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The MicroRNA miR773 Is Involved in the *Arabidopsis* Immune Response to Fungal Pathogens

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MicroRNAs (miRNAs) are 21- to 24-nucleotide short noncoding RNAs that trigger gene silencing in eukaryotes. In plants, miRNAs play a crucial role in a wide range of developmental processes and adaptive responses to abiotic and biotic stresses. In this work, we investigated the role of miR773 in modulating resistance to infection by fungal pathogens in *Arabidopsis thaliana*. Interference with miR773 activity by target mimics (in *MIM773* plants) and concomitant upregulation of the miR773 target gene *METHYLTRANSFERASE 2* (*MET2*) increased resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum*, *Colletototrichum higginianum*) fungal pathogens. By contrast, both *MIR773* overexpression and *MET2* silencing enhanced susceptibility to pathogen infection. Upon pathogen challenge, *MIM773* plants accumulated higher levels of callose and reactive oxygen species than wild-type plants. Stronger induction of defense-gene expression was also observed in *MIM773* plants in response to fungal infection. Expression analysis revealed an important reduction in miR773 accumulation in rosette leaves of plants upon elicitor perception and pathogen infection. Taken together, our results show not only that miR773 mediates pathogen-associated molecular pattern-triggered immunity but also demonstrate that suppression of miR773 activity is an effective approach to improve disease resistance in *Arabidopsis* plants.

Plants have evolved diverse mechanisms to defend themselves against pathogenic microorganisms. The initiation of defense responses typically begins with the recognition of conserved pathogen-associated molecular patterns (PAMPs) by host pattern-recognition receptors (PRR), which leads to PAMP-triggered immunity (PTI) (Jones and Dangl 2006; Boller and He 2009). During PTI, plants express a diverse array of immune responses, such as callose deposition and generation of reactive oxygen species (ROS), activation of Ca^{2+} signaling and protein phosphorylation cascades, which ultimately leads to the dynamic reprogramming of host gene expression and accumulation of pathogenesis-related (PR) proteins (Bigeard et al. 2015; van Loon et al. 2006). Among the PAMPs triggering defenses against pathogens are the bacterial (flagellin) and fungal (chitosan) elicitors (Luna et al. 2011; Zipfel et al. 2004). However, in order to survive, successful pathogens must produce and deliver effectors that effectively suppress PTI, resulting in disease susceptibility. As a counteractive strategy, plants have evolved a repertoire of immune receptors, or resistance (R) proteins, that recognize pathogen effectors to mount an immune response, the so-called effector-triggered immunity (ETI) (Cui et al. 2015; Jones and Dangl 2006). The plant hormones ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) play central roles in the regulation of plant immune responses (De Vleesschauwer et al. 2014; Denancé et al. 2013; Glazebrook 2005; Robert-Seilaniantz et al. 2011). While many of these responses rely on gene expression changes due to transcriptional activation or repression, there is increasing evidence for posttranscriptional regulation of immune responses, with host endogenous small RNAs being essential in both PTI and ETI (Huang et al. 2016; Katiyar-Agarwal and Jin 2010; Pumplin and Voinnet 2013; Staiger et al. 2013; Seo et al. 2013).

MicroRNAs (miRNAs) are a class of short noncoding small RNAs that direct posttranscriptional gene silencing in a sequence-specific manner through cleavage or translational repression mechanisms (Axtell 2013; Brodersen et al. 2008; Llave et al. 2002; Rogers and Chen 2013). miRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in *Arabidopsis* (Li et al. 2013). MiRNAs play a critical role in controlling developmental processes (Li and Zhang 2016; Rubio-Somoza and Weigel 2011) and adaptation to different types of abiotic (e.g., nutrient deficiency, drought, cold, and salinity) and biotic stresses (Campo et al. 2013; Chiou et al. 2006; Huang et al. 2016; Jagadeeswaran et al. 2009; Li et al. 2014; Navarro et al. 2006; Shivaprasad et al. 2012; Sunkar et al. 2012). Our knowledge of miRNA functions in plant immunity is, however, still limited.

The role of miRNAs in disease resistance was first described in the interaction of *Arabidopsis thaliana* with the bacterial pathogen *Pseudomonas syringae* (Navarro et al. 2006). Here, perception of flg22, a flagellin-derived peptide, promotes miR393 accumulation resulting in downregulation of auxin receptors and in bacterial resistance. Subsequently, several other miRNAs have been shown to play a role during defense in *Arabidopsis*, such as miR393, miR396, miR398b, miR40l0, miR472, miR844, and miR863 (Boccara et al. 2014; Lee et al. 2015; Li et al. 2010; Navarro et al. 2006; Niu et al. 2016; Park et al. 2014; Soto-Suárez et al. 2017). miRNAs might function as

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positive or negative regulators in PTI and ETI by targeting negative or positive defense regulators. Although an important fraction of the *Arabidopsis* miRNAome is known to be regulated during pathogen infection (Baldrich et al. 2014; Jagadeeswaran et al. 2009), the function of most of these pathogen-regulated miRNAs and their contribution to *Arabidopsis* immunity remains elusive. Additionally, most studies so-far carried out for the identification of miRNAs affecting disease resistance defense have been executed in the interaction of *Arabidopsis* with the bacterial pathogen *Pseudomonas syringae*, and our knowledge of miRNAs controlling immune responses to infection by fungal pathogens in *Arabidopsis* is still scarce.

In the present work, we examined the role of miR773 in *Arabidopsis* immunity to fungal infection. We show that blocking miR773 activity through the expression of a miR773 target mimic in *MIM773* lines and the concomitant upregulation of the miR773 target gene *MET2* (Fahlgren et al. 2007; Li et al. 2010) confer resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum* f. sp. *conglutinans*, *Colletotrichum higginsianum*) fungal pathogens. Conversely, overexpression of *MIR773A* and silencing of *MET2* increased susceptibility to fungal infection. Resistance to infection by *P. cucumerina* in *MIM773* lines is associated with a stronger expression of basal defense responses upon pathogen attack, including higher accumulation

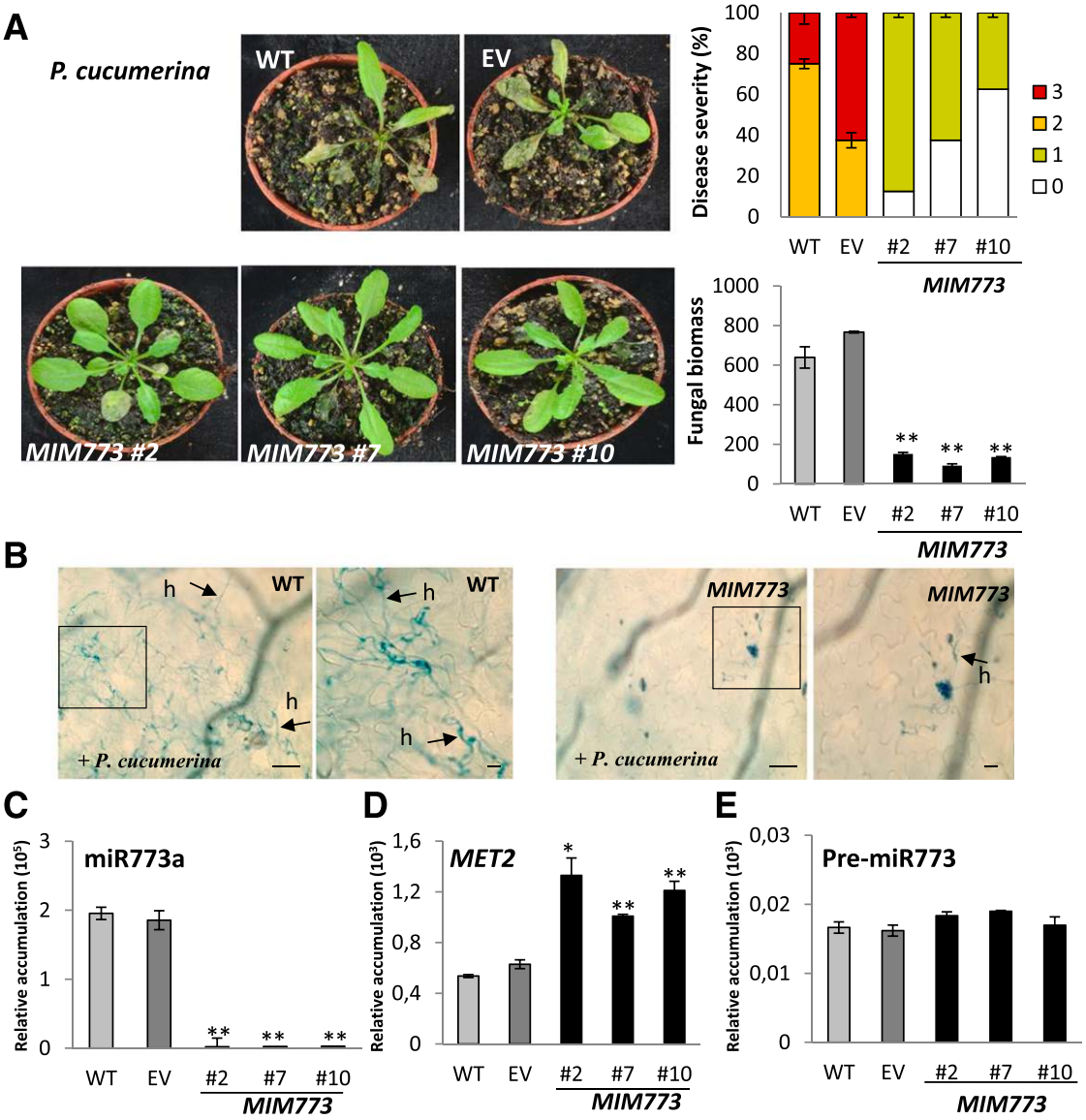

Fig. 1. *MIM773* plants show resistance to infection by *Plectosphaerella cucumerina*. Three-week old plants were inoculated with fungal spores. Results are from one of three independent experiments performed with three independent *MIM773* lines (*MIM773#2*, *MIM773#7*, and *MIM773#10*) and control plants (WT, empty vector) which gave similar results. At least 12 plants per genotype were assayed in each experiment. Histograms show the mean ± standard deviation. Statistical significance (analysis of variance test, asterisks [**] indicate $P \leq 0.01$). **A,** Plants 10 days after spray-inoculation with *P. cucumerina* spores (1×10^6 spores per milliliter, 0.5 ml per plant). Disease severity was scored as the number of leaves showing disease symptoms: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 100%. Quantification of fungal biomass was carried out by quantitative polymerase chain reaction (qPCR) using specific primers of *P. cucumerina β-tubulin* at 3 days postinoculation (Sánchez-Vallet et al. 2010). Values of fungal DNA were normalized to the *Arabidopsis UBIQUITIN21* gene (At5g25760). **B,** Trypan blue staining of *P. cucumerina*–inoculated leaves at 24 h postinoculation. Right panels show the boxed region at higher magnification (scale bar, 200 µm); h = hyphae. **C,** Accumulation of mature miR773 was determined by stem-loop reverse transcription qPCR. **D,** Accumulation of the miR773 target *MET2*, as determined by reverse transcription (RT)-qPCR. The single asterisk indicates a significance of $P < 0.05$. **E,** Accumulation of miR773a precursor transcripts, as determined by RT-qPCR.

of H_2O_2 and callose and superactivation of defense gene expression. Collectively, our results support a role of miR773 in controlling disease resistance in *Arabidopsis*.

RESULTS

Resistance to infection by fungal pathogens in *MIM773* plants.

To identify miRNAs contributing to disease resistance in *A. thaliana*, we initially assayed resistance to infection by *P. cucumerina* in a set of miRNA target mimicry (*MIM*) lines in which the activity of miRNAs is suppressed and the activity of miRNA targets correspondingly increased (Todesco et al. 2010). *P. cucumerina* (previously known as *Fusarium tabacinum*; anamorph *Plectosporium tabacinum*) (Palm 1995) is a necrotrophic fungus that causes sudden death and blight in crops, such as melon, soybean, snap bean, pumpkin, squash, zucchini, or white lupine, and that can also infect *Arabidopsis* (Llorente et al. 2005; Sánchez-Vallet et al. 2012; Ton and Mauch-Mani 2004).

Among the various *MIM* lines assayed (Supplementary Table S1), *MIM773* plants were consistently more resistant to infection by *P. cucumerina* than wild-type and empty vector controls (Fig. 1A). Disease severity was defined as the number

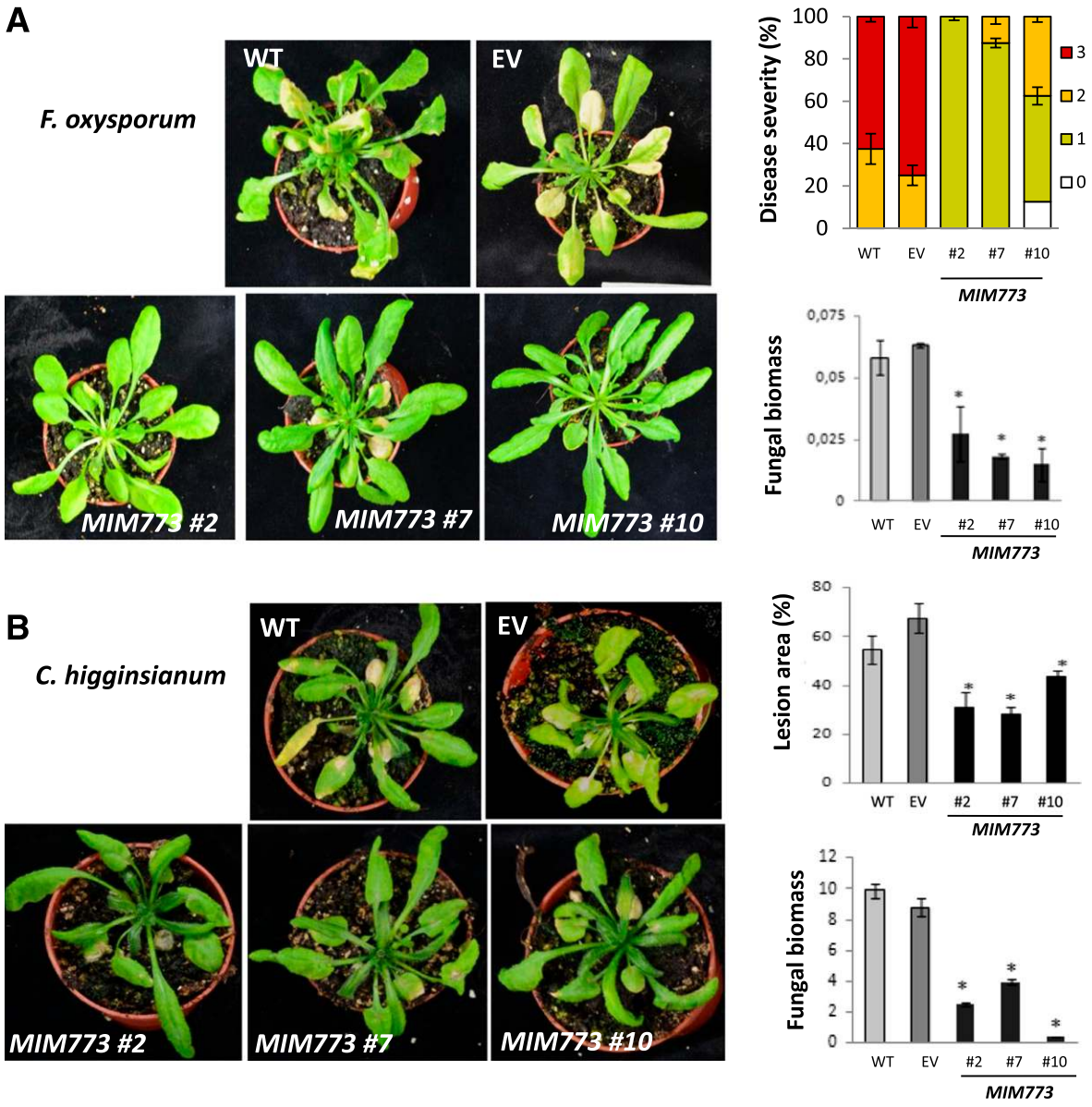

Fig. 2. *MIM773* plants show resistance to infection by hemibiotroph fungal pathogens. Three-week-old plants were inoculated with fungal spores. Results are from one out of three independent experiments performed with three independent *MIM773* lines (*MIM773#2*, *MIM773#7*, and *MIM773#10*) and control plants (wild-type, empty vector), which gave similar results. At least 12 plants per genotype were assayed in each experiment. Histograms show the mean ± standard deviation. Statistical significance (analysis of variance test, asterisks [*] indicate $P \leq 0.05$). **A,** Plants 17 days after inoculation with *Fusarium oxysporum* f. sp. *conglutinans*. Inoculation was carried out by applying the fungal spores (1×10^6 spores per milliliter) to soil. Disease severity was determined as described above. Quantification of *F. oxysporum* DNA was carried out by quantitative polymerase chain reaction (qPCR), using specific primers of the *F. oxysporum chitinase class III* gene at 3 days postinoculation (dpi) (Bravo Ruiz et al. 2016). Values are fungal DNA levels normalized against the *Arabidopsis UBIQUITIN21* gene (At5g25760). **B,** Plants at 9 days after inoculation with *Colletotrichum higginsianum.* Leaves were locally inoculated with a spore suspension at 5×10^5 spores per milliliter. Diseased leaf area was quantified using image analysis software (Assess 2.0). Quantification of *C. higginsianum* DNA was carried out by qPCR using specific primers for the *C. higginsianum* ITS2 (Internally transcribed spacer 2) gene at 3 dpi. Values are fungal DNA levels normalized against the *Arabidopsis UBIQUITIN21* gene.

of plants falling into disease categories based on the number of diseased leaves. At 10 days after infection, the *MIM773* plants had a disease score of 0 (plants with no symptoms) and 1 (plants with up to 25% of diseased leaves). By the same time, control plants scored in categories 2 (26 to 50% of diseased leaves) and 3 (51 to 100%) (Fig. 1A). Measurements of fungal biomass by quantitative polymerase chain reaction (qPCR) (Fig. 1A) and trypan blue staining (Fig. 1B) confirmed reduced fungal growth in leaves of *P. cucumerina*–inoculated *MIM773* plants compared with *P. cucumerina*–inoculated control plants.

To ascertain that the observed effects were due to changes in miR773 activity, we assayed miR773 accumulation and miR773 target gene expression. In *Arabidopsis*, there are two *MIR773* family members, with miR773a having been shown to target *MET2* (At4g14140) (Fahlgren et al. 2007; Li et al. 2010). As expected, *MIM773* plants accumulated lower levels of mature miR773 as a consequence of mimicry-triggered miRNA degradation (Todesco et al. 2010) (Fig. 1C; Supplementary Fig. S1). As a control, miR396 accumulation was found not to be affected in *MIM773* plants relative to control (wild-type and empty vector plants) (Supplementary Fig. S2). The observed reduction in mature miR773 levels was paralleled by an increase in *MET2* transcript levels (Fig. 1D). *MIR773A* precursor transcripts accumulated at similar levels in *MIM773* and wild-type plants (Fig. 1E). Finally, the *MIM773* plants were similar to wild-type plants in terms of growth and rosette leaf number and diameter (Supplementary Fig. S3).

Next, we challenged *MIM773* plants with the hemibiotrophic fungi *Fusarium oxysporum* f. sp. *conglutinans* and *Colletotrichum higginsianum*, the causal agents of wilt and of anthracnose leaf spot disease on a broad range of cultivated plant species as well as *Arabidopsis* (Mauch-Mani and Slusarenko 1994; O'Connell et al. 2004). Upon *F. oxysporum* f. sp. *conglutinans* inoculation, chlorosis appeared by 5 to 7 days postinoculation in leaves of control plants and progressively progressed over time, whereas by *MIM773* lines exhibited much milder symptoms (Fig. 2A). Disease severity scores and fungal biomass supported a suppressive effect in *MIM773* plants (Fig. 2A, right panels). *MIM773* plants also were more resistant to *C. higginsianum* (Fig. 2B), with a smaller diseased leaf area and reduced fungal biomass accumulation (Fig. 2B, right panels).

Taken together, these results demonstrate that reduced miR773 activity enhances resistance to infection by both necrotrophic and hemibiotrophic fungi.

MIM773 plants exhibit stronger PTI upon *P. cucumerina* infection.

A common feature of basal resistance to pathogen infection is the accumulation of callose and ROS production at the infection sites (Luna et al. 2011; Morales et al. 2016; Pastor et al. 2013; Pétriacq et al. 2016; Torres et al. 2006). Callose functions as the first line of defense impeding fungal penetration, whereas ROS act as antimicrobial agents and signaling molecules for the activation of defense responses. Aniline blue staining indicated that *MIM773* plants inoculated with *P. cucumerina* accumulated more callose than did wild-type plants and produced more H_2O_2 (Fig. 3A and B). It is noteworthy that, in *MIM773* plants, callose and H_2O_2 were not detected before inoculation with

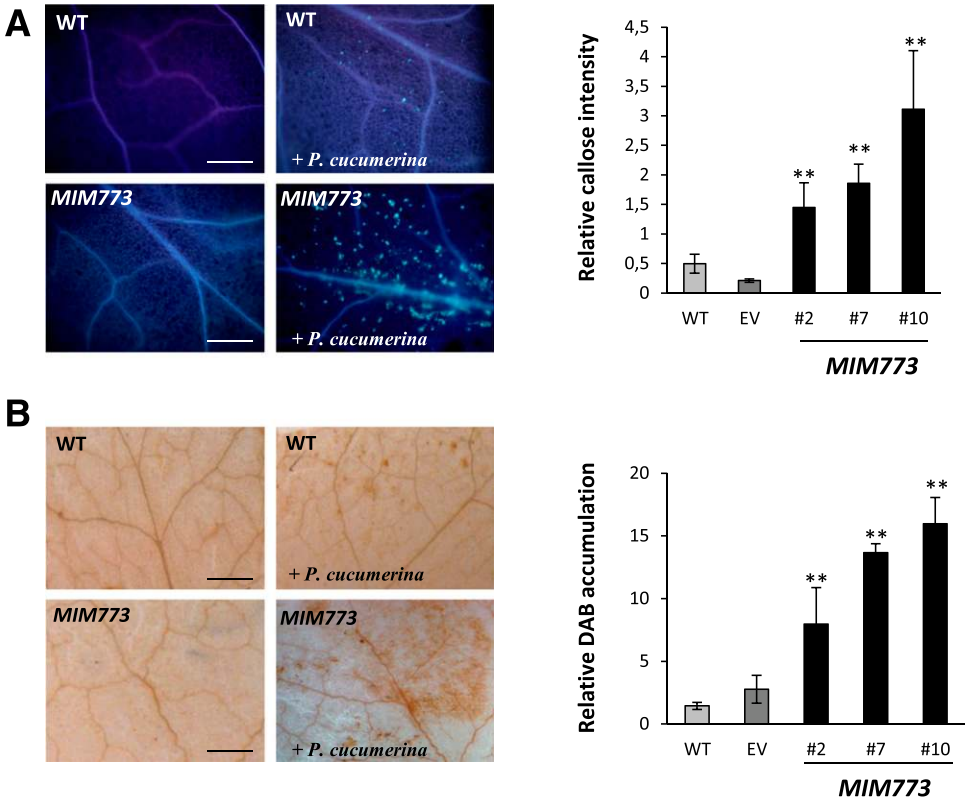

Fig. 3. Callose deposition and H_2O_2 production in *Plectosphaerrella cucumerina*–infected leaves of wild-type and *MIM773* plants. In vitro–grown plants were sprayed with a *P. cucumerina* spore suspension (1×10^6 spores per milliliter, 2 ml per plate). Statistical significance was determined by analysis of variance (asterisks [**] indicate $P \leq 0.01$). Representative results of one of three independent experiments that produced similar results are presented (three independent lines and 15 plants per independent line were assayed, and four leaves per plant were examined). Histograms (right panels) represent mean ± standard deviation. **A,** Callose deposition in mock-inoculated (left panels) and *P. cucumerina*–inoculated (right panels) plants was examined by aniline blue staining at 24 h postinoculation (hpi). Callose deposition was quantified by determining the number of callose-corresponding pixels relative to the total number of pixels covering plant material on digital photographs. Bars (left panels) represent 50 µm. **B,** H_2O_2 production was visualized by 3,3′-diaminobenzidine (DAB) staining at 24 hpi. Patches of brown precipitate of oxidized DAB are prominent in *MIM773* plants.

P. cucumerina, indicating that these immune responses were not constitutively active in *MIM773* plants. The enhanced callose and H_2O_2 accumulation in leaves of *MIM773* plants might well help to arrest pathogen growth in these plants.

MIR773A overexpression and *MET2* silencing confers enhanced susceptibility to *P. cucumerina*.

Since *MIM773* plants exhibited enhanced resistance to fungal infection, we reasoned that either constitutive expression of *MIR773A* or silencing of *MET2* expression might result in susceptibility to pathogen infection. We generated plants overexpressing *MIR773A* (*MIR773A* OE), which accumulated higher levels of mature miR773 and had reduced levels of *MET2* transcripts (Fig. 4A). Northern blot analysis confirmed miR773 accumulation in *MIR773* OE plants. In the absence of infection, *MIR773A* OE plants showed no obvious differences in growth, leaf number, or rosette diameter to nontransgenic plants (Supplementary Fig. S4).

Three-week-old *MIR773A* OE and control plants were challenged with *P. cucumerina*. *MIR773A* overexpression increased susceptibility to *P. cucumerina* (Fig. 4B). Disease susceptibility in *MIR773A* OE plants was further confirmed by quantifying disease severity and fungal biomass in the infected plants (Fig. 4C). The opposite effects of depleting miR773 in *MIM773* plants and overexpression of miR773 in *MIR773A* OE plants are in agreement with miR773 negatively regulating resistance to *P. cucumerina*.

We confirmed that, in two independent T-DNA insertion lines for *MET2* (*met2-1*, SALK_010893 and *met2-2*, SALK_010896), the *MET2* locus is disrupted by insertions in the second exon (Supplementary Fig. 5A). *MET2* transcripts were not detected in the *met2* mutants, supporting that they are knockout mutants for *MET2*. As expected, *MIR773A* precursor expression was unaffected in the *met2* mutants, which showed no obvious morphological defects in the absence of infection.

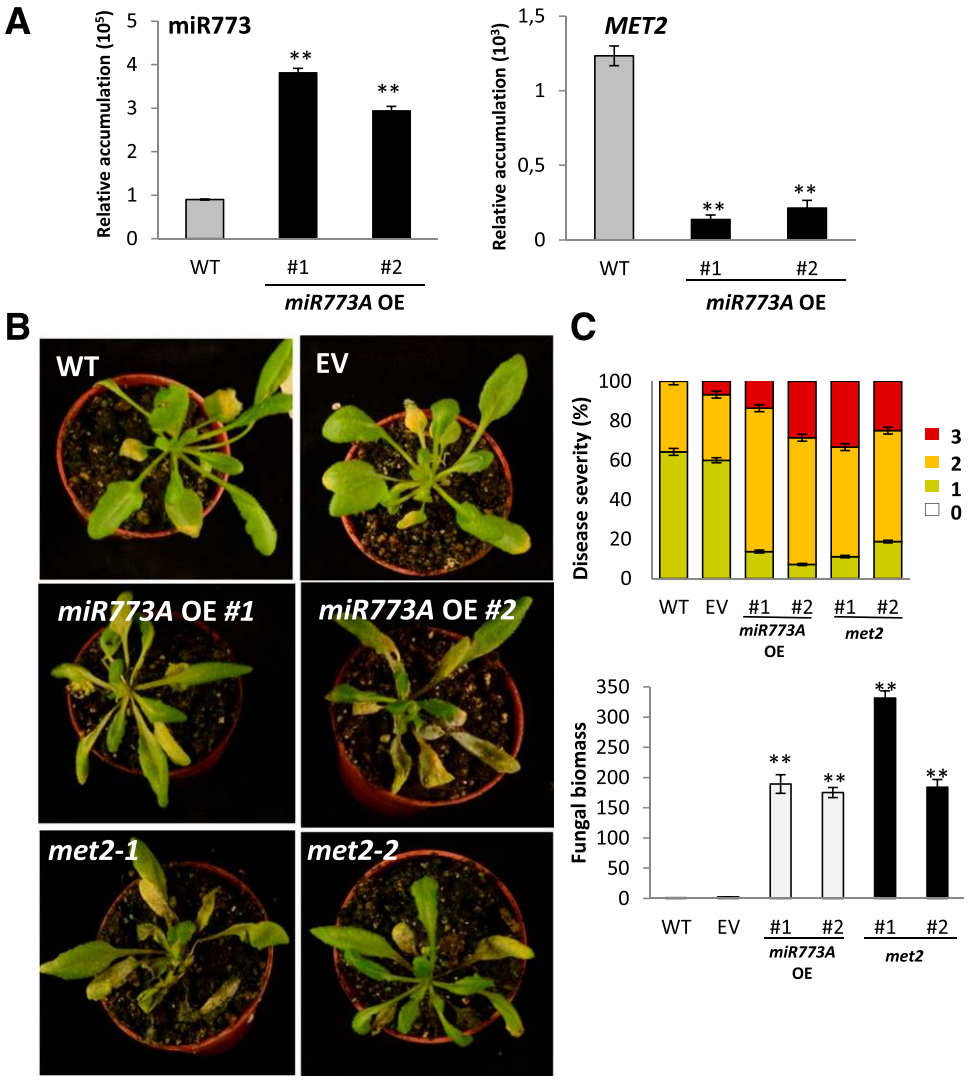

Fig. 4. Enhanced susceptibility to infection by *Plectosphaerella cucumerina* in transgenic plants overexpressing (OE) *MIR773A* and *met2* loss-of-function mutants. Three independent homozygous *MIR773A* OE plants, *met2*, and control plants (EV, empty vector; WT, wild-type) were analyzed. Three independent experiments were carried out, each with at least 12 plants per line. Results for two *MIR773A* OE lines are shown (similar results were obtained for the third *MIR773A* OE line). **A,** Accumulation of miR773 (left panel) and *MET2* transcripts (right panel) in OE miR773a plants. The histogram shows the mean ± standard deviation (analysis of variance test; asterisks [**] indicate $P \leq 0.01$). **B,** Disease susceptibility of OE miR773a and *met2* plants at 15 days postinoculation with *P. cucumerina* spores (1×10^4 spores per milliliter). **C,** Disease severity was scored as the number of leaves showing disease symptoms: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 100%. Quantification of fungal biomass was carried out by quantitative polymerase chain reaction (qPCR) using specific primers of *P. cucumerina β-tubulin* at 3 days postinoculation (Sánchez-Vallet et al. 2010). Values of fungal DNA were normalized to the *Arabidopsis UBIQUITIN21* gene (At5g25760). Statistical significance was determined by analysis of variance (asterisks [**] indicate $P \leq 0.01$, overexpressor or mutant lines vs. wild type).

Upon challenge with *P. cucumerina*, both *met2* mutant lines exhibited enhanced susceptibility to infection, supported by visual inspection, disease severity scoring, and quantification of fungal biomass in the infected plants (Fig. 4B and C). This finding is consistent with the observed phenotype of susceptibility to *P. cucumerina* in *MIR773A* OE plants and provides additional support for the involvement of the miR773/*MET2* pair in disease resistance.

MiR773 accumulation decreases as part of PTI responses against fungal pathogens.

Because altering miR773 activity has an effect on the host response to pathogen infection but does not appear to have consequences in the absence of pathogens, we sought to determine whether miR773 accumulation was itself pathogen-responsive. As shown in Figure 5A, steady-state levels of *MIR773* transcripts and mature miR773 rapidly decreased after infection with *P. cucumerina*, while expression of the miR773 target *MET2* was increased (Fig. 5B).

In PTI, the induction of defense mechanisms relies on the detection of PAMPs (or elicitors). We therefore investigated whether miR773 might be induced by a crude preparation of fungal elicitors. Similar to *P. cucumerina* infection, elicitor treatment rapidly reduced both miR773 precursor and mature miRNA (Fig. 5C) and, at the same time, enhanced *MET2* expression (Fig. 5D). These observations indicate that miR773 is PAMP-responsive and functions in PTI.

For a better understanding of miR773 regulation, we investigated miR773 accumulation in root and leaf tissues of wild-type plants in response to fungal infection. In unchallenged plants,

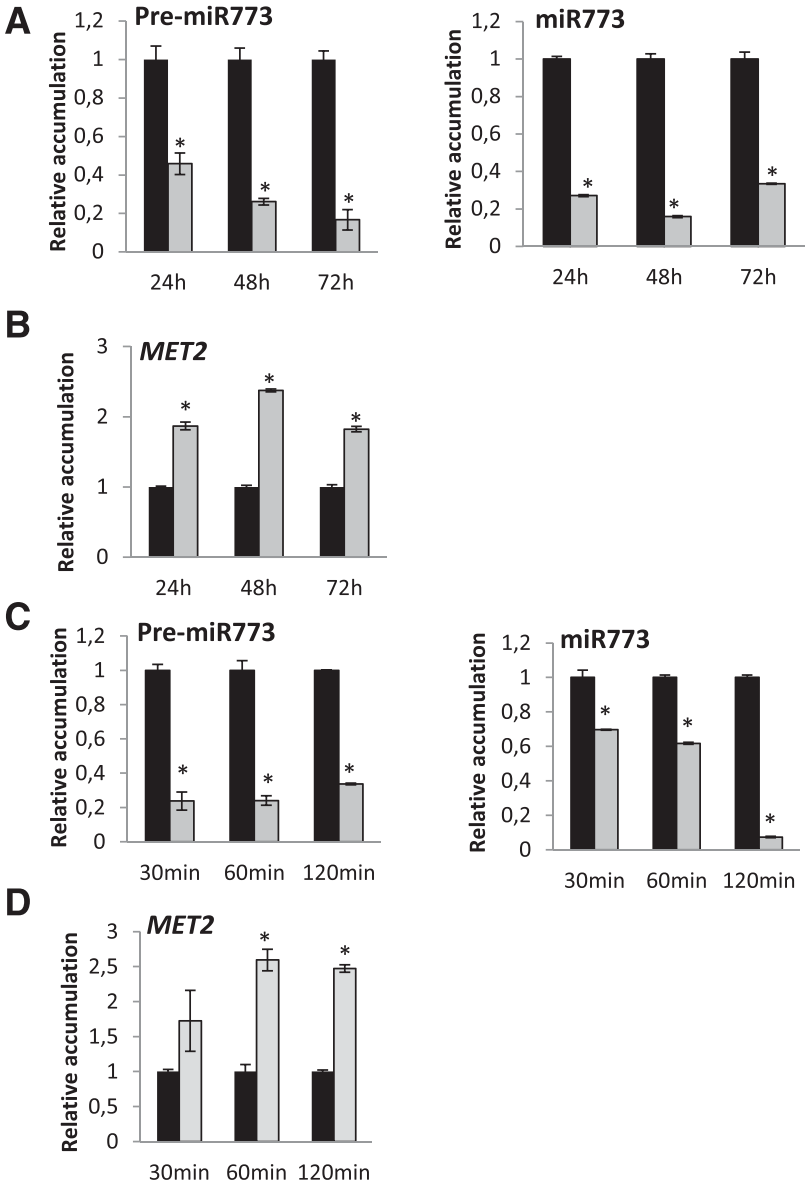

Fig. 5. Accumulation of miR773 and *MET2* transcripts in rosette leaves in response to infection by *Plectosphaerrella cucumerina* or treatment with elicitors obtained from *P. cucumerina*. The values represent changes in the accumulation of miR773 sequences at the indicated times after inoculation with fungal spores, treatment with fungal elicitors, or mock-inoculation. Black and gray bars correspond to mock-inoculated and *P. cucumerina*–inoculated plants, respectively. The expression level in mock-inoculated plants was set to 1.0. Histograms show the mean ± standard deviation of one of three biological replicates with similar results, each replicate with 12 plants per genotype (asterisks [*] indicate $P \leq 0.05$; analysis of variance test). **A,** Accumulation of premiR773 and mature miR773 sequences after inoculation with *P. cucumerina* spores, as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and stem-loop RT-qPCR analysis, respectively. **B,** Accumulation of *MET2* in response to inoculation with *P. cucumerina* spores. **C,** Accumulation of premiR773 and mature miR773 sequences at the indicated times of treatment with *P. cucumerina* elicitors. **D,** Accumulation of *MET2* in response to treatment with *P. cucumerina* elicitors.

miR773 expression was much higher in leaves than in roots (Fig. 6). Infection with either *P. cucumerina* or *C. higginsianum* significantly decreased miR773 accumulation in leaves but not in roots of wild-type plants (Fig. 6A, left and middle panels). In contrast, infection with the root pathogen *F. oxysporum* f. sp. *conglutinans* causes a reduction in miR773 levels in both tissues (leaves and roots) (Fig. 6A, right panel). The in-situ hybridization revealed miR773 accumulation in leaf mesophyll cells of noninfected plants (Fig. 6B, upper left panel), while miR773 was barely detectable in leaves of either uninfected and *P. cucumerina*–infected *MIM773* plants or infected wild-type plants (Fig. 6B, upper panels). Collectively, these results demonstrate that miR773 accumulation in mesophyll cells of rosette leaves decreases in response to infection by *P. cucumerina*.

miR773 reduction leads to stronger induction of defense gene expression.

To ascertain the molecular basis of the enhanced resistance to *P. cucumerina* in *MIM773* plants, we studied the expression of defense marker genes associated with JA/ET (*PDF1.2*), JA (*VSP2*), and SA (*PR1*, *NPR1*) signaling. Defense gene expression was induced in response to fungal infection in wild-type plants (Fig. 7A and B). Most importantly, transcripts of all defense genes accumulated to higher levels in *P. cucumerina*–inoculated *MIM773* plants than in *P. cucumerina*–inoculated wild-type plants (Fig. 7A and B). Contrary to this, a lower induction (*VSP2*, *PR1a*) or no induction (*PDF1.2*) of defense gene expression was observed in fungal-infected *met2* plants compared with the fungal-infected wild-type plants (Supplementary Fig. S6). These observations support the idea that, upon challenge with *P. cucumerina*, the *MIM773* plants show stronger activation of defense responses that are regulated by ET, JA, or SA, consistent with the enhanced disease resistance in these plants. In addition, under non-infection conditions, the expression of *PDF1.2* was slightly higher in *MIM773* plants (Fig. 7A).

When examining the expression of genes involved in ET biosynthesis in *MIM773* plants, namely *ACS1* (*1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 1*) and *ACO2* (*ACC OXIDASE 2*), they were found to be induced more strongly in *MIM773* plants than in wild-type plants, in response to *P. cucumerina* infection (Fig. 7C). In the absence of pathogen infection, *ACS1* and *ACO2* expression was already slightly elevated in *MIM773* plants (Fig. 7C).

These pieces of evidence support that *MIM773* plants respond more strongly to pathogen infection through the super-induction of defense genes whose expression is regulated by defense-related hormones. An opposite response occurs in *met2* plants, these plants exhibiting a weaker induction (even no induction) of defense gene expression in response to fungal infection.

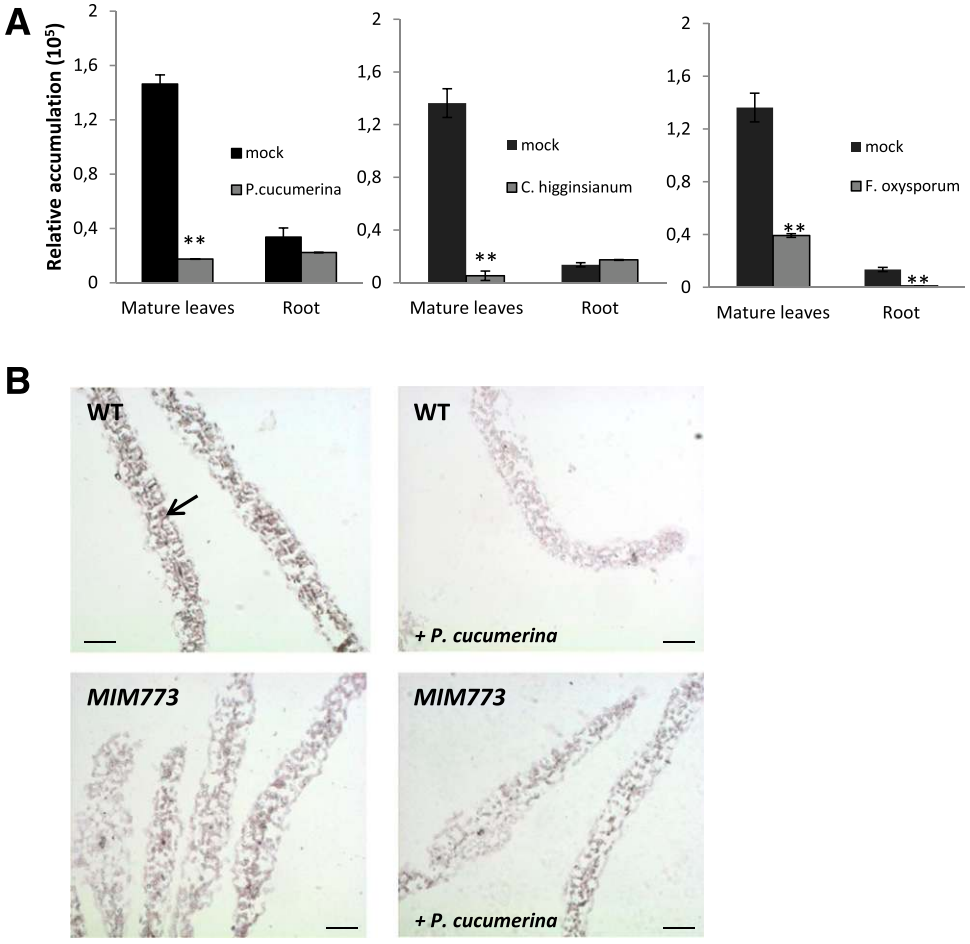

Fig. 6. Detection of miR773 in wild-type and *MIM773* plants. **A,** Accumulation of miR773 was determined by stem-loop reverse transcription-quantitative polymerase chain reaction analysis in leaves and roots of in vitro–grown *Arabidopsis* plants at 24 h postinoculation with fungal spores (*Plectosphaerrella cucumerina*, 1×10^6 spores per milliliter; *Colletotrichum higginsianum*, 5×10^5 spores per milliliter; *Fusarium oxysporum* f. sp. *conglutinans*, 1×10^6 spores per milliliter) (gray bars). Control plants were mock-inoculated (black bars). Histograms represent the mean ± standard deviation of one of three biological replicates with similar results, each replicate with 24 plants per genotype. Statistical significance was determined by analysis of variance (asterisks (**) indicate $P \leq 0.01$, infected vs. mock-inoculated). **B,** In situ miR773 hybridization in mature leaves of *P. cucumerina*– and mock-inoculated plants. Hybridization was carried out using a 3′ digoxygenin–labeled locked nucleic acid miR773a probe (scale bar, 500 μm).

DISCUSSION

In this study, we provide evidence that miR773a functions as a negative regulator of *Arabidopsis* immunity against necrotrophic (*P. cucumerina*) and hemibiotrophic (*F. oxysporum* f. sp. *conglutinans*, *C. higginsianum*) fungal pathogens. Our results with *MIM773* plants, in which miR773 activity is attenuated, and with plants deficient for the miR773 target *MET2* extends previous findings that flagellin treatment results in downregulation of miR773 and that *MIR773A* overexpression increases susceptibility to infection by the hemibiotrophic bacterial pathogen *P. syringae* (Li et al. 2010).

MIM773 plants apparently mount ET/JA- and SA- dependent defense responses. *Arabidopsis* plants are known to deploy JA/ET-, SA-, abscisic acid, and auxin-dependent defenses against *P. cucumerina* (García-Andrade et al. 2011; Llorente et al. 2008; Pétriacq et al. 2016; Sánchez-Vallet et al. 2012). Pathogen-induced downregulation of miR773 and upregulation of its target *MET2* may, thus, lead to a potentiated activation of defense responses. Although we provide evidence for a function of miR773 in the regulation of ET/JA- and SA-dependent defense responses, our current understanding does not allow us to determine how miR773 and its target gene *MET2* modulate the hormone-dependent activation of defense responses.

Given that *MET2* is targeted by miR773, the question arises as to what role miR773-guided regulation of *MET2* expression has in immunity. DNA methyltransferases and DNA demethylases function in an antagonistic manner in controlling the

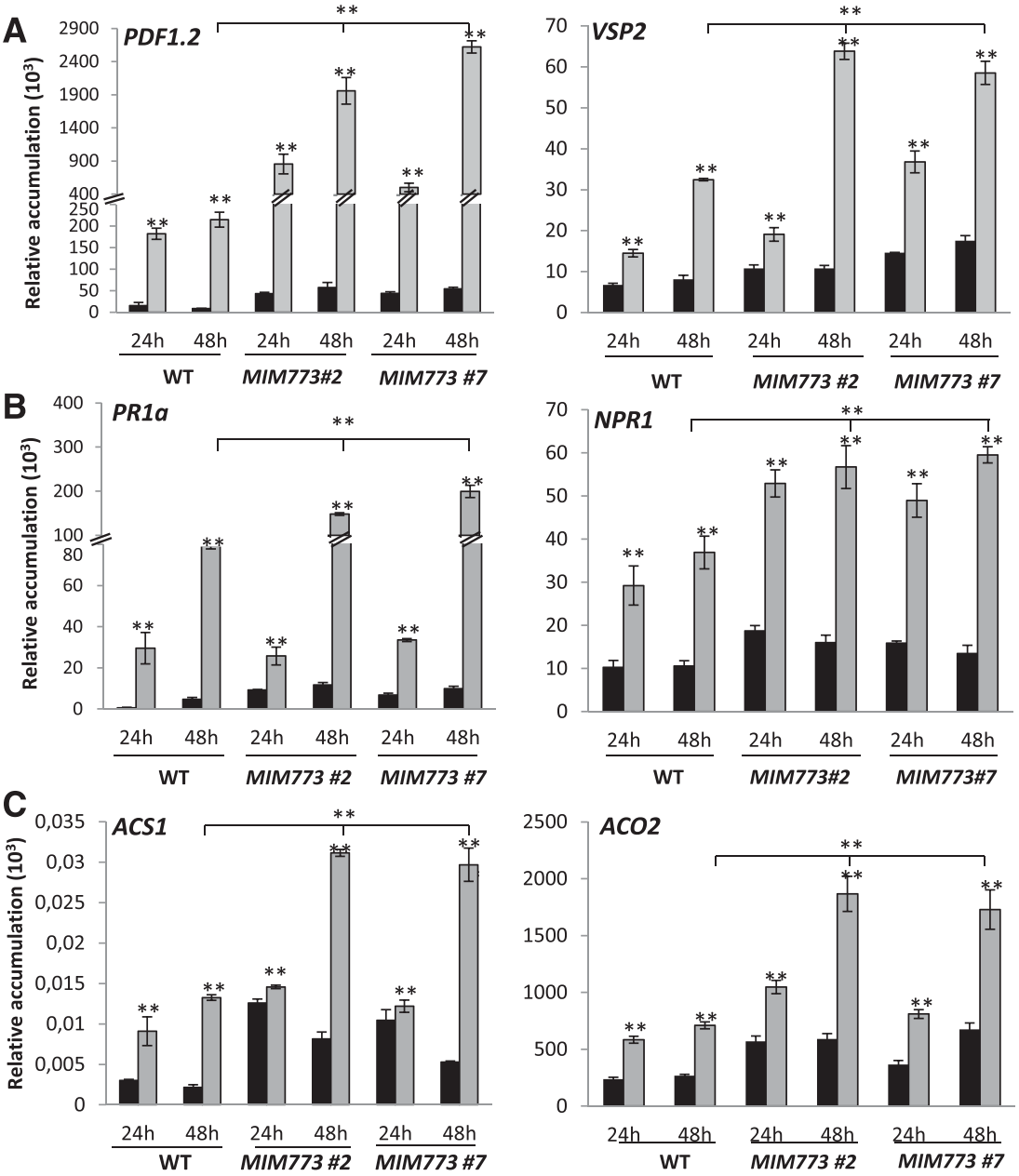

Fig. 7. Accumulation of defense marker genes in wild-type and *MIM773* plants. **A** and **B**, Reverse transcription-quantitative polymerase chain reaction analysis showing the accumulation of transcripts for genes that are regulated by the jasmonic acid and ethylene (JA/ET)-dependent (*PDF1.2*), JA-dependent (*VSP2*), and salicylic acid–dependent (*PR1a*, *NPR1*) signaling pathways at the indicated timepoints after inoculation with *Plectosphaerrella cucumerina* spores. **C**, Analysis of the *ACS1* and *ACO2* genes involved in ET biosynthesis is shown. Mock- and *P.cucumerina*–inoculated plants are represented by black and gray bars, respectively. Results from two of the three *MIM773* lines analyzed are shown (similar results were obtained for the third line). Histograms show the mean ± standard deviation of three biological replicates, each with 12 plants per genotype. Statistical significance was determined by analysis of variance (asterisks [**] indicate $P \leq 0.01$, *P. cucumerina*– vs. mock-inoculated; wild-type *P. cucumerina*–inoculated 48 h vs. *MIM773 P. cucumerina*–inoculated 48 h).

overall status of DNA methylation (Deleris et al. 2016). Typically, DNA methylation represses gene expression, whereas hypo-methylation is frequently associated with transcriptional upregulation. The dynamic regulation and functional consequences of DNA methylation are, however, substantially more complex. For instance, in flowering plants there are at least five distinct classes of methylated genes, including gene body methylated (gbM) genes in which there is an enrichment of CG DNA methylation within the transcribed regions and depletion at the transcriptional start and termination sites (Bewick and Schmitz 2017). Although the function of gbM remains elusive, it was proposed that gbM serves to regulate splicing and gene expression (Regulski et al. 2013; Zilberman et al. 2008). To note, infection of *Arabidopsis* plants with *P. syringae* results in dynamic changes in DNA methylation, which in turn lead to the transcriptional activation of defense-related genes (Dowen et al. 2012; Pavet et al. 2006; Yu et al. 2013). Similarly, about half of all genes that respond to infection by the oomycete pathogen *Hyaloperonospora arabidopsidis* are also altered in mutants with DNA methylation and gene silencing defects (López Sánchez et al. 2016). Such mutants are also affected in pathogen resistance, but they can be either more resistant or more susceptible, depending on the pathogen lifestyle. The hyper-methylated *ros1* (*REPRESSOR OF SILENCING 1*) mutant (affected in DNA demethylation) has increased susceptibility to the biotrophic oomycete *H. arabidopsidis* but enhanced resistance to the necrotrophic fungus *P. cucumerina* (López-Sánchez et al. 2016). Conversely, the hypo-methylated *nrpe1* RdDM (defective in RNA-directed DNA methylation) mutant exhibited enhanced susceptibility to *P. cucumerina* infection but resistance to *H. arabidopsidis* (López Sánchez et al. 2016).

Consistent with results reported by López Sánchez et al. (2016), upregulation of *MET2* through interference with miR773 activity confers resistance to *P. cucumerina*, whereas downregulation of *MET2* expression through *MIR773* overexpression increases susceptibility to infection. These observations reinforce the interpretation that DNA methylation controls basal resistance against *P. cucumerina* in *Arabidopsis*. DNA methylation may exert persistent control over defense genes in the absence of infection, with infection triggering both DNA methylation and gene-expression changes. DNA methylation might modulate immune responses, either by modifying the activity of gene promoters or bodies (or both) or by regulating the transcriptional status of transposable elements at specific loci. For instance, the methylation status of the COPIA retrotransposon has been shown to control the expression of *RPP7* (*RECOGNITION OF PERONOSPORA PARASITICA 7*), a disease resistance gene encoding an immune receptor conferring resistance to *H. arabidopsidis* in *Arabidopsis* (Tsuchiya and Eulgem 2013).

To conclude, our results lend further support to findings that miR773a is an important component in the regulation of PTI responses. Given the absence of obvious defects in noninfected plants with altered miR773 or miR773 target activity, manipulation of miR773 or its targets may provide a new strategy for engineering crop protection.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis (*Arabidopsis thaliana*) Col-0 plants were grown on a mixture of soil, perlite, and vermiculite (2:1:1) under neutral day conditions (12 h of light and 12 h of dark), at 22°C day and 20°C night temperatures, and 60% relative humidity. The production of *MIM* lines (Col-0 background) was previously described (Todesco et al. 2010). Rosette diameter and leaf number were determined in 3-week-old plants, using Image J. The *Arabidopsis* mutants used in this work, *met2-1* and *met2-2* (SALK_010893 and SALK_010896, respectively) were grown as described above. Primers used for genotyping the T-DNA insertion mutants are indicated in Supplementary Table S2.

For *MIR773A* overexpression, a 364-bp genomic sequence containing the miR773a precursor was obtained by PCR from *Arabidopsis* genomic DNA, using gene-specific primers. The miR773a precursor sequence was cloned into the pCAMBIA1300 vector under the control of the *35S CaMV* promoter. The floral dip method was used for *Arabidopsis* (Col-0) transformation. As a control, *Arabidopsis* plants were transformed with the pCAMBIA1300 empty vector.

Infection assays and elicitor treatment.

Fungi were grown at 28°C on PDA (potato dextrose agar) supplemented with chloramphenicol. Spores were collected by adding sterile water to the surface of the mycelium, adjusting to the appropriate concentration using a Bürker counting chamber. For infection experiments, control (wild-type, empty vector) and *MIM* plants (homozygous lines) were grown for 3 weeks and were then spray-inoculated with a spore suspension of *P. cucumerina* at the appropriate concentration or were mock-inoculated. Progression of the infection was examined at different days postinoculation. In the case of *F. oxysporum* f. sp. *conglutinans*, the spore suspension was applied to the soil near the roots (200 µl, 1×10^6 spores per milliliter). Infections with *C. higginsianum* were performed by drop-inoculation of the leaves (10 µl, 5×10^5 spores per milliliter). Fungal- and mock-inoculated plants were allowed to continue growth under high humidity. Statistically significant differences among the inoculated *Arabidopsis* genotypes were determined by one-way analysis of variance (ANOVA). Disease severity and lesion area were determined using digital imaging software (Assess 2.0, American Phytopathological Society). The relative quantification of fungal DNA in infected leaves was carried out by qPCR as described (Sánchez-Vallet et al. 2010; Soto-Suárez et al. 2017). For elicitor treatment, *Arabidopsis* plants were sprayed with an elicitor suspension obtained from *P. cucumerina* (300 µg/ml) as previously described (Coca and San Segundo 2010).

Plant tissue staining.

Trypan blue staining was performed as previously described (Ramírez et al. 2010). Stained material was observed using a Zeiss Axiophot microscope under bright-field illumination. At least 15 plants per genotype were analyzed.

Callose deposition was determined by aniline blue staining. 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 an epifluorescence microscope (Zeiss Axiophot microscope) under UV illumination. Callose deposition was quantified by determining the relative number of callose-corresponding pixels relative to the total number of pixels covering the plant material on digital photographs (Luna et al. 2011).

DAB (3,3′-diaminobenzidine) staining was used to determine H_2O_2 levels. For this, *Arabidopsis* plants were immersed in a DAB solution (1 mg/ml) with vacuum for 30 min, were maintained in the dark overnight, and were washed with 70% ethanol for 1 h. Plants were observed using an Olympus DP71 microscope under bright-field illumination. DAB staining intensities were quantified from digital photographs by the number of dark-brown DAB pixels relative to the leaf surface.

Callose measurements and H_2O_2 quantifications were made on at least 15 different seedlings per genotype. Statistically significant differences were determined by one-way ANOVA test.

Gene expression analyses.

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from TURBO DNAse (Ambion) treated total RNA (1 µg) with High Capacity cDNA reverse transcription kit (Applied Biosystems) and oligo-dT. Reverse transcription (RT)-qPCR tests were performed in optical 96-well plates, in a Light Cycler 480, using SYBR green (Roche). Primers were designed using Oligo Analyzer software (Integrated DNA Technologies). The *β-tubulin2* (At5g05620) expression was used to normalize the transcript level in each sample. Three independent biological replicates with three technical replicates were analyzed. An ANOVA test was used to evaluate differences in gene expression. Accumulation of mature miR773 sequences was determined by stem-loop RT-qPCR (Varkonyi-Gasic et al. 2007). Nucleotide sequencing confirmed the specific amplification of miR773 sequences.

For Northern blot analysis, the small RNA fraction obtained from total RNA (600 µg) was fractioned in a 17.5% polyacrylamide gel containing 8 M urea and was transferred to nylon membranes. Probes were 3′-labeled with digoxigenin (DIG), using the DIG oligonucleotide 3′-end labeling kit, following manufacturer's instructions (Roche).

MiR773 in situ hybridization.

Plants grown in in vitro conditions for 15 days were inoculated with *P. cucumerina* spores or were mock-inoculated. Mature leaves were harvested at 24 h postinoculation and were analyzed separately for in situ miR773 detection. Fixation and sample processing was performed as described by Wang et al. (2008). Hybridization was carried out with a 3′ DIG–labeled locked nucleic acid miR773a probe (Exiqon) overnight at 37°C. Detection of the hybridized probe was performed using an antibody solution (anti-DIG, alkaline phosphatase conjugated antibody) and the color substrate NBT (4-nitroblue-tetrazolium chloride).

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