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Loss-of-function of *NITROGEN LIMITATION ADAPTATION* confers disease resistance in Arabidopsis by modulating hormone signaling and camalexin content

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ABSTRACT

Phosphorus is an important macronutrient required for plant growth and development. It is absorbed by the roots in the form of inorganic phosphate (Pi). Under Pi limiting conditions, plants activate the Phosphate Starvation Response (PSR) system to enhance Pi acquisition. The *NITROGEN LIMITATION ADAPTION* (*NLA*) gene is a component of the Arabidopsis PSR, and its expression is post-transcriptionally regulated by miR827. We show that loss-of-function of *NLA* and *MIR827* overexpression increases Pi level and enhances resistance to infection by the fungal pathogen *Plectosphaerella cucumerina* in Arabidopsis. Upon pathogen infection, high Pi plants (*e.g. nla* plants and wild type plants grown under high Pi supply) showed enhanced callose deposition. High Pi plants also exhibited superinduction of camalexin biosynthesis genes which is consistent with increased levels of camalexin during pathogen infection. Pathogen infection and treatment with fungal elicitors, triggered up-regulation of *MIR827* and down-regulation of *NLA* expression. Under non-infection conditions, the *nla* plants showed increased levels of SA and JA compared with wild type plants, their levels further increasing upon pathogen infection. Overall, the outcomes of this study suggest that *NLA* plays a role in Arabidopsis immunity, while supporting convergence between Pi signaling and immune signaling in Arabidopsis.

1. Introduction

Phosphorous (P) is one of the most important macronutrients required for plant growth and development. It is a key component of fundamental biomolecules, such as nucleic acids phospholipids and ATP, the principal molecule for storing and transferring energy in cells. P is also a central regulator in numerous metabolic reactions, including signaling pathways and protein activity modulation. Plants absorb phosphorus from the soil in the form of inorganic form (Pi). Although P levels in soils are usually high, its bioavailability is often extremely low which represents a limiting factor for plant growth.

To cope with Pi limitation, plants have evolved multiple molecular mechanisms that allow the plant to enhance the acquisition of Pi from the soil. This includes the activation of the Pi starvation response (PSR) leading to the induction of phosphate transporter genes of the *PHT1* family (Yang et al., 2021; Paz-Ares et al., 2022). In Arabidopsis, the transcription factor PHR1 (PHOSPHATE STARVATION RESPONSE 1) acts in the Pi starvation signaling pathway by regulating a group of Pi starvation response genes by binding to a conserved *cis*-element, the P1BS (PHR1 specific binding sequence element) which is present in the promoter regions of many Pi starvation-induced genes (Rubio et al., 2001). The involvement of two microRNAs (miRNAs), namely miR399 and miR827, in the Arabidospsis PSR is well documented (Delhaize and Randall, 1995; Chiou et al., 2006; Lin et al., 2013). MiR399 down-regulates *PHOSPHATE 2* (*PHO2*, encoding an E2 ubiquitin conjugase), while miR827 negatively regulates *NITROGEN LIMITATION*

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ADAPTATION (*NLA*, encoding an E3 ubiquitin ligase). PHO2 and NLA mediate the ubiquitination and degradation of the plasma-membrane-localized PHT1 transporters. In this way, miRNA-mediated down-regulation of *PHO2* and *NLA* relieves the negative regulation over PHT1 transporters, thus, increasing Pi uptake in the roots (Bari et al., 2006; Kant et al., 2011). Whereas considerable progress has been made in characterizing the regulatory mechanisms that underlie adaptation of plants to Pi limiting conditions, less is known about adaptive mechanisms to Pi excess condition.

The plant immune system consists of interconnected processes that are induced upon recognition of the pathogen. Depending on the molecules that are recognized by the host plant, two immune systems have been defined. Plants recognize pathogen epitopes, known as Pathogen-Associated Molecular Patterns (PAMPs, also known as elicitors). This recognition activates a general defense response referred to as PAMP-triggered immunity (PTI) leading to the induction of defense-related genes, including *Pathogenesis-Related* (*PR*) genes (Jones and Dangl, 2006; Boller and Felix, 2009; Li et al., 2020). Pathogens adapted to their host have evolved effectors that are delivered to plant cells and suppress PTI leading to disease susceptibility. In turn, plants have evolved another immune system in which pathogen effectors, or host proteins modified by these effectors, are recognized by proteins encoded by resistance (*R*) genes, called Effector-triggered Immunity (ETI). Whereas PTI contributes to resistance to diverse pathogens, ETI is pathogen strain or race specific. Phytohormones also play an essential role in the regulation of plant immune responses. The involvement of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in the regulation of defense responses to pathogens in plants has long been recognized (Denancé et al., 2013; Berens et al., 2017; Aerts et al., 2021). Synergistic and antagonistic interactions between hormone signaling pathways allow fine-tuned responses to different pathogens.

Phytoalexins are low molecular weight antimicrobial compounds that also take part in the defense system used by plants against pathogens (Ahuja et al., 2012). The major phytoalexin in Arabidopsis is camalexin, a tryptophan-derived secondary metabolite, and its accumulation has been correlated with resistance to necrotrophic fungi such as *Plectosphaerella cucumerina*, *Botrytis cinerea* and *Alternaria brassicicola* (Simone et al., 2003; Majse et al., 2007; Sanchez-Vallet et al., 2010).

Increasing evidence supports that PSR modulates the Arabidopsis immune system (Castrillo et al., 2017; Chan et al., 2021). Under low Pi conditions, PHR1 was found to negatively regulate defense responses, which correlated with enhanced resistance of *phr1* mutants to infection by bacterial and oomycete pathogens (*P. syringae* DC3000 and *Hyaloperonospora arabidopsidis*, respectively) (Castrillo et al., 2017). PHR1, however, does not necessarily compromise the entire immune system as immune responses are still activated by certain pathogens, even in low Pi conditions (Hiruma et al., 2016). In other studies, transgenic expression of a phytoplasma effector (SAP11) in Arabidopsis was found to trigger Pi starvation responses and subsequent Pi accumulation (Lu et al., 2014). The SAP11 transgenic plants were more susceptible to *P. syringae* infection (Lu et al., 2014). On the other hand, Pi accumulation caused by high Pi fertilization or miR399 overexpression in transgenic rice was found to compromise expression of the defense-related genes, thus, resulting in a phenotype of susceptibility to infection by the blast fungus *Magnaporthe oryzae* (Campos-Soriano et al., 2020). Contrary to this, we recently reported that Pi accumulation promotes resistance to infection by fungal pathogens in Arabidopsis (Val-Torregrosa et al., 2022). Thus, the impact of Pi content on disease resistance might differ depending on the interacting partners. Although adaptation to Pi supply conditions and immunity appear not to be independent processes, the molecular mechanisms by which Pi signaling and immune signaling interact each other still remain unclear.

NLA was originally described as a positive regulator in the adaptive response of Arabidopsis plants to nitrogen (N) limitation (Peng et al., 2007). Nowadays, it is widely recognized that *NLA* also plays a pivotal role in the regulation of Pi homeostasis (Lin et al., 2013; Park et al., 2014). *NLA* encodes a RING-type ubiquitin ligase responsible of degradation of PHT1 transporters *via* ubiquitination (Kant et al., 2011; Hsieh et al., 2009). Suppression of *NLA* by the Pi-starvation inducible miR827 relieves its negative regulation of PHT1, resulting in increased Pi uptake.

The objective of this work was to investigate whether *NLA* plays a role in disease resistance in Arabidopsis. We show loss-of-function of *nla*, as well as *MIR827* overexpression, is accompanied by an increase in Pi content and confers resistance to infection by fungal pathogens. Transcriptional activation of *MIR827,* and subsequent down-regulation of *NLA*, occurs during pathogen infection as well as in response to treatment with fungal elicitors. Compared with wild type plants, *nla* mutant plants showed higher deposition of callose. We also show that loss-of-function of *NLA*, as well as growing Arabidopsis plants under high Pi supply, promotes the accumulation of camalexin. Resistance to pathogen infection in *nla* plants is also associated with modulation of the SA- and JA-dependent defense pathways. Overall, the results presented here suggest that *NLA* regulates Arabidopsis immunity.

2. Materials and methods

2.1. Plant material infection assays and elicitor treatment

Arabidopsis thaliana (ecotype Col 0) were grown under a 12 h light/12 h dark photoperiod and 60 % humidity a temperature of 22 °C ± 2 °C. The *nla* mutant and miR827 OE plants used in this work were previously described (Lin et al., 2013; Kant et al., 2011; Peng et al., 2007), for *nla*; (Kant et al., 2011), for miR827 OE). For disease resistance assays and elicitor treatment experiments, Arabidopsis plants were grown in a mixture of soil:perlite:vermiculite (2:1:1) and modified Hoagland half strength medium, under neutral photoperiod (12 h light / 12 h dark), 60 % of humidity and a temperature of 22ºC ± 2ºC for three weeks. The fungus *P. cucumerina* was grown on PDA (Potato Dextrose Agar) plates with chloramphenicol (34 μg/ml). *Colletotrichum higginsianum* was grown on Oatmeal agar plates in darkness. Fungal spores were collected by adding sterile water to the surface of the mycelium, and adjusted to the desired final concentration using a Bürker counting chamber. Plants were spray-inoculated with a spore suspension of *P. cucumerina* (5×10^5 spores/ml), or mock-inoculated. *C. higginsianum* was locally-inoculated with a spore suspension at 4×10^6 spores/ml (10 μl/leaf and 5 leaves/plant). Fungal-inoculated and mock-inoculated plants were maintained under high humidity and disease symptom development was followed with time. Lesion area was measured with software ImageJ (National Institute of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/). Three independent experiments were performed with at least 12 plants per genotype in each experiment. Statistically significant differences were determined by t-test. For *in vitro* experiments, two-week old Arabidopsis plants were spray-inoculated with *P.cucumerina* (4×10^6 spores/ml). Fungal biomass was quantified by real-time PCR using specific primers for the corresponding fungus and the Arabidopsis *UBIQUITIN21* (At5g25760) gene as the internal control (Soto-Suárez et al., 2017). PCR primers are listed in Suppl. Table S1. Elicitor treatment was performed by spraying plants with an elicitor extract obtained from *P. cucumerina* (300 μg ml^{-1}) as described (Casacuberta et al., 1992).

For Pi treatment experiments, plants were grown *in vitro* on meshes placed on agar plates with modified Hoagland half strength medium containing 0.25 mM KH_2PO_4 for one week. Seedlings were then transferred to fresh agar-medium at the desired concentration of Pi (0.05, 0.25, or 2 mM Pi). The plants were allowed to continue growing for one more week under each Pi regime. The *in vitro*-grown plants were then inoculated with a spore suspension of *P. cucumerina*, or mock-inoculated.

2.2. Plant tissue staining

For trypan blue staining, leaves were fixed by vacuum infiltration for

1 h in ethanol:formaldehyde:acetic acid (80:3.5:5 v/v), stained with lactophenol blue solution for one hour, and then washed with chloral hydrate for 15 min. Leaves were placed on glass slides with glycerol and observed with a Leica DM6 microscope under bright field.

Aniline blue staining was used to determine callose deposition. For this, chlorophyll was removed, with 70 % ethanol, from leaves that were then incubated in 70 mM phosphate buffer (pH 9.0) supplemented with aniline blue (0.01 % wt/vol) with vacuum for 30 min. Samples were maintained in dark conditions for 2 h. Leaves were observed with Leica DM6 microscope under UV illumination. Callose deposition was quantified by determining the relative number of callose-corresponding pixels on digital photographs relative to the total number of pixels using ImageJ software (Luna et al., 2011).

For H_2DCFDA staining, the Arabidopsis leaves were placed on a solution of H_2DCFDA (at a concentration of 10 μM), vacuum infiltrated during 5 min, and then maintained in darkness for 10 min. Two washes with distillated water were performed. Photographs were taken on a Leica DM6 microscope to visualize green fluorescence. DCFDA staining was quantified by determining the relative number of DCFDA-corresponding pixels relative to the total number of pixels using ImageJ software.

2.3. Agrobacterium-mediated transformation of Arabidopsis

To obtain the *MIR827promoter:GUS* construct, the DNA sequence of the *MIR827* promoter region was extracted from the NCBI (http://www.ncbi.nlm.nih.gov). The transcription start site was identified by using the transcription start site identification program for plants (http://linux1.softberry.com/) and *cis* elements were determined using the PLACE database (https://www.dna.affrc.go.jp/PLACE). The DNA sequence covering 1.2 kb upstream of the transcription start site of *MIR827* was amplified by PCR from genomic DNA, and cloned into the pKGWFS7 plant vector. The PCR product was verified by sequencing. The plant expression vector was transferred to the *Agrobacterium tumefaciens* strain GV3101. Arabidopsis (Col-0) plants were transformed using the floral dip method.

2.4. Analysis of GUS activity

Histochemical staining of GUS enzyme activity was performed according to (Jefferson et al., 1987). Leaves were placed on glass slides with glycerol and observed using a Leica DM6 microscope. Hormone treatment with salicylic acid (SA) was performed in a SA solution of 100 μM during 12 h and darkness.

2.5. Measurements of Pi

The Pi content of Arabidopsis plants was determined as previously described (Versaw and Harrison, 2002). For each experiment four biological replicates (three plants/replicate) were used. Statistical *t* test analysis was used to analyze the data.

2.6. Gene expression analyses

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 μg) with reverse transcriptase and oligo-dT (High Capacity cDNA reverse transcription kit, Applied Biosystems). RT-qPCR was performed in optical 96-well plates using SYBR® green in a Light Cycler 480 (Roche). Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *β-tubulin2* gene (At5g05620) was used to normalize the transcript level in each sample. Primers used for RT-qPCR and stem-loop RT-qPCR are listed in Suppl. Table S1. Accumulation of mature miR827 was determined by stem-loop reverse transcription quantitative PCR (Varkonyi-Gasic et al., 2007). Northern blot analysis of miR827 was carried out at previously described (Campo et al., 2013) using a P^{32}-labeled oligonucleotide complementary to miR827 sequence (indicated in Suppl. Table S1). At least 3 biological replicates were analyzed per genotype and condition, each replicate consisting of leaves from at least 3 independent plants. Two-way analysis of variance (ANOVA) was used to analyze data.

2.7. Determination of hormones and camalexin levels

The rosettes of three-week-old WT (Col-0) and *nla* plants were analyzed by LC-MS for SA, SAG, JA and OPDA content as previously described (Sánchez-Bel et al., 2018). Briefly, 30 mg of freeze dried material was extracted with MeOH:H2O (30:70) containing 0.01 % of HCOOH containing a mixture of 10 ug. L^{-1} of the internal standards salicylic acid-d 5 (SA-d5) and dehydrojasmonic acid (Sigma-Aldrich). Following extraction, samples were centrifuged (15.000 rpm, 15 min) and filtered through regenerated cellulose filters. An aliquot of 20 ul was injected into a UPLC (Waters Aquity) interfaced with a Xevo TQ-S Mass Spectrometer (TQS, Waters). Hormones were quantified by contrasting with an external calibrarion curve of pure chemical standards of SA, SAG, JA and OPDA. Camalexin levels were determined by using 10 μg/L indole acetic acid-d5 (IAA-d5) as internal standard and a curve of the pure chemical standard in a range from 1 to 150 $\mu g.L^{-1}$ were injected under the same chromatographic conditions as the plant samples. Sample separation was performed with a LC Kinetex C18 analytical column of a 5 μm particle size, 2.1 100 mm (Phenomenex). Chromatographic and TQS conditions were performed as described in (Sánchez-Bel et al., 2018). At least 6 biological replicates were analyzed per genotype and condition, each replicate consisting of leaves from at least 3 independent plants. The plant material was lyophilized prior analysis. Two-way analysis of variance (ANOVA) followed by HSD (Honestly-Significant-Difference) Tukey's test was used to analyze data.

3. Results

3.1. Resistance to infection by fungal pathogens in nla and miR827 overexpressor plants

To investigate whether *NLA* plays a role in disease resistance in Arabidopsis, *nla* mutant plants were challenged with the fungal pathogen fungal pathogen *Plectosphaerella cucumerina.* The Arabidopsis/*P. cucumerina* pathosystem is a well-established model for studies on basal resistance to infection by necrotrophic fungi (Sanchez-Vallet et al., 2010). The *nla* mutant used for these studies (*nla-1*, Col-0 background) is a RING-domain deletion mutant in which the third and fourth exon were deleted (Lin et al., 2013; Kant et al., 2011; Peng et al., 2007) (Suppl. Fig. S1A). Compared with wild type plants, the *nla* mutant plants showed down-regulation of *NLA* expression and higher Pi accumulation in leaves (Suppl. Fig. S1B), which is consistent with results previously reported (Lin et al., 2013). In this work we also examined disease resistance in Arabidopsis plants overexpressing *MIR827* (henceforth miR827 OE). As miR827 down-regulates *NLA* expression, the loss-of-function mutation of *nla* is expected to have a disease phenotype similar to that of transgenic lines overexpressing miR827. The production and characterization of miR827 OE plants was previously described (Kant et al., 2011). As expected, miR827 OE plants accumulated pre-miR827 and mature miR827 transcripts (Suppl. Fig. S1C). Compared with wild type plants, the miR827 OE plants showed down-regulation in *NLA* expression and accumulated higher amounts of Pi in leaves (Suppl. Fig. S1D).

Three-week-old plants, a developmental stage in which no phenotypic differences were observed between *nla,* miR827 OE and wild type plants, were inoculated with *P. cucumerina* spores (Suppl. Fig. S2). Adult miR827 OE plants, however, displayed a reduced size compared with wild type plants which is in agreement with results previously reported (Hewezi et al., 2016). To exclude the possibility that differences in development caused by miR827 OE could interfere with disease

resistance assays, all infection experiments were carried out on 3 week-old plants (wild type, miR827 OE, *nla* plants).

Compared with wild type plants, *nla* and miR827 OE plants consistently showed enhanced resistance to infection by *P. cucumerina* (Fig. 1A). Quantification of fungal biomass and determination of plant survival confirmed resistance to *P. cucumerina* in *nla* and miR827 OE plants (Fig. 1B, C). Moreover, trypan blue staining of infected leaves revealed extensive fungal growth in leaves of wild type plants, but not in leaves of *nla* and miR827 OE plants (Fig. 1D). The *nla* plants also exhibited resistance to infection by the hemibiotrophic fungus *Colletotrichum higginsianum*, which was confirmed by quantification of fungal biomass and percentage of diseased leaves (Suppl. Fig. S3). *C. higginsianum* causes the anthracnose leaf spot disease of *Brassica* species, including *A. thaliana* (O'Connell et al., 2004). Subsequent studies in this work were conducted on *nla* mutant plants infected with *P. cucumerina*.

3.2. Callose accumulation in nla plants in response to P. cucumerina infection

Callose deposition is a hallmark of the induction of PTI (Luna et al., 2011; Wang et al., 2021). It functions as a defense mechanism for reinforcing plant cell walls to arrest pathogen infection, as also observed during *P. cucumerina* infection in Arabidopsis. Fungal elicitors are also known to function as potent PAMPs for the induction of callose deposition (Wang et al., 2021).

In this work, aniline blue was used to visualize callose accumulation in leaves of wild type and *nla* plants that have been inoculated with *P. cucumerina* spores, or mock-inoculated. Neither wild type nor *nla* plants showed callose deposition in the absence of pathogen infection

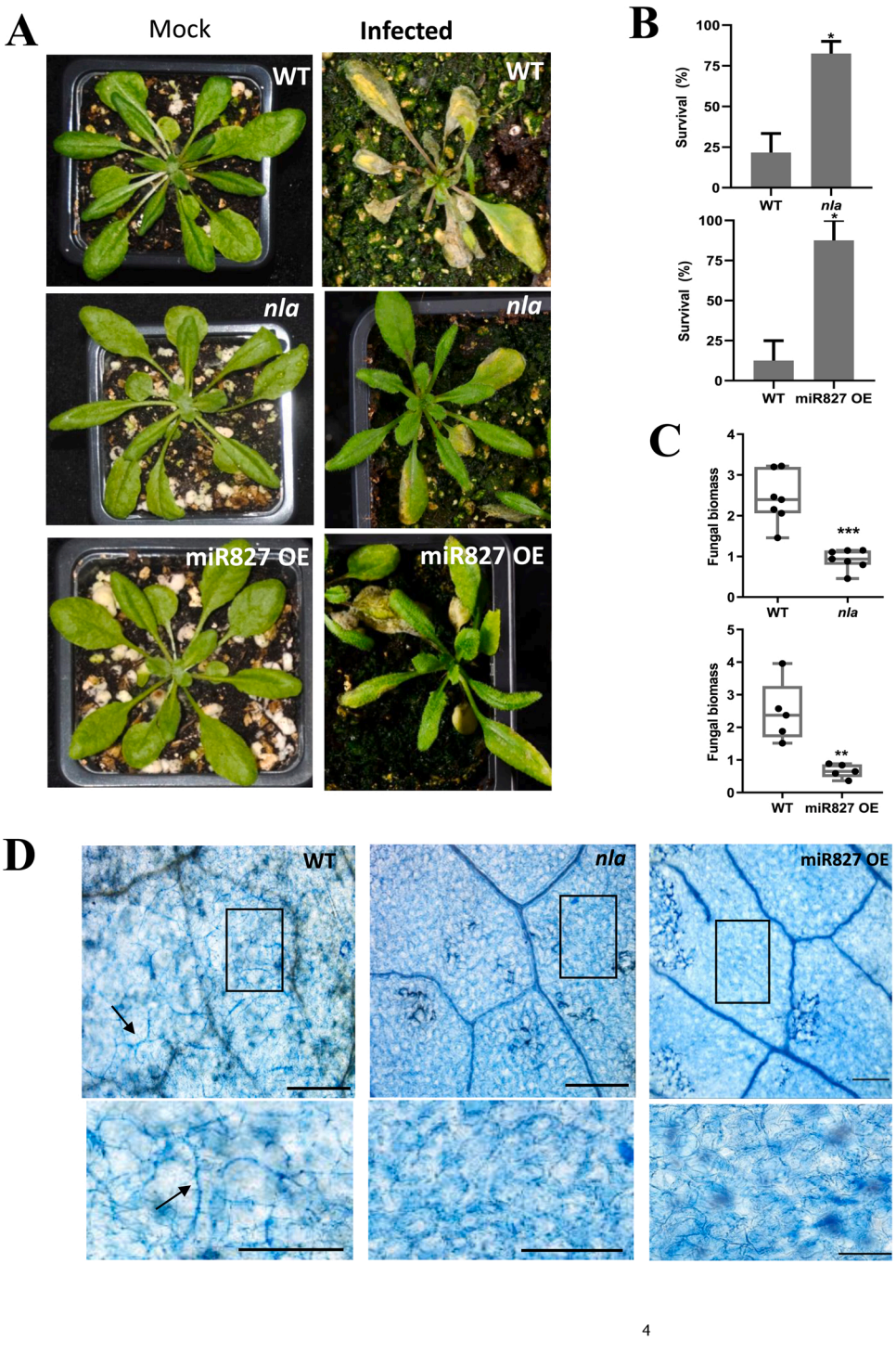

Fig. 1. Resistance of *nla* and miR827 OE plants to infection by necrotrophic fungal pathogen *P. cucumerina*. Three-week-old plants were mock-inoculated or inoculated with *P. cucumerina* (5×10^5 spores/ml). Three independent infection experiments were carried out with similar results (at least 24 plants per genotype each experiment). (A) Phenotype of mock-inoculated and *P. cucumerina*-inoculated wild type, *nla* and miR827 OE plants at 7 days post-inoculation (dpi). (B) Fungal biomass of *nla* and miR827 OE plants at 7 dpi. Quantification of fungal DNA was performed by qPCR using specific primers of *P. cucumerina* (Soto--Suárez et al., 2017). (C) Survival of *nla* and miR827 OE plants at 7 dpi. (D) Trypan blue staining of leaves from *P. cucumerina*-infected wild-type, *nla* and miR827 OE plants at 7 dpi. Arrows indicate fungal hyphae. Scale bars represent 200 μm. Higher magnifications are shown (right panels; bars, 100 μm).

(mock conditions) (Fig. 2A, Mock). Upon infection with *P. cucumerina*, both wild type and *nla* plants accumulated callose. Notably, *P. cucumerina*-infected *nla* plants showed significantly higher callose deposits compared with *P. cucumerina*-infected wild type plants (Fig. 2A, + *P. cucumerina*). Quantification of callose deposition confirmed higher accumulation in *nla* plants than in wild type plants during pathogen infection (Fig. 2A, right panel). The observation that callose does not accumulates in *nla* plants in the absence of pathogen infection suggest that this immune response is not constitutively active in *nla* plants. Presumably, enhanced accumulation of callose during pathogen infection might contribute to arrest pathogen invasion in *nla* plants.

Knowing that *nla* plants accumulated more callose after *P. cucumerina* infection, and that these plants also accumulated Pi in leaves, it was of interest to determine whether callose accumulation is affected by Pi supply. Towards this end, wild type and *nla* plants were grown under different Pi concentrations (0.05 mM, 0.25 mM and 2 mM Pi (hereinafter $P_{0.05}$, $P_{0.25}$ and P_2, respectively). Measurement of Pi content confirmed higher leaf Pi content when increasing Pi supply to wild type and *nla* plants (Suppl. Fig. S4). In the absence of pathogen infection, aniline blue staining revealed small fluorescent callose spots in wild type and *nla* plants that have been grown at the highest Pi condition (2 mM Pi) (Fig. 2B, mock). To note, callose spots had a larger size in *nla* plants compared with wild type plants. Upon pathogen challenge, callose deposits were more abundant and had a larger size in *P. cucumerina*-infected *nla* plants grown under high Pi supply (P_2 plants) compared with wild type plants also grown under high Pi (Fig. 2B, + *P. cucumerina*). Quantification of callose deposition confirmed that callose accumulated at substantially higher levels in the fungal-infected *nla* plants compared with the fungal-infected wild type plants (Fig. 2C). These results demonstrated that an increase in Pi content caused by high Pi supply or loss-of-function of *nla* promotes callose deposition in Arabidopsis.

3.3. NLA and MIR827 expression is regulated during fungal infection

The genome of *Arabidopsis thaliana* contains a single copy of the *MIR827* gene, whose expression is induced under Pi limiting conditions which, in turn, results in down-regulation of *NLA* expression (Hsieh et al., 2009). In this work, we investigated whether infection by *P. cucumerina* has an effect on *MIR827* and *NLA* expression. As shown in Fig. 3A, miR827 precursor and mature transcripts accumulated at significantly higher levels in *P. cucumerina*-infected wild type plants compared with non-infected plants, while *NLA* expression decreased in response to fungal infection. Northern blotting also revealed an increase in the accumulation of mature miR827 species in wild type plants in response to pathogen infection (Suppl. Fig. S5). Treatment with a crude preparation of *P. cucumerina* elicitors also triggers up-regulation of *MIR827* expression and down-regulation of *NLA* expression (Fig. 3B).

We further examined fungal-responsiveness of *MIR827* potentially contributing to the observed reduction in *NLA* expression. For this, transgenic Arabidopsis plants overexpressing the *MIR827* promoter were generated (*MIR827prom::GUS* lines). As expected, higher levels of precursor and mature miR827 transcripts, and lower levels of *NLA* expression, were observed in plants that have been grown under low Pi condition ($P_{0.05}$) (Suppl. Fig. S6, $P_{0.05}$). By increasing Pi supply, *MIR827* expression decreased, while *NLA* expression increased (Suppl. Fig. S6, $P_{0.25}$).

In *MIR827prom::GUS* lines plants grown under low Pi supply ($P_{0.05}$), GUS activity was observed in leaves and roots (Fig. 4, mock). Of interest, treatment with fungal elicitors caused a substantial increase in the activity of the *MIR827* promoter (Fig. 4, elicitors), which is in agreement with results obtained by expression analysis (Fig. 3B). Furthermore, a remarkable increase in *MIR827* promoter activity occurred in SA-treated *MIR827prom::GUS* plants (Fig. 4, SA). When *MIR827prom::GUS* lines were grown under high Pi supply, GUS activity was drastically reduced as it was only detected at the distal part of the leaves, and not in roots (Suppl. Fig. S7A). Neither Pi treatment, nor elicitor treatment or SA-treatment, had an effect on GUS activity in transgenic Arabidopsis plants expressing the *GUS* reporter gene under the control of the *35 S Cauliflower Mosaic Virus 35 S* promoter (*35Sprom::GUS* plants) (Suppl. Fig. S7B and (Suppl. Fig. S7C).

Further supporting a pathogen-induced transcriptional regulation of *MIR827* expression, multiple sequence motifs (*cis*-elements) that are known to be involved in the pathogen-responsiveness of protein coding genes were identified in the *MIR827* promoter (Suppl. Fig. S8; Suppl. Table S2). They included, W box TGAC core elements that mediate the transcriptional activation of pathogen- and elicitor-regulated genes, as well as SA-responsive *cis*-elements (Suppl. Fig. S8; Suppl. Table S2). As expected, the P1BS element present in phosphate starvation responsive genes was also identified in the *MIR827* promoter (Suppl. Fig. S8; Suppl. Table S2).

From these results it is concluded that *MIR827* expression is transcriptionally activated during fungal infection and treatment with fungal elicitors. Since miR827 down regulates *NLA* expression by targeting cleavage of *NLA* transcripts, the transcriptional activation of *MIR827* expression during infection and elicitor treatment might account, at least in part, for down-regulation of *NLA* expression in the fungal-infected and elicitor-treated Arabidopsis plants.

3.4. Loss-of-function of NLA promotes the accumulation of camalexin

The antifungal activity of camalexin against phytopathogens is well documented (Simone et al., 2003; Thomma et al., 1999; Van Baarlen et al., 2007). In particular, camalexin accumulation was found to correlate with resistance to infection by *P. cucumerina* (Sanchez-Vallet et al., 2010). In this work, we investigated whether loss-of-function of *nla* has an effect on the accumulation of the phytoalexin camalexin.

Camalexin originates from tryptophan, and its biosynthesis involves the activity of several cytochrome P450 enzymes (Fig. 5A). They are: *CYP79B2*, *CYP79B3*, *CYP71A13* and *CYP71B15/PAD3*. Infection with *P. cucumerina* was found to induce the expression of genes involved in camalexin biosynthesis in *nla* and wild type plants (Fig. 5B). Upon pathogen challenge, however, all camalexin biosynthesis genes reached higher expression levels in *nla* plants compared with wild type plants (Fig. 5B). Consistent with the observed super-induction of camalexin biosynthesis genes, the *nla* plants accumulated higher levels of camalexin during pathogen infection than wild type plants (Fig. 5C). Finally, growing wild type plants under high Pi conditions resulted in higher accumulation of camalexin, thus, establishing a link between Pi level and camalexin production (Fig. 5D). On this basis, Pi accumulation caused by loss-of-function of *nla* or by treatment with high Pi might be responsible of camalexin accumulation which, in turn, would enhance resistance to *P. cucumerina* infection.

3.5. NLA silencing is associated with accumulation of SA and JA

Evidence exists that components of the JA signaling pathway can modulate camalexin formation (Rowe et al., 2010). For instance, *A. thaliana* mutants deficient in JA synthesis or JA perception have reduced camalexin accumulation during pathogen infection (Rowe et al., 2010). Resistance to pathogen infection in Arabidopsis plants was also reported to be dependent not only on camalexin biosynthesis, but also on JA and SA signaling (Simone et al., 2003).

Knowing that *nla* plants accumulate camalexin during *P. cucumerina* infection, we examined the expression of SA-regulated and JA-regulated defense genes in mock-inoculated and *P. cucumerina*-inoculated *nla* plants (at 48 hpi). Compared with wild type plants, the SA markers *PR1* and *NPR1* showed a higher expression in *nla* plants in the absence of pathogen infection (Fig. 6A, black bars). In response to *P. cucumerina* infection, *PR1* and *NPR1* expression reached similar levels in wild type and *nla* plants (Fig. 6A).

Next, we examined the expression of genes in the two branches of the

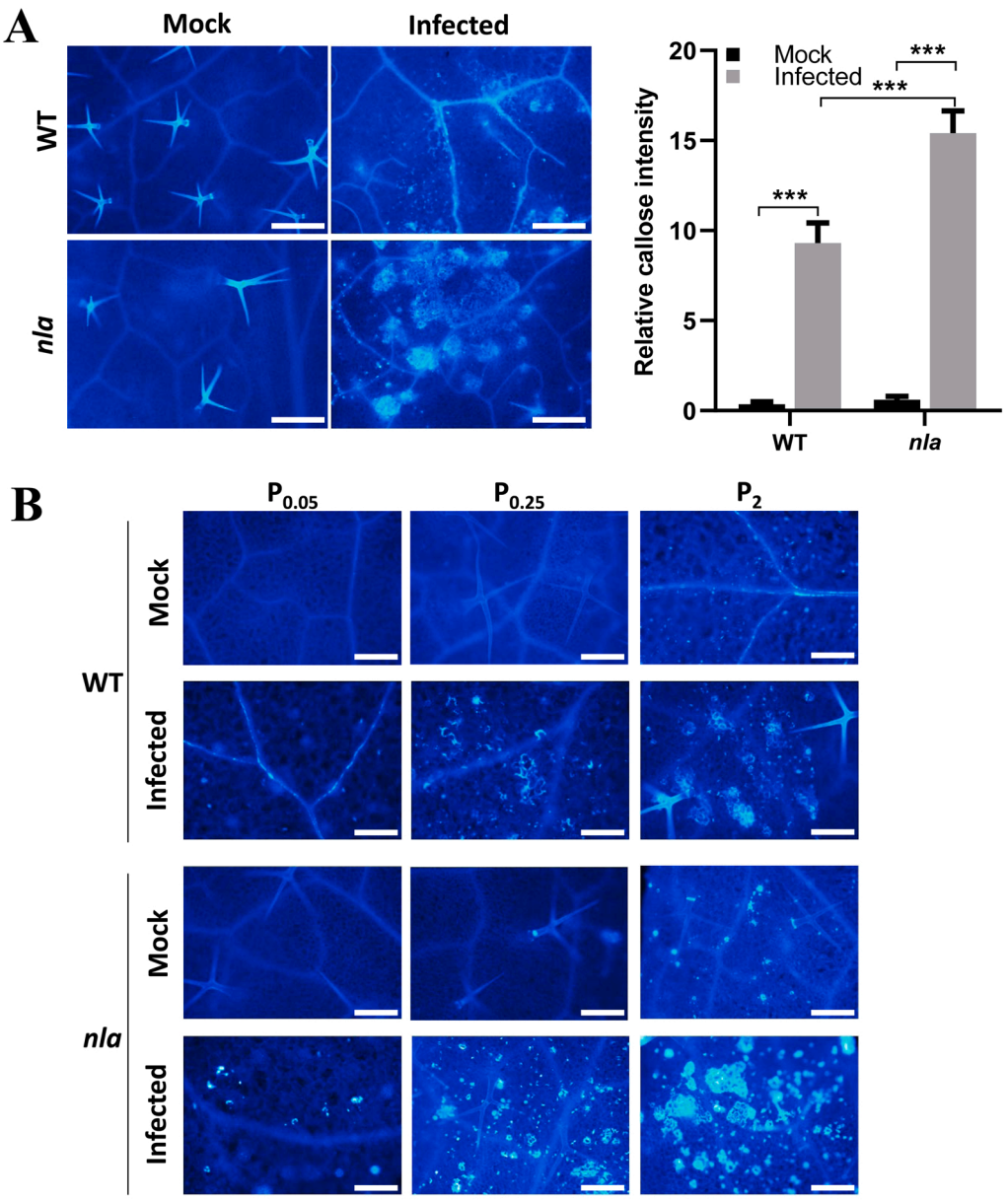

Fig. 2. Callose deposition in *P. cucumerina*-infected leaves of wild type and *nla* plants. Wild type and *nla* plants were inoculated with *P. cucumerina* spores (5×10^5 spores/ml), or mock-inoculated. Aniline blue staining was carried out at 48 hpi. Representative results from one of three independent infection experiments that gave similar results are shown (at least 12 plants each experiment). (A) Micrographs of aniline blue-stained leaves (left panels). Bars represent 200 μm. Pathogen-induced callose deposition was quantified by determining the relative number of fluorescent pixels on digital micrographs of infected leaves (right panel). Bars represent mean ± SEM. (B) Callose accumulation in wild type and *nla* plants that have been grown *in vitro* under increasing Pi supply (0.05 mM, 0.25 mM and 2 mM Pi) for 7 days, and then inoculated with *P. cucumerina* spores, or mock-inoculated. (C) Quantification of callose deposition in wild type and *nla* plants (same conditions as in B).

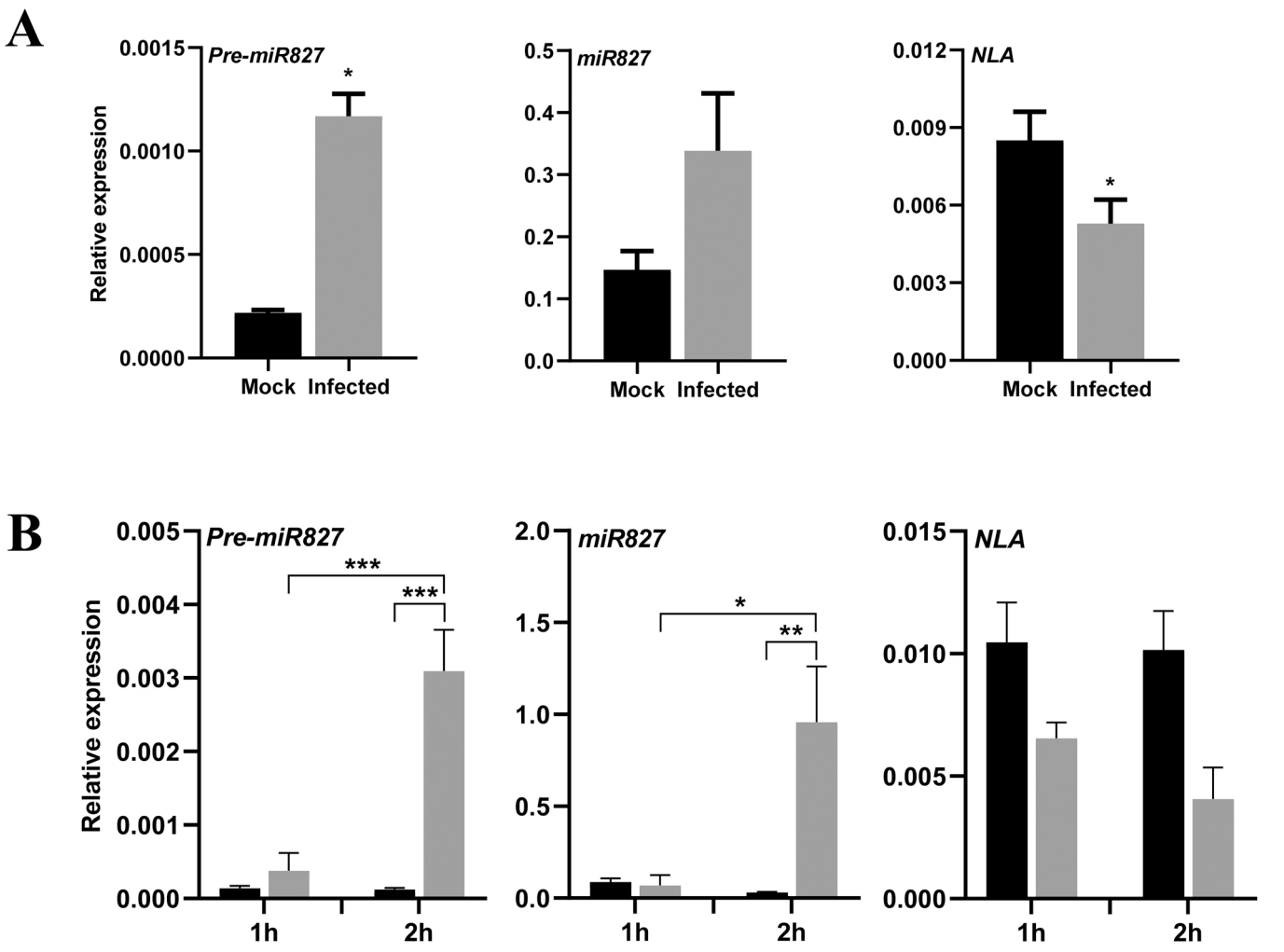

Fig. 3. *MIR827* and *NLA* expression in response to infection with *P. cucumerina* and treatment with *P. cucumerina* elicitors. Plants were grown in soil for three weeks and then inoculated with fungal spores (5 $\times 10^5$ spores/ml) or treated with elicitors obtained from this fungus (300 μg/ml elicitors). (A) Accumulation of pre-miR827, mature miR827 and *NLA* transcripts in *P. cucumerina*-inoculated or mock-inoculated plants at 48hpi. (B) Accumulation of pre-miR827, mature miR827 and NLA transcripts in elicitor-treated and mock-inoculated plant. The level of transcripts was determined by RT-qPCR (pre-miR827) and stem-loop RT-qPCR (miR827). Black bars, mock-inoculated plants. Gray bars, *P. cucumerina*-inoculated or elicitor-treated plants. Histograms show the mean ± SEM. Statistical significance was determined by ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

JA signaling pathway, the MYC2 branch (regulated by the AtMYC2 transcription factor), and the ERF branch (regulated by the AtERF1 transcription factor) (Lorenzo et al., 2004). The marker gene commonly used for the ERF branch is *PDF1.2* (*Plant Defensin 1.2*), whereas the induction of *VSP2* (*Vegetative Storage Protein 2*) serves as marker of the MYC branch (Lorenzo et al., 2004; Pieterse et al., 2012; Wasternack and Hause, 2013; Zhang et al., 2017). Under non-infection conditions, *PDF1.2* expression was significantly higher in *nla* plants compared with wild type plants (as for *ERF1,* a tendency to a higher expression of in *nla* plants was observed). Upon *P. cucumerina* infection, *ERF1* and *PDF1.2* expression was further induced, the expression of these genes reaching a higher level in the infected *nla* plants than in the infected wild type plants (Fig. 6B). Regarding the MYC2/VSP2 branch of the JA pathway, *P. cucumerina* infection activated *MYC2* expression to a similar level in wild type and *nla* plants. However, an opposite response to pathogen infection was observed in *VSP2* expression between wild type and *nla* plants (Fig. 6**B**). Thus, *VSP2* expression slightly increased in *P. cucumerina*-infected wild type plants (compared with non-infected wild type plants), but in *nla* plants, *VSP2* expression substantially decreased in response to pathogen infection (Fig. 6B). As it is observed in *nla* plants, *P.cucumerina* infection also down-regulated *VSP2* expression in *pho2* mutant plants accumulating Pi (Val-Torregrosa et al., 2022).

Levels of SA (and the SA glucoside SAG) and JA (and its biosynthetic precursor OPDA) were measured in wild type and *nla* plants, inoculated with *P. cucumerina* or mock-inoculated. In the absence of pathogen infection, *nla* plants accumulated higher levels of SA and JA compared to wild type plants (Fig. 6C). The pathogen-induced SA level was similar in wild type and *nla* plants, but *nla* plants had a higher content of SAG, the storage form of SA, than wild type plants during infection, pointing to a tight control of SA content in *nla* plants. To note, *P. cucumerina* infection results in higher accumulation of JA in *nla* plants compared with wild type plants (Fig. 6C). The *nla* plants also showed a higher level of OPDA during pathogen infection than wild type plants. On this, it is worth mentioning that treatment with high Pi in wild type plants was recently reported to increase SA and JA levels which was accompanied by up-regulation of SA- and JA-dependent defense gene expression (Val-Torregrosa et al., 2022).

Collectively, this study revealed that *nla* plants accumulate JA under non infection and infection conditions. It is tempting to hypothesize that, in addition to camalexin accumulation, resistance to *P. cucumerina* in *nla* plants involves JA-dependent defense responses. Furthermore, an opposite regulation of the two branches of the JA signaling pathway occurs in *nla* plants during *P. cucumerina* infection (e.g up-regulation of *PDF1.2* and down-regulation of *VSP2*), a response that also occurs in the

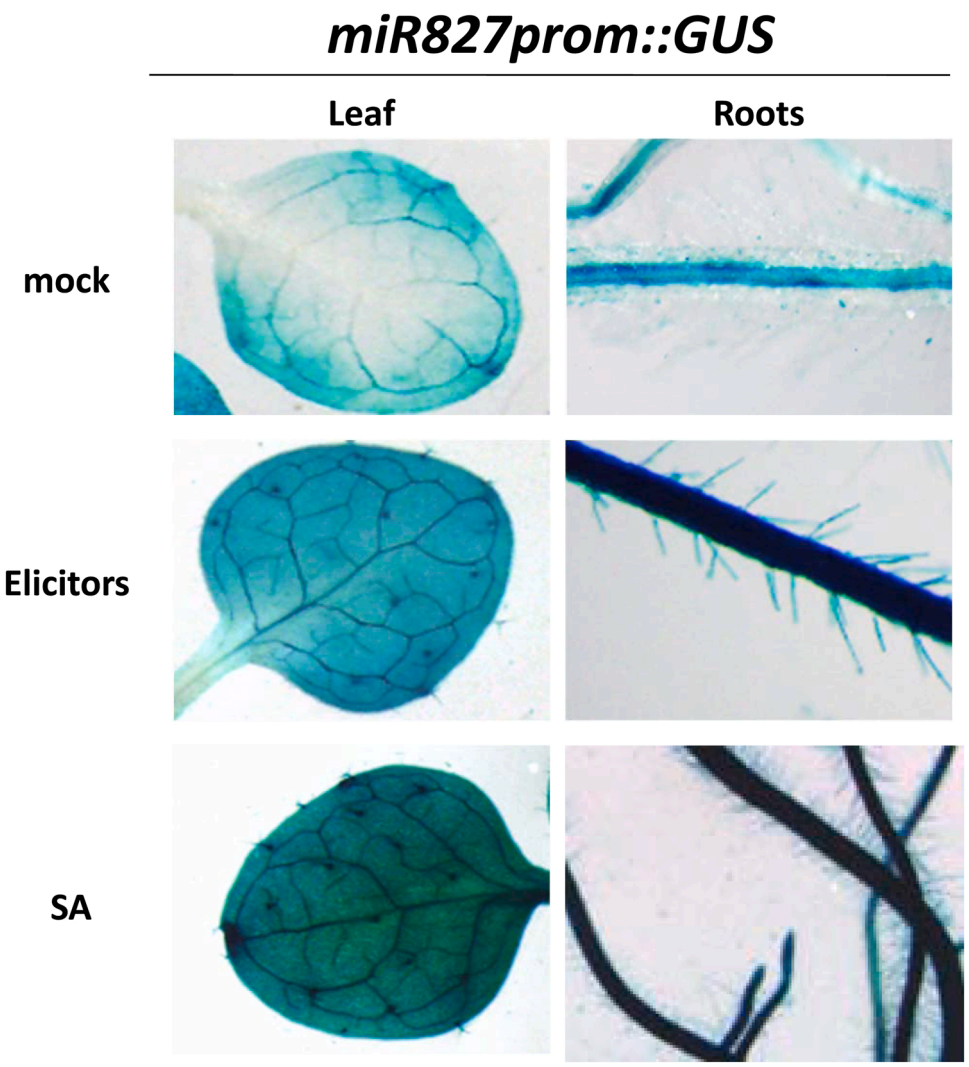

Fig. 4. Histochemical analysis of GUS activity in leaves and roots of *miR827prom::GUS* plants that have been grown under low Pi conditions (0.05 mM Pi). Plants were mock-inoculated or treated with *P. cucumerina* elicitors (300 μg/ml elicitors; 60 min of treatment), or SA (0.1 mM SA; 12 h of treatment). Representative images are shown. Two independent lines were assayed which gave similar results.

Pi accumulator mutant *pho2* of Arabidopsis. These findings together with those previously reported on treatment of wild type plants with high Pi suggest that Pi accumulation fosters immune responses and confers resistance to pathogen infection in Arabidopsis.

4. Discussion

Upon pathogen attack, plants deploy effective mechanisms to arrest the infection. However, resistance to pathogens can be attenuated or strengthened with co-occurrence of additional stress factors, such Pi stress. Increasing evidence support that, under Pi limiting conditions, plants coordinate the PSR and immune responses to alleviate Pi starvation stress (Castrillo et al., 2017; Finkel et al., 2019). In this work, we show that increasing Pi content in Arabidopsis leaves by loss-of-function of *NLA* or *MIR827* overexpression confers resistance to infection by fungal pathogens in Arabidopsis. These findings indicated that functioning of the miR827/*NLA* pair plays a role in Arabidopsis immunity.

Several lines of evidence support that the *NLA* contributes to disease resistance in Arabidopsis by regulating immune responses. First, resistance to pathogen infection in *nla* plants is associated with a greater accumulation of callose. Equally, growing Arabidopsis plants under high Pi supply, fosters callose accumulation during pathogen infection. The relevance of callose deposition in resistance to pathogen infection in Arabidopsis, including *P. cucumerina* infection, is well established (Luna et al., 2011; Wang et al., 2021; Pastor-Fernández et al., 2019).Second, *nla* plants showed an increase SA and JA content under non-infection, and accumulated higher levels of JA under infection conditions. Similarly, treatment with high Pi of wild type plants results in an increase in SA and JA levels and upregulation of SA- and JA-regulated defense genes (Val-Torregrosa et al., 2022). To note, an opposite regulation of genes in the two branches of the JA signaling pathway occurs in *nla* and wild type plants in response to *P. cucumerina* infection. Whereas pathogen infection induces *PDF1.2* (marker of the ERF1 branch) in *nla* plants, *VSP2* expression (maker of the MYC2 branch) is down regulated by infection in these plants. Third, *nla* plants accumulated higher levels of camalexin during pathogen infection, which could be also inferred from the observed superactivation of camalexin biosynthesis genes in the *nla* plants. Camalexin plays a key role in Arabidopsis defense against *P. cucumerina*, and the *in vitro* antifungal activity of camalexin against *P. cucumerina* has been demonstrated (Sanchez-Vallet et al., 2010). Also, *cyp79B2 cyp79B3* mutants were found to be more susceptible to *P. cucumerina* infection than wild type plants (Sanchez-Vallet et al., 2010). Superinduction of camalexin biosynthesis genes during pathogen infection, and subsequent accumulation of camalexin might explain, at least in part, the observed phenotype of disease resistance in *nla* mutant plants.

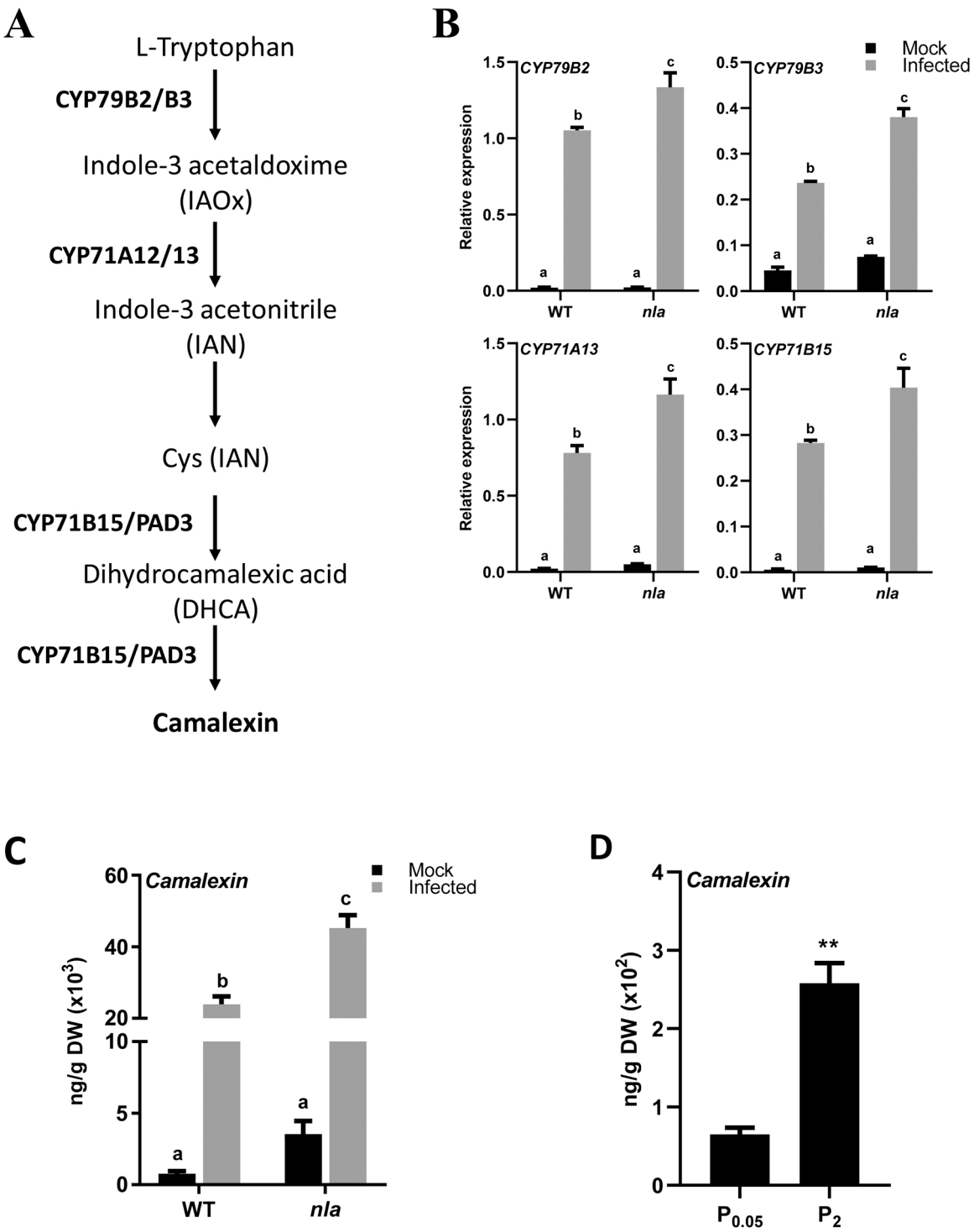

Fig. 5. Camalexin accumulation wild type and *nla* plants during infection with *P. cucumerina* or Pi treatment. (A) Camalexin biosynthesis pathway. (B) Expression of camalexin biosynthesis genes (*CYP79B2*, *CYP79B3*, *CYP71A13* and *CYP71B15*) in soil-grown wild type and *nla* plants. Three-week old plants were inoculated with *P. cucumerina* spores, or mock inoculated. Transcript levels were determined at 48 hpi. Six biological replicates (three plants per replicate were examined). Bars represent mean ± *SEM*. Letters indicate statistically significant differences (ANOVA, HSD Tukey's test, $P \leq 0.05$). (C) Accumulation of camalexin in wild type and *nla* plants under infection and non- infection conditions. (D) Camalexin accumulation in wild type plants grown under different Pi conditions. Plants were grown on modified Hoagland half strength medium for 1 week and then one more week at the desired Pi concentration ($P_{0.05}$ or P_2 mM Pi) as previously described (Val--Torregrosa et al., 2022). Bars represent mean ± *SEM* (six biological replicates and three plants per replicate).

Regarding interactions between SA- and JA-mediated signaling pathways it is generally assumed that these signaling pathways are antagonistic. In the literature, however, there are also examples indicating that SA and JA (and ET) can act synergistically to influence defense gene expression and disease resistance in Arabidopsis, as it is also observed in our work. In particular, a co-operation between SA-dependent and JA/ET-dependent pathways for the induction of resistance against *P. cucumerina* in Arabidopsis was previously reported (Berrocal-Lobo et al., 2002). In other studies, it was described that components of the JA/ET signaling pathway can function in

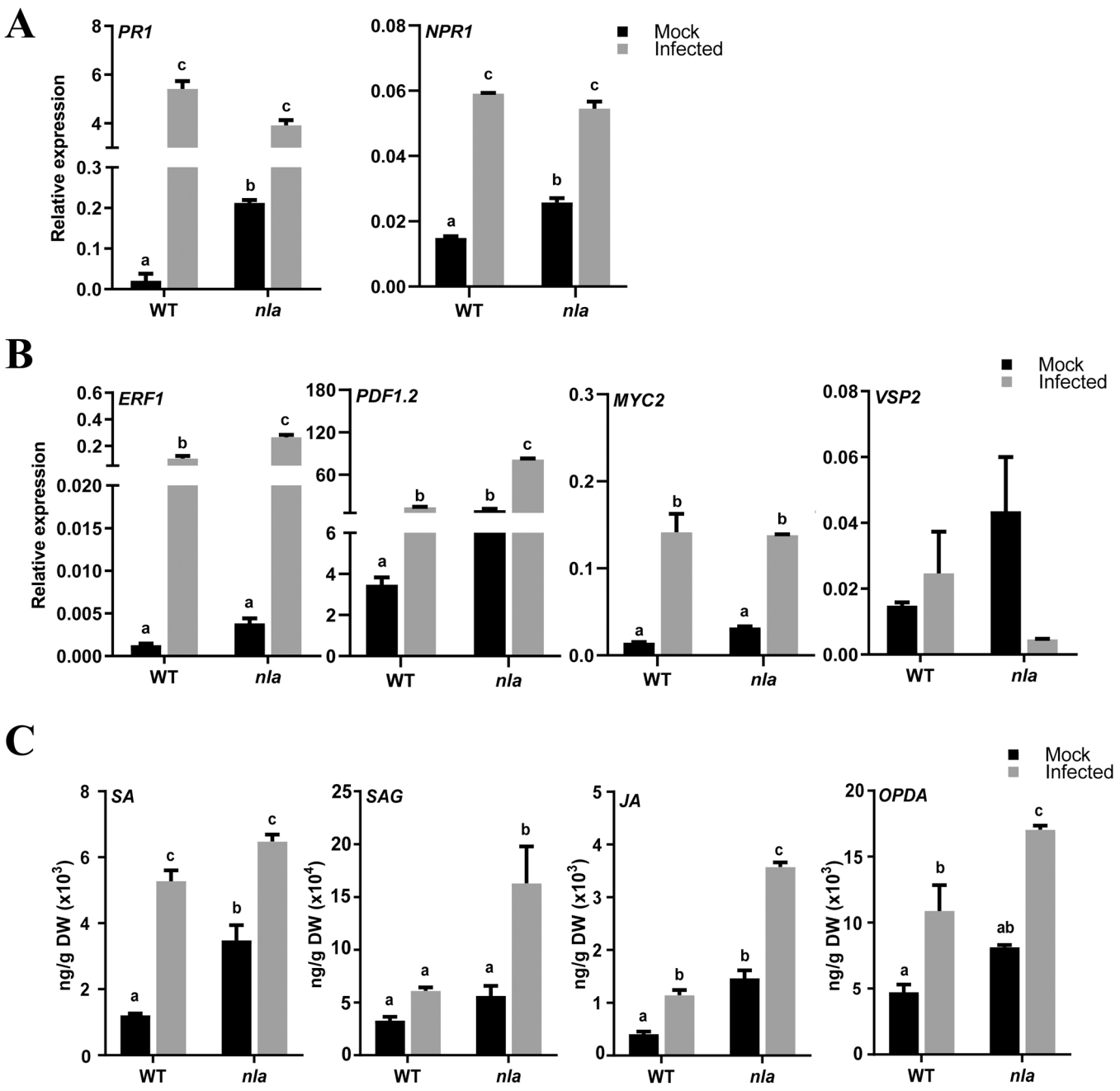

Fig. 6. Expression of defense marker genes of the SA and JA signaling pathways and accumulation of SA and JA in wild type and *nla* plants. Transcript levels in (A) and (B) were determined by RT-qPCR in mock-inoculated and *P. cucumerina*-inoculated plants at 48 hpi (black and gray bars, respectively). Letters indicate statistically significant differences (ANOVA, HSD Tukey's test, $P \leq 0.05$). (A) Genes in the SA signaling pathway (*PR1*, *NPR1*). (B) Genes in the ERF1/PDF1.2 and MYC2/VSP2 branch in the JA signaling pathway. Bars represent mean ± *SEM* (three biological replicates and three plants per replicate). **C.** Levels of SA, SAG, JA and OPDA. Bars represent mean ± *SEM* (six biological replicates and three plants per replicate).

combination with the SA-mediated (NPR1-independent) response of Arabidopsis against *P. syringae* and *P. parasítica* (Clarke et al). It is also known that defense gene expression and resistance against pathogens in the *hrl1* (*hypersensitive response-like lesions 1*) mutant of Arabidopsis is regulated synergistically by SA and ET/JA defense pathways (Devadas et al., 2002). Gene expression analysis of Arabidopsis plants treated with SA, JA or ET also revealed overlapping responses to these hormones (Schenk et al., 2000). Indeed, the outcome of JA-SA interactions has been proposed to depend on the relative concentration of JA and SA (Mur et al., 2006). Together, this piece of information supports that both antagonistic and synergistic interactions between SA and JA/ET signaling pathways might occur in Arabidopsis. Most probably, Arabidopsis plants have evolved antagonistic or synergistic interactions between these regulatory mechanisms depending on the type of pathogen, and perhaps also depending on the nutritional status of the host plant. In this way, the plant will be able to tailor the most appropriate response to a particular pathogen under a particular condition.

As a further confirmation of the implication of *NLA* in disease resistance, both fungal infection and treatment with fungal elicitors is accompanied by up-regulation of *MIR827* expression and down-regulation of *NLA*. Not only by treatment with fungal elicitors but also by treatment with SA results in transcriptional activation of *MIR827*, which is consistent with the presence of elicitor- and SA-responsive *cis* elements in the *MIR827* promoter. Several Pi starvation-responsive

genes have been reported to contain SA-inducible elements in their promoters (Baek et al., 2017). Taken together, results here presented indicated that *NLA* plays a role in Arabidopsis immunity while further supporting cross-talk between phosphate signaling and disease resistance.

Regarding the processes by which loss-of-function of *NLA,* and subsequent accumulation of Pi, might confer resistance to pathogen infection, several possibilities, not mutually exclusive, can be considered. Increasing supply of Pi might improve the physiological and metabolic status of the plant which would then allow the plant to mount an effective immune response during pathogen infection. Another possibility is that Pi accumulation might have a toxic effect on fungal growth, an aspect that has not been investigated in this work. The possibility that Pi accumulation might affect disease resistance depending on the type of pathogen and/or pathogen lifestyle should be also considered. However, the observation that *nla* plants exhibit resistance to fungal pathogens with a necrotrophic (*P. cucumerina*) or hemibiotrophic (*C. higginsianum*) lifestyle suggest that *nla*-mediated disease resistance, likely, is not dependent on the pathogen lifestyle. It is worth mentioning that, contrary to what is observed in Arabidopsis, Pi accumulation increases susceptibility to infection by the blast fungus *M. oryzae* in rice (Campos-Soriano et al., 2020). Therefore, there is not a general model to predict the role of Pi in plant-pathogen interactions. In other studies, inactivation of *NLA* expression was found to increase susceptibility to the cyst nematode *Heterodera schachtii* (Hewezi et al., 2016).

Alterations in the expression of phosphate transporter genes have been also shown to affect immune signaling in plants further supporting connections between Pi homeostasis and immunity. In particular, the expression of genes associated to PTI was found to be suppressed in rice plants overexpressing the phosphate transporter *OsPT8*, these plants being more susceptible to infection by fungal and bacterial rice pathogens (Dong et al., 2019). That plant defense responses and Pi homeostasis are integrated is also supported by recent findings indicating that activation of PTI signaling negatively regulates PHT1-mediated Pi transport across the plasma membrane in Arabidopsis (Dindas et al., 2021). Further studies will be needed to characterize how signaling pathways controlling Pi homeostasis converge with pathogen-induced signaling pathways in different plant species. Understanding these processes is of paramount importance in designing strategies to improve disease resistance in plants.

5. Conclusions

Taken together, this study demonstrated that loss-of-function of *NLA,* and subsequent accumulation of Pi, is accompanied by stronger immune responses during pathogen infection, such as higher callose deposition, camalexin accumulation and JA content. These observations indicated that *NLA* regulates Arabidopsis immunity. This work offered a framework for understanding the impact of Pi accumulation in disease resistance and laid a foundation for future studies to understand interconnected regulations between Pi homeostasis and immunity in plants.

CRediT authorship contribution statement

BV-T performed the experiments with the help of MB and MDM. VF performed hormone and camalexin analyses. BSS and T-JC conceived the study and designed the experiments. BSS and BV-T wrote the article with contributions from MB. All authors have read and approved the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111374.

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