeast two-hybrid screens

Bait plasmid construction and yeast two-hybrid screens were performed as described in the BD Matchmaker Library Construction and Screening Kits user manual (Clontech). The coding sequence of the wild type and modified *NLA* sequences ($^{RING}NLA^{C279A}$) was amplified and cloned into pGBKT7 bait vector using *Eco*RI and *Pst*I sites, to generate pGBKT7-NLA and pGBKT7-$^{RING}NLA^{C279A}$ bait constructs, respectively. The yeast (*Saccharomyces cerevisiae*) strain Y187 was then transformed with the bait constructs to create the bait strains. Screening for putative interactors was performed using three prey libraries prepared from Arabidopsis roots inoculated with *H. schachtii* and generated in yeast strain AH109 (Hewezi *et al.*, 2008b). Positive clones were selected on the synthetic dropout (SD/-Leu/-Trp/-Ade/-His) medium and by visualizing the activity of *MEL1* reporter gene. Specificity of the interaction was performed using yeast co-transformation assays as described by Clontech protocols. Primers used for cloning are listed in Table S2.

Accession numbers

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Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR) database under the following accession numbers: miR827 (AT3G59884), *NLA* (AT1G02860), *PR1* (AT2G14610), *PR3* (AT3G12500) *PR4* (AT3G04720), *PR5* (AT1G75040), *PDF1.2* (AT5G44420), *PAD4* (AT3g52430), *EDS1* (AT3G48090), and *Actin8* (AT1G49240).

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Author contributions

T.H. and T.J.B. conceived and designed the experiments. T.H., S.P., M.Q., M.B., and J.H.R. performed the experiments. T.H., S.P., and M.B. analyzed the data. T.H. wrote the article.

Short legends for Supporting Information

Figure S1. GUS activity of the miR827 promoter in non-infected 2-week-old plants under Pi-sufficient conditions.

Figure S2. GUS activity of transgenic plants expressing *pNLA:GUS* and *pNLA*$_{miR827}$*:GUS* constructs in non-infected 2-week-old plants under Pi-sufficient conditions.

Figure S3. qPCR quantification of *H. schachtii*–induced posttranscriptional degradation of *NLA* transcripts in wild-type plants.

Figure S4. Root phenotypes of *35S:MIM827*, *nla-3*, *35S:wtNLA*, and *35S:rNLA* plants.

Figure S5. Quantification of *NLA* expression levels in overexpression lines.

Table S1. List of 51 NLA-interacting clones identified by Y2H screens.

Table S2. Primers sequences used in the current study.

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Figure legends

Figure 1. Upregulation miR827 in the *Heterodera schachtii* – induced syncytia.
(a) Histochemical localization of GUS activity of *PmiR827:GUS* plants in the initial feeding cells at 1 dpi.
(b, c, d) Histochemical localization of GUS activity of *PmiR827:GUS* plants in the developing syncytia at 2, 3 and 4 dpi, respectively.
(e, f) Histochemical localization of GUS activity of *PmiR827:GUS* plants in the fully developed syncytia at 7 and 10 dpi, respectively.
(g, h) Histochemical localization of GUS activity of *PmiR827:GUS* plants in the syncytia of J4 females and males, respectively. Arrows point to the initial feeding cells in (a) and to developing syncytia in (b, c, and d). N indicates nematode, and S indicates syncytium. Bars = 100 μm

Figure 2. Post-transcriptional silencing of *NLA* gene by miR827 in the syncytium.
(a)–(d) Strong GUS activity of transgenic lines expressing the *pNLA:GUS* construct was detected in the syncytia induced by *H. schachtii* at J2 (a), early J3 (b), late J3 (c), and J4 stages (d).
(e)–(h) GUS activity of the transgenic lines expressing $pNLA_{miR827}$*:GUS* construct indicated robust downregulation of *NLA* in the syncytia throughout J2 (e), early J3 (f), late J3 (g), and J4 (f) stages of nematode infection. N indicates nematode, and S indicates syncytium. Bars = 100 μm.

Figure 3. Overexpression of miR827 in Arabidopsis altered plant morphology and increased plant susceptibility to *H. schachtii*.

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(a, b) Phenotypes of three-week-old transgenic plants overexpressing miR827 showing developmental defects in leaves and reduced plant size (a) and normal roots (b). Data of root lengths are presented as means ± SE (n = 30).

(c) Transgenic Arabidopsis lines overexpressing miR827 showed significant downregulation of *NLA* transcripts. Expression level of *NLA* was quantified in root tissues by qPCR using *Actin8* as internal control. Data are average of three biological samples, each with four technical replicates ± SE.

(d) Transgenic Arabidopsis lines overexpressing miR827 showed hyper-susceptibility to *H. schachtii*. Nematode susceptibility assays were repeated at least three times, each with 20 replications and similar results were obtained. Mean values significantly different from the wild-type Col-0 were determined by paired t tests ($P < 0.05$) and are indicated by asterisks.

Figure 4. Inactivation of miR827 expression altered plant morphology and reduced plant susceptibility to *H. schachtii*.

(a) Design strategy for modifying the miR399 complementary sequence in the IPS1 (*INDUCED BY PHOSPHATE STARVATION1*) gene into a mimic sequence for miR827 (MIM827).

(b) Phenotypes of three-week-old transgenic plants overexpressing MIM827 showing developmental defects in leaves and reduced plant size.

(c) Downregulation of mature miR827 in the transgenic Arabidopsis lines overexpressing MIM827. Expression levels of mature miR827 were measured in root tissues by qPCR using U6 as internal control. Data are average of three biological samples, each with four technical replicates ± SE.

(d) Transgenic Arabidopsis lines overexpressing MIM827 showed reduced susceptibility to *H. schachtii*. Mean values significantly different from the wild-type Col-0 were determined using unadjusted paired t tests ($P < 0.05$) and are indicated by asterisks. Nematode susceptibility assays were repeated at least three times, each with 20 replications and similar results were obtained. Data from one representative experiment are shown.

Figure 5. NLA regulates Arabidopsis susceptibility to *H. schachtii*.

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(a)–(c) Phenotypes of three-week-old *nla-3* mutant (a), transgenic plants overexpressing the wild-type mRNA of *NLA* (*P35S:wtNLA*) (b) or a miR827-resistant non-cleavable mRNA variant (*P35S:rNLA*) (c).

(d)–(f) Nematode susceptibility assays of *nla-3* mutant, and transgenic plants overexpressing *P35S:wtNLA* or *P35S:rNLA* constructs. The *nla-3* mutant phenocopied miR827 overexpression plants of increased susceptibility to *H. schachtii* (d). Transgenic lines overexpressing *P35S:wtNLA* construct did not significantly impact plant susceptibility to *H. schachtii* (e). In contrast, transgenic lines overexpression *P35S:rNLA* construct exhibited significant decreases in plant susceptibility to *H. schachtii* (f). Data are presented as means ± SE (n = 20). Mean values significantly different from the wild-type Col-0 or Ws were determined using unadjusted paired t tests ($P < 0.05$) and are indicated by asterisks. Nematode susceptibility assays were repeated at least three times and similar results were obtained. Data from one representative experiment are shown.

Figure 6. miR827–NLA regulatory module impaired plant immune system.

(a) qPCR quantification of the indicated PR genes in transgenic plants overexpressing the *P35S:rNLA* construct (line 6-4) and in the *nla-3* knockout mutant.

(b) qPCR quantification of the indicated PR genes in transgenic plants overexpressing the *P35S:miR827* construct (line 18-9) or the *P35S:wtNLA* construct (line 13-2). The expression levels were quantified in the root tissues of these lines relative to the corresponding wild-type plants using *Actin8* as internal control. Data are the average of three biological samples, each consisting of four technical replicates ± SE.

(c) Overexpression of miR827 promoted bacterial survival in planta. Binary plasmids for overexpressing miR827, MIM827 and *NLA* were introduced into *A. tumefaciens* strain GV3101 and used for transient expression in *N. benthamiana* leaves by Agro-infiltration, one day prior to inoculation with *P. fluorescens* strain EtHAn. *A. tumefaciens* strain GV3101 transformed with empty vector was used as a negative control. EtHAn bacteria were quantified at day 0 and 3. Data represent the mean values of the numbers of colony-forming unit (CFU) per cm^2 ± SE of

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three biological samples. The experiment was repeated three times and similar results were obtained.

Figure 7. Identification of putative NLA-interacting proteins.

(a) NLA interacts specifically with UBC8 and UCB10 in yeast. Yeast competent cells (strain AH109) were transformed individually with UBC8 and UCB10 prey plasmids in combination with the NLA bait vector, the empty bait vector, or bait vector containing the human Lamin C gene to test for interaction specificity. Yeast co-transformant cells were plated on the non-selective SD/-Leu/-Trp medium (left panel) and on the selective SD/-Leu/-Trp/-Ade/-His medium (right panel). The specificity of the interactions was visualized by differential growth of yeast cells containing NLA bait and UBC8 and UCB10 prey plasmids on the selective medium, whereas yeast cells containing the prey plasmids along with the empty bait vector or Lamin C bait failed to grow on the selective medium.

(b) Schematic representation of the $^{RING}NLA^{C279A}$ bait used in Y2H screens.

(c) Interactions between $^{RING}NLA^{C279A}$ (bait) and the indicated Arabidopsis proteins (preys) in yeast co-transformation assay. Interaction specificity was tested as indicated in (a) and observed by differential yeast growth on the selective medium.

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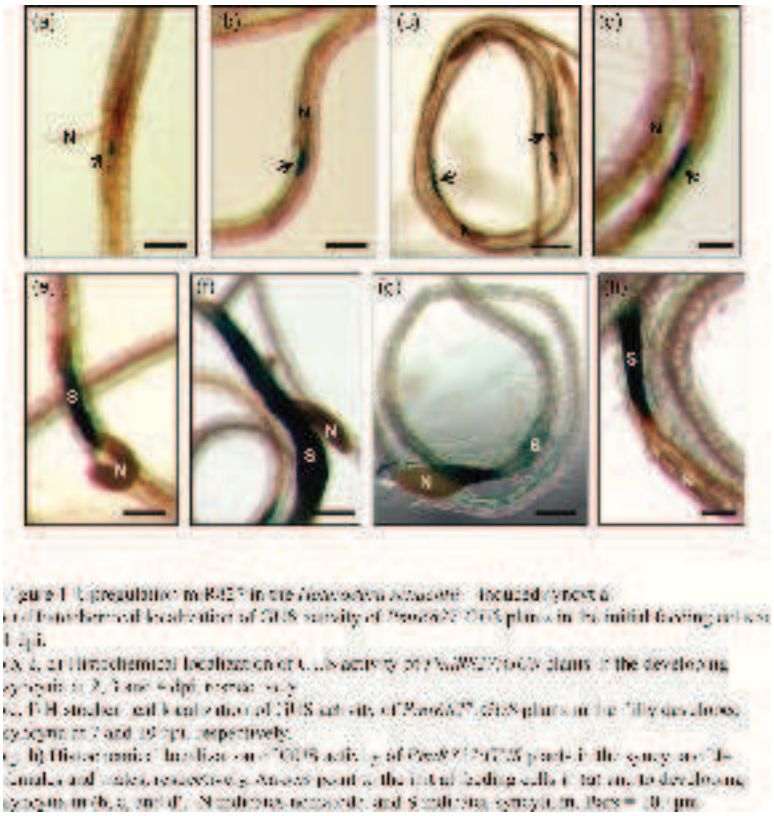

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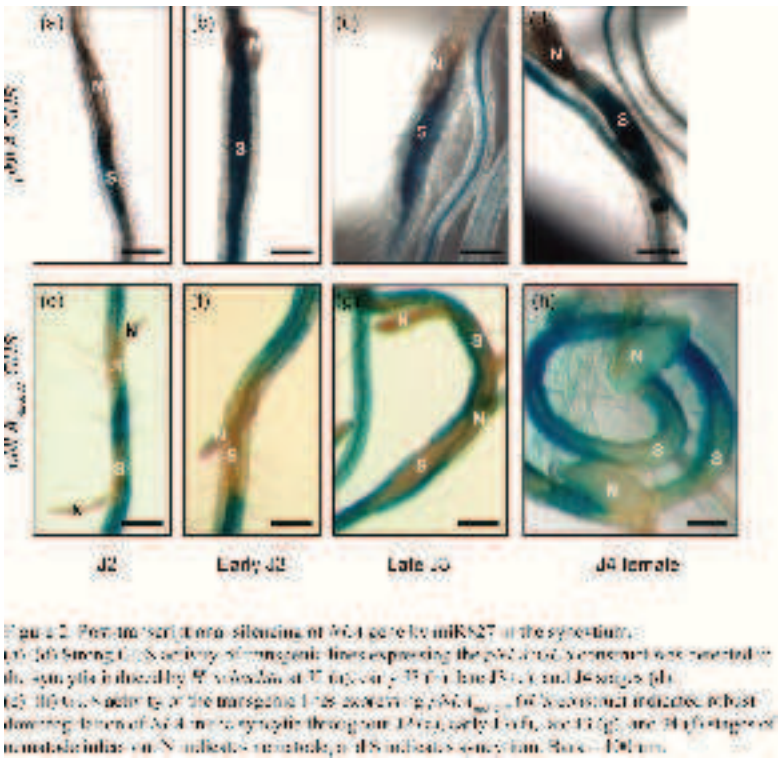

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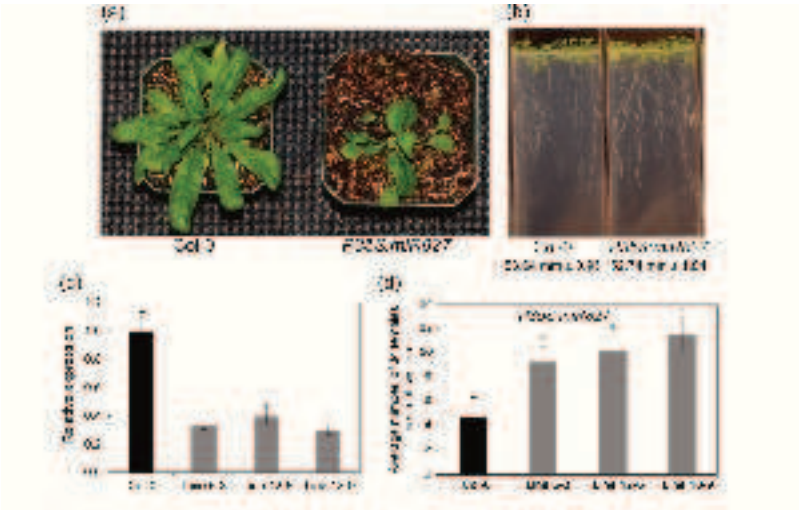

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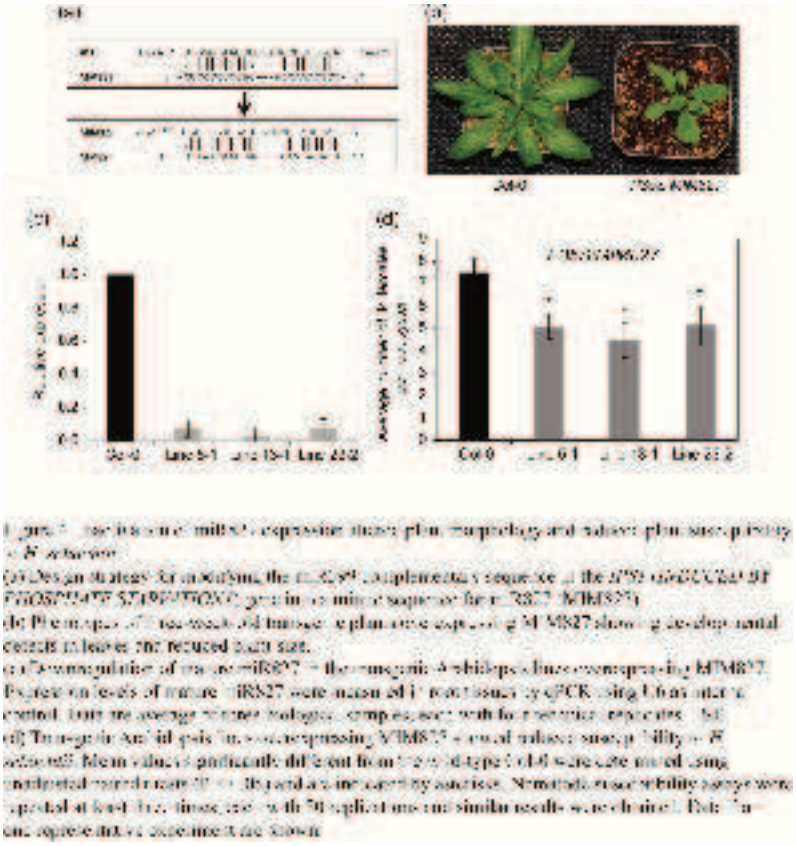

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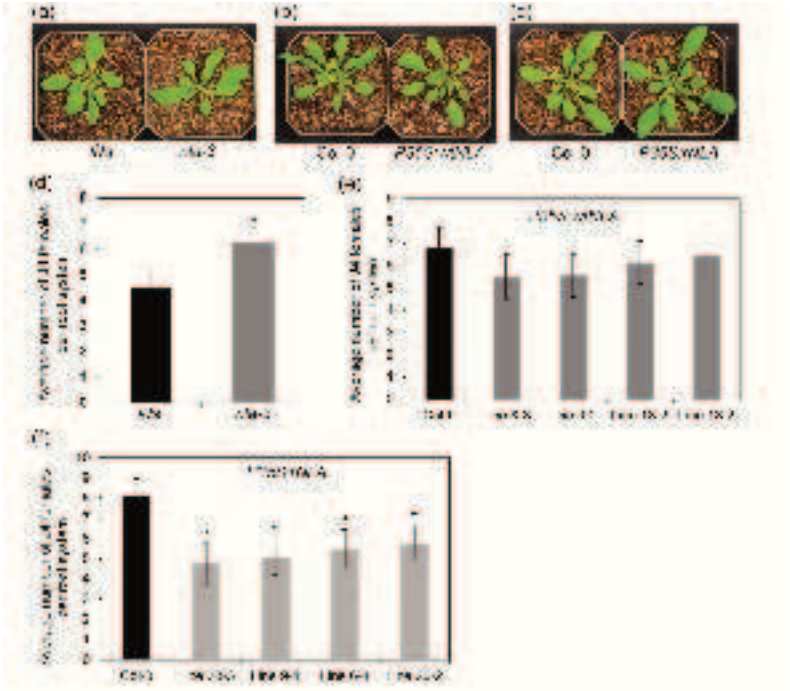

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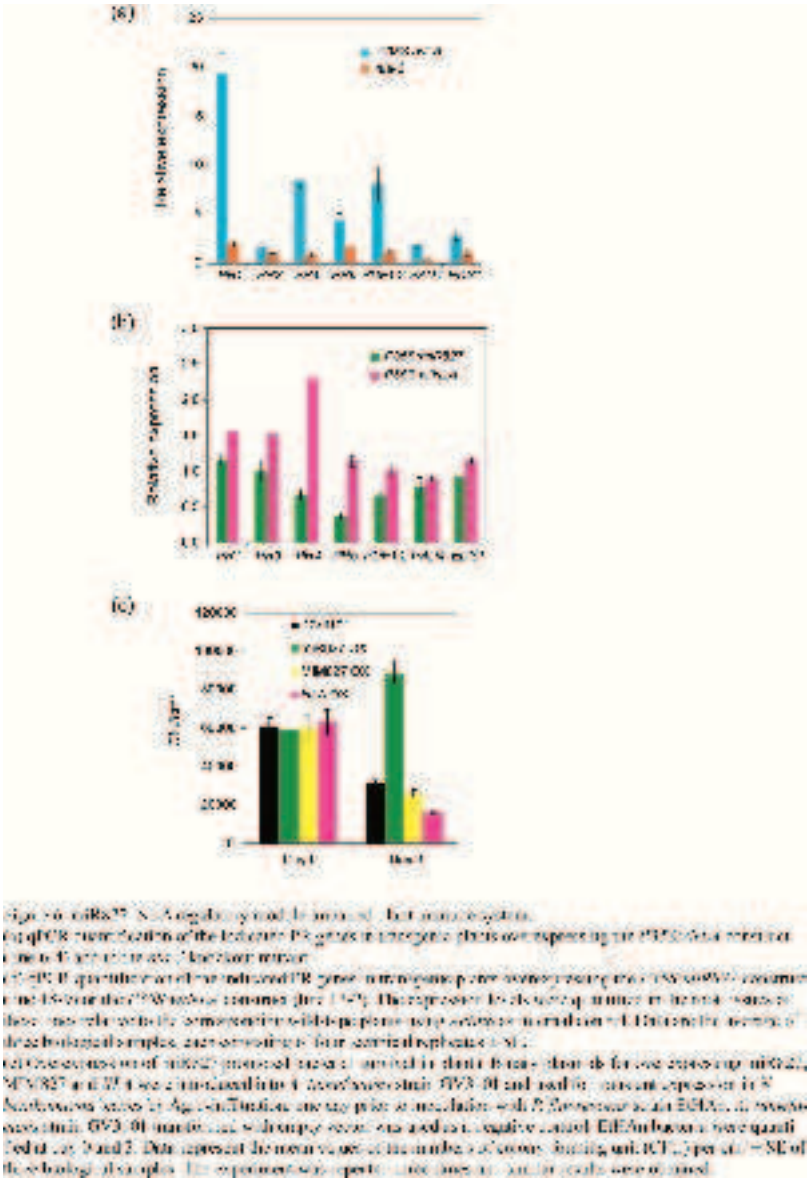

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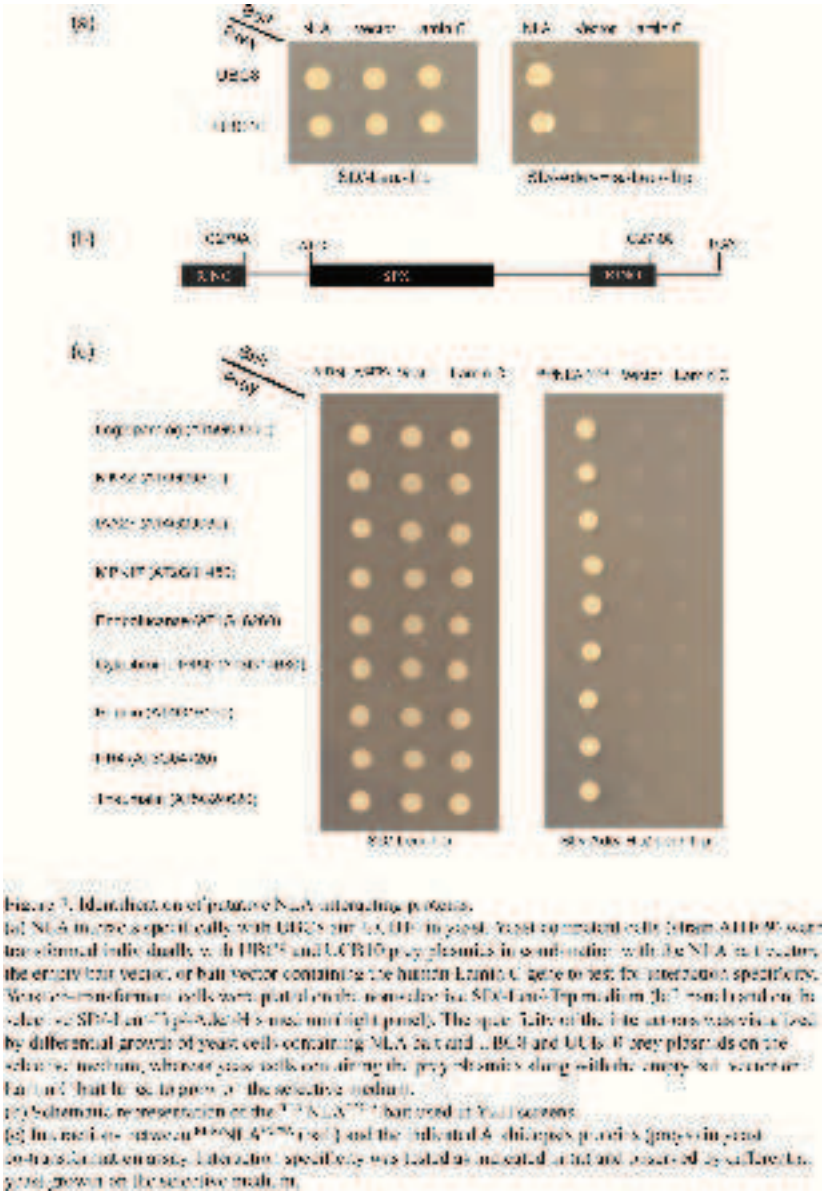

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