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Cytidine-to-Uridine RNA Editing Factor NbMORF8 Negatively Regulates Plant Immunity to *Phytophthora* Pathogens[1]

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Mitochondria and chloroplasts play key roles in plant-pathogen interactions. Cytidine-to-uridine (C-to-U) RNA editing is a critical posttranscriptional modification in mitochondria and chloroplasts that is specific to flowering plants. Multiple organellar RNA-editing factors (MORFs) form a protein family that participates in C-to-U RNA editing, but little is known regarding their immune functions. Here, we report the identification of *NbMORF8*, a negative regulator of plant immunity to *Phytophthora* pathogens. Using virus-induced gene silencing and transient expression in *Nicotiana benthamiana*, we show that *NbMORF8* functions through the regulation of reactive oxygen species production, salicylic acid signaling, and accumulation of multiple Arg-X-Leu-Arg effectors of *Phytophthora* pathogens. NbMORF8 is localized to mitochondria and chloroplasts, and its immune function requires mitochondrial targeting. The conserved MORF box domain is not required for its immune function. Furthermore, we show that the preferentially mitochondrion-localized NbMORF proteins negatively regulate plant resistance against *Phytophthora*, whereas the preferentially chloroplast-localized ones are positive immune regulators. Our study reveals that the C-to-U RNA-editing factor *NbMORF8* negatively regulates plant immunity to the oomycete pathogen *Phytophthora* and that mitochondrion- and chloroplast-localized NbMORF family members exert opposing effects on immune regulation.

Mitochondria and chloroplasts, which serve as energy conversion sites within cells, play key roles in plant-pathogen interactions. Mitochondria and chloroplasts are important sources of reactive oxygen species (ROS), which may act as key defense molecules in plant immune responses and as signaling molecules during the spread of the hypersensitive response (HR; Amirsadeghi et al., 2007; Colombatti et al., 2014). The production of several plant hormones involved in immunity, such as jasmonic acid, salicylic acid (SA), and abscisic acid, depends on chloroplast metabolism (Apel and Hirt, 2004; Mittler et al., 2004; Nomura et al., 2012; Serrano et al., 2016). Due to the significant role played by mitochondria and chloroplasts in plant immunity, plant pathogens secrete many virulence effectors that are targeted to chloroplasts and mitochondria to modulate their effect on host immunity (Block et al., 2010; Rodríguez-Herva et al., 2012; de Torres Zabala et al., 2015). However, it remains largely unclear how mitochondrial and chloroplast proteins achieve modulation of the plant immune system.

Cytidine-to-uridine (C-to-U) RNA editing in mitochondria and chloroplasts, which is mainly regulated by nucleus-encoded RNA-editing factors, is a critical posttranscriptional modification specific to flowering plants (Gray and Covello, 1993; Takenaka et al., 2013; Barkan and Small, 2014; Shikanai, 2015; Yan et al., 2018). C-to-U RNA editing usually changes the first or second position of nucleic acid triplet codons, leading to altered protein sequences. Five groups of proteins participating in C-to-U RNA editing have been identified: pentatricopeptide repeat (PPR) proteins, multiple organelle RNA-editing factors (MORFs; also known as RNA-editing factor interacting proteins [RIPs]), organelle RNA recognition motif-containing proteins, protoporphyrinogen IX oxidase1, and organelle zinc finger1 (Zhang et al., 2014; Sun et al., 2015, 2016; Yan et al., 2018). Increasing evidence supports the

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conclusion that this type of posttranscriptional modification plays important roles in plant metabolism, adaptations to the environment, and signal transduction (Zsigmond et al., 2008; Yang et al., 2017; He et al., 2018; Yan et al., 2018).

PPR proteins directly interact with mRNA to determine the specificity of RNA editing, and a PPR protein specifically recognizes one or several editing sites (Barkan and Small, 2014). In land plants, the PPR family is greatly expanded; Arabidopsis (*Arabidopsis thaliana*) contains more than 400 PPR proteins, whereas there are many fewer PPR proteins in fungi, protists, and animals (Barkan and Small, 2014). In contrast, the MORF family only has nine members in Arabidopsis: two MORFs are targeted to plastids (AtMORF2 and AtMORF9), six are targeted to mitochondria (AtMORF1, AtMORF3, AtMORF4, AtMORF5, AtMORF6, and AtMORF7), and one (AtMORF8) localizes to both organelles (Bentolila et al., 2012; Takenaka et al., 2012). The role of MORFs is to interact with other RNA-editing factors to form an RNA editosome (Bentolila et al., 2012; Härtel et al., 2013; Takenaka et al., 2013; Brehme et al., 2015; Glass et al., 2015; Hackett et al., 2017; Ma et al., 2017). For example, MORF8 has been shown to be a component in multiple editing complexes. Unlike the PPR proteins, each MORF protein participates in multiple RNA-editing sites. In addition, MORFs usually form homomers or heteromers to aid in editosome formation (Zehrmann et al., 2015). Sequence alignment of all nine known MORF family proteins in Arabidopsis showed that they share a conserved motif, the so-called MORF box, which is approximately 100 amino acid residues from the N terminus of the protein (Takenaka et al., 2012). Given the large number of RNA-editing factors (~400 PPR proteins, nine MORFs, and six organelle RNA recognition motif-containing proteins in Arabidopsis; Yan et al., 2018), our understanding of their roles in regulating plant immunity is limited. To date, there has only been one report on the role of RNA editing-related factors in plant immunity, that being for the Arabidopsis chloroplast-located protein OCP3, which regulates resistance to the necrotrophic pathogen *Plectosphaerella cucumerina* by regulating the RNA editing of the chloroplast gene *ndhB* (García-Andrade et al., 2013).

To detect and respond to invading pathogens, plants have evolved pattern recognition receptors to recognize conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (Jones and Dangl, 2006). PAMP-triggered immunity (PTI) is the basal immune response to broad-spectrum pathogens. To overcome this basal immune system, pathogens have developed a variety of effectors. In turn, plants have developed a second group of receptors, nucleotide-binding-leucine-rich repeat receptors (NLRs), to detect the presence of effectors, resulting in effector-triggered immunity (ETI; Jones and Dangl, 2006). Effectors can be directly or indirectly recognized by corresponding NLR proteins, leading to strong and fast cell death, called the HR, hence restricting pathogen growth (Jones and Dangl, 2006; Schwessinger and Ronald, 2012). The effectors that are recognized by the NLR proteins are called avirulence (AVR) proteins. Many *Avr* and *Resistance* (*R*) gene pairs have been cloned from *Phytophthora* spp. pathogens and their hosts, such as the *Phytophthora infestans Avr* genes *PiAvr3a* (Armstrong et al., 2005), *PiAvrblb1* (Vleeshouwers et al., 2008), and *PiAvrVnt1* (Pel et al., 2009) and the corresponding potato (*Solanum tuberosum*) *R* genes *R3a* (Huang et al., 2005), *RB* (Song et al., 2003), and *RpiVnt1* (Foster et al., 2009).

Phytophthora represent a unique group of plant pathogens called oomycetes that are phylogenetically distant from true fungi. Nearly all 120 *Phytophthora* species identified in the genus are plant pathogens, capable of infecting hundreds of plant species, including many important crops, and causing devastating diseases leading to huge economic losses every year (Kamoun et al., 2015). The most well-known is *P. infestans*, the causal agent of potato late blight and the Great Irish Famine in the 19th century. While the recognition of pathogen effectors is genetically well known, molecularly well characterized, and widely used for the development of genotype-specific disease resistance, little is known about the genetic basis of plant susceptibility to *Phytophthora* pathogens.

Analysis of the roles of *MORF* genes, beyond their RNA-editing function, has been hampered by lethal or growth retardation consequences resulting from their in planta expression suppression. In this study, we employ virus-induced gene silencing (VIGS) and *Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana benthamiana* to investigate the immune function of *NbMORF* genes that were responsive to infection by *Phytophthora parasitica*. We showed that NbMORF8 was localized in chloroplasts and mitochondria and played a substantial role in immunity by negatively regulating plant resistance against *Phytophthora* pathogens. Its immune function involves the regulation of ROS burst, the SA signaling pathway, and the accumulation of multiple RXLR (Arg-X-Leu-Arg) effectors of *Phytophthora* pathogens. Furthermore, we found that the immune function of *NbMORF8* is independent of its conserved MORF box domain. We also found that mitochondrion-preferred NbMORF proteins (NbMORF1a and NbMORF1b) negatively regulated plant resistance to *Phytophthora*, whereas chloroplast-preferred MORF proteins (NbMORF2b, NbMORF2c, and NbMORF9) positively regulated plant immunity.

RESULTS

Mitochondrion- and Chloroplast-Localized MORF Proteins Exert Opposite Immune Functions against *P. parasitica*

A VIGS-based approach was employed to identify negative regulators of plant resistance to *P. parasitica*. This led to the identification of NbMORF8, an ortholog of AtMORF8 as revealed by rigorous phylogenetic

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analysis, which is a MORF family protein. We also found that the expression of multiple annotated *NbMORF* members was up-regulated in *N. benthamiana* during *P. parasitica* infection, as shown in our RNA sequencing data (Jia, 2017). These results suggested that *MORF* family genes may participate in the interaction between *P. parasitica* and *N. benthamiana*. Hence, we decided to further explore the potential immune role of *NbMORF* genes in *N. benthamiana*.

To characterize putative MORF members in *N. benthamiana*, we performed a BLASTP search against the predicted gene open reading frames of *N. benthamiana* using Arabidopsis MORF proteins as queries to identify candidate *NbMORF* genes. Twenty candidate *NbMORF* genes were obtained (Supplemental Table S1). We further cloned eight candidate genes, using PCR amplification from cDNA libraries: *NbMORF1a*, *NbMORF1b*, *NbMORF2a*, *NbMORF2b*, *NbMORF2c*, *NbMORF8a*, *NbMORF8b*, and *NbMORF9*. The genes were named according to the Arabidopsis orthologs. *NbMORF8a* was the negative regulator that we identified using VIGS. A phylogenetic tree was constructed. All NbMORF proteins contain a conserved MORF box sequence of ~100 amino acid residues, like their Arabidopsis orthologs (Fig. 1; Supplemental Fig. S1). However, NbMORF2a lacks an N-terminal amino acid sequence preceding the MORF box. NbMORF8b is truncated from the C-terminal end to within the MORF box when compared with NbMORF8a (Fig. 1; Supplemental Fig. S1).

To test the immune functions of these *NbMORF* genes, we performed VIGS assays on *N. benthamiana* followed by *P. parasitica* inoculation. *NbMORF1a* and *NbMORF1b* or *NbMORF2b* and *NbMORF2c* showed high sequence similarity, so we cosilenced *NbMORF1a/1b* and *NbMORF2b/2c*, respectively (Supplemental Fig. S2). Leaves detached from plants 14 d after inoculation with VIGS constructs were inoculated with *P. parasitica* zoospores. These results showed that the *TRV-NbMORF2a*

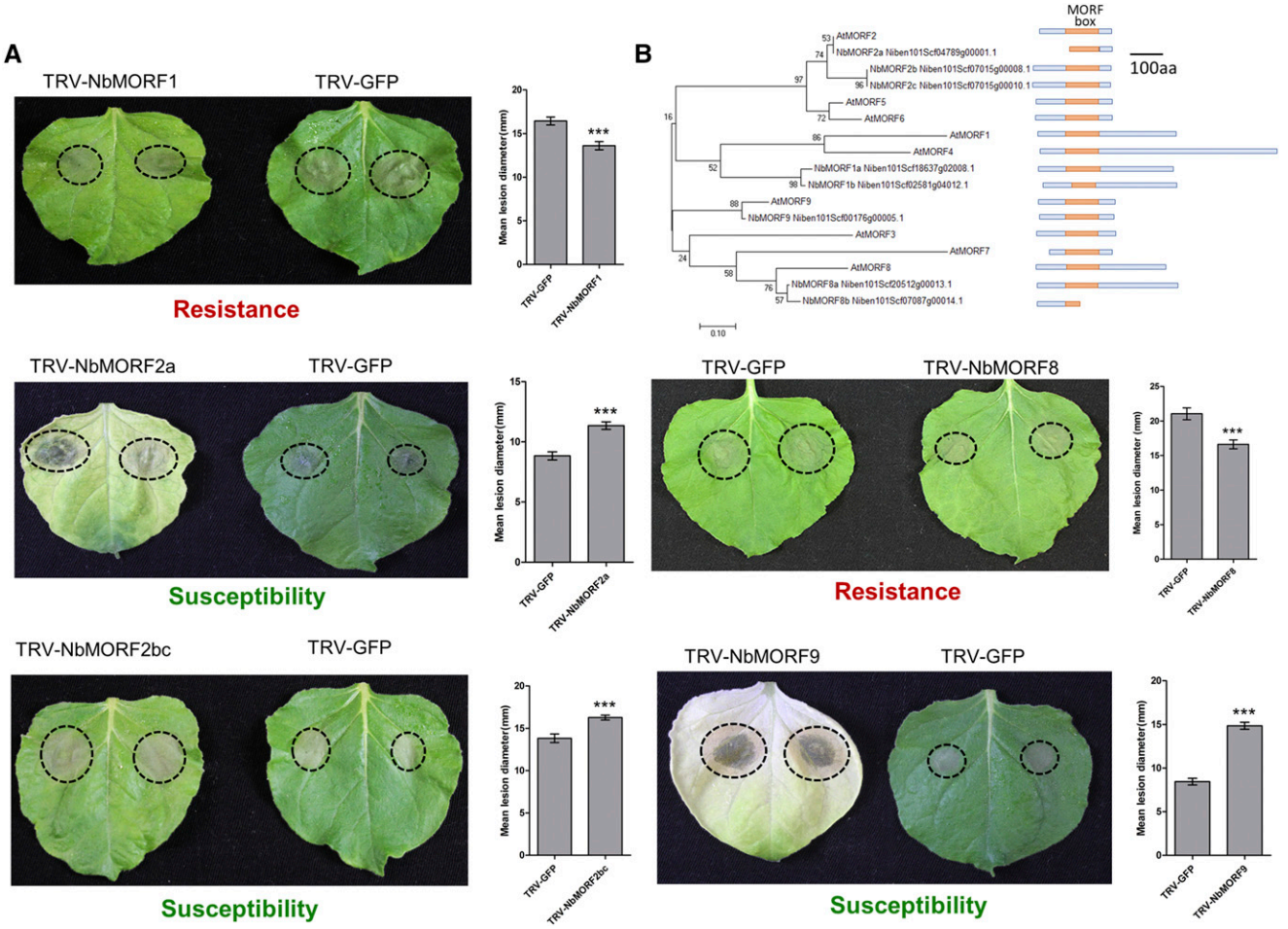

Figure 1. MORF proteins play different immune roles in response to *Phytophthora* pathogens in *N. benthamiana*. A, Silencing *NbMORF1a/1b*, *NbMORF2a*, *NbMORF2b/c*, *NbMORF8*, or *NbMORF9* in *N. benthamiana* led to different responses to *P. parasitica*. Images were taken at ~40 h after inoculation with *P. parasitica* zoospores. Results are means ± SE of 20 infections from at least 10 leaves. Statistical significance was assessed by Student's *t* test (***$P < 0.001$). Similar results were observed in three independent experiments. B, Cladogram of similarities between the AtMORF and NbMORF proteins. The phylogenetic tree was constructed by using the neighbor-joining method. All NbMORF proteins share a conserved MORF box. aa, Amino acids.

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and *TRV-NbMORF9* plants exhibited bleached leaves and were more susceptible to *P. parasitica* (Fig. 1; Supplemental Fig. S2). *TRV-NbMORF2b/2c* plants were more susceptible but did not exhibit any bleaching phenotype (Fig. 1; Supplemental Fig. S2). Silencing *NbMORF1a/1b* or *NbMORF8* enhanced resistance to pathogens and showed reduced plant height, malformed leaves and flowers, and infertility when the plants began forming flowers (Fig. 1; Supplemental Figs. S2 and S3). Quantitation of gene expression confirmed that *NbMORF* genes in VIGS plants were at least 80% reduced (Supplemental Fig. S2). As silencing of *NbMORF2a* or *NbMORF9* resulted in bleached leaves, we did not analyze them further. PTI and ETI are the two major layers of the plant immune system. To examine whether *NbMORF* genes participate in PTI- or ETI-induced HR, we transiently expressed the *P. infestans* elicitin gene *INF1*, *Bax*, *P. infestans* RXLR effector genes, and cognate potato *R* genes *R3a/Avr3a*^{KI}, *RB/Avrblb1*, and *RpiVnt1/AvrVnt1* in the *NbMORF*-silenced leaves. The results showed that *NbMORF1ab*- or *NbMORF2bc*-silenced leaves had no influence on HR (Supplemental Fig. S4). However, silencing *NbMORF8* suppressed HR induced by R/AVR recognition but not by INF1 or Bax (Fig. 2). These results suggest that *NbMORF8* participates in the ETI-induced HR response.

To further confirm the role of *NbMORF* genes in immunity, *NbMORF1a*, *NbMORF1b*, *NbMORF2b*, *NbMORF2c*, *NbMORF8a*, and *NbMORF8b* were overexpressed in *N. benthamiana* leaves followed by inoculation with *P. parasitica*. The results showed that overexpression of *NbMORF1a*, *NbMORF1b*, or *NbMORF8a* enhanced plant susceptibility to *P. parasitica* (Fig. 3; Supplemental Fig. S5), while overexpression of *NbMORF2b* or *NbMORF2c* led to increased resistance. Overexpression of *NbMORF8b* had no effect on plant immunity (Supplemental Fig. S5). We also found that the transcript levels of *NbMORF1a*, *NbMORF1b*, *NbMORF2a*, *NbMORF2b*, *NbMORF2c*, *NbMORF8a*, and *NbMORF9* were all induced during infection (Supplemental Fig. S6; primers to distinguish *NbMORF2b* and *NbMORF2c* could not be designed because of the high sequence similarity). These results implied that *NbMORF* genes, with the exception of *NbMORF8b*, are involved in plant immune response to *Phytophthora* infection.

It was reported that all AtMORF family proteins are targeted to mitochondria or chloroplasts (Bentolila et al., 2012; Takenaka et al., 2012). To examine whether NbMORF proteins have similar subcellular localization as their Arabidopsis orthologs, we performed transient expression of GFP-tagged NbMORF proteins in *N. benthamiana* and monitored fluorescence using confocal microscopy. The localization of six NbMORF proteins (NbMORF1a, NbMORF1b, NbMORF2b, NbMORF2c, NbMORF8a, and NbMORF8b) was dually targeted to mitochondria and chloroplasts (Fig. 3; Supplemental Fig. S7). NbMORF9 was detected only in chloroplasts (Supplemental Fig. S7). However, the mean density analysis, which showed the fluorescence intensities of mitochondria and chloroplasts, indicated that NbMORF1a and NbMORF1b were preferentially targeted to mitochondria, while NbMORF2b and NbMORF2c were preferentially targeted to chloroplasts. NbMORF8a and NbMORF8b were targeted to both mitochondria and chloroplasts without preferences (Fig. 3). NbMORF2a, which lacks a leading peptide, was targeted to the cytoplasm and nucleus (Supplemental Fig. S7).

To determine whether the localization of NbMORF proteins (NbMORF1a, NbMORF1b, NbMORF2b, NbMORF8a, and NbMORF2c) to either plastids or plastids and mitochondria is required for their immune functions, we targeted GFP-tagged NbMORF proteins to chloroplasts (CpNbMORF) by replacing the NbMORF leading peptides with AtMORF9's leading peptide, which is reported to target the protein to the chloroplast (Takenaka et al., 2012). NbMORF2b and NbMORF2c only differ in the leading peptide. The results showed that the GFP-derived fluorescence was detected exclusively in the chloroplasts (Fig. 3; Supplemental Fig. S8). The pathogen inoculation assay showed that the targeted chloroplast localization of NbMORF1a, NbMORF1b, and NbMORF8a abolished their ability to enhance plant susceptibility (Supplemental Fig. S9). However, NbMORF2b/2c still enhanced the resistance (Supplemental Fig. S9). These results demonstrate that mitochondrion-preferred NbMORF members (NbMORF1a and NbMORF1b) are negative regulators of host immunity, while chloroplast-preferred NbMORF members (NbMORF2b, NbMORF2c, and NbMORF9) are positive regulators. NbMORF8a, targeted to both organelles, functions in mitochondria and acts as a negative regulator of plant immunity to *P. parasitica*.

NbMORF8 Is a Negative Regulator of Plant Immunity to Multiple *Phytophthora* Pathogens

As *NbMORF8* silencing not only showed enhanced resistance to *P. parasitica* but also attenuated HR induced by ETI, we chose *NbMORF8* for further analysis. To examine whether *NbMORF8* silencing conferred resistance to different *Phytophthora* pathogens, we also inoculated *TRV-NbMORF8* leaves with *P. infestans* and *Phytophthora capsici* (Supplemental Fig. S10). The results consistently showed that silencing *NbMORF8* conferred enhanced resistance to all tested *Phytophthora* pathogens. Both the lesion diameter of these two pathogens and the sporulation of *P. infestans* were significantly reduced (Supplemental Fig. S10).

While *NbMORF8*-silenced plants were morphologically indistinguishable from the *TRV-GFP* control plants at the point of pathogen inoculation (Supplemental Fig. S3), they started to show altered growth phenotypes, including reduced plant height, malformed leaves and flowers, and infertility when progressing from vegetative growth to reproductive stages (Supplemental Fig. S3), suggesting a role in plant development. *NbMORF8*-silenced plants exhibited fewer flowers compared with the *TRV-GFP*

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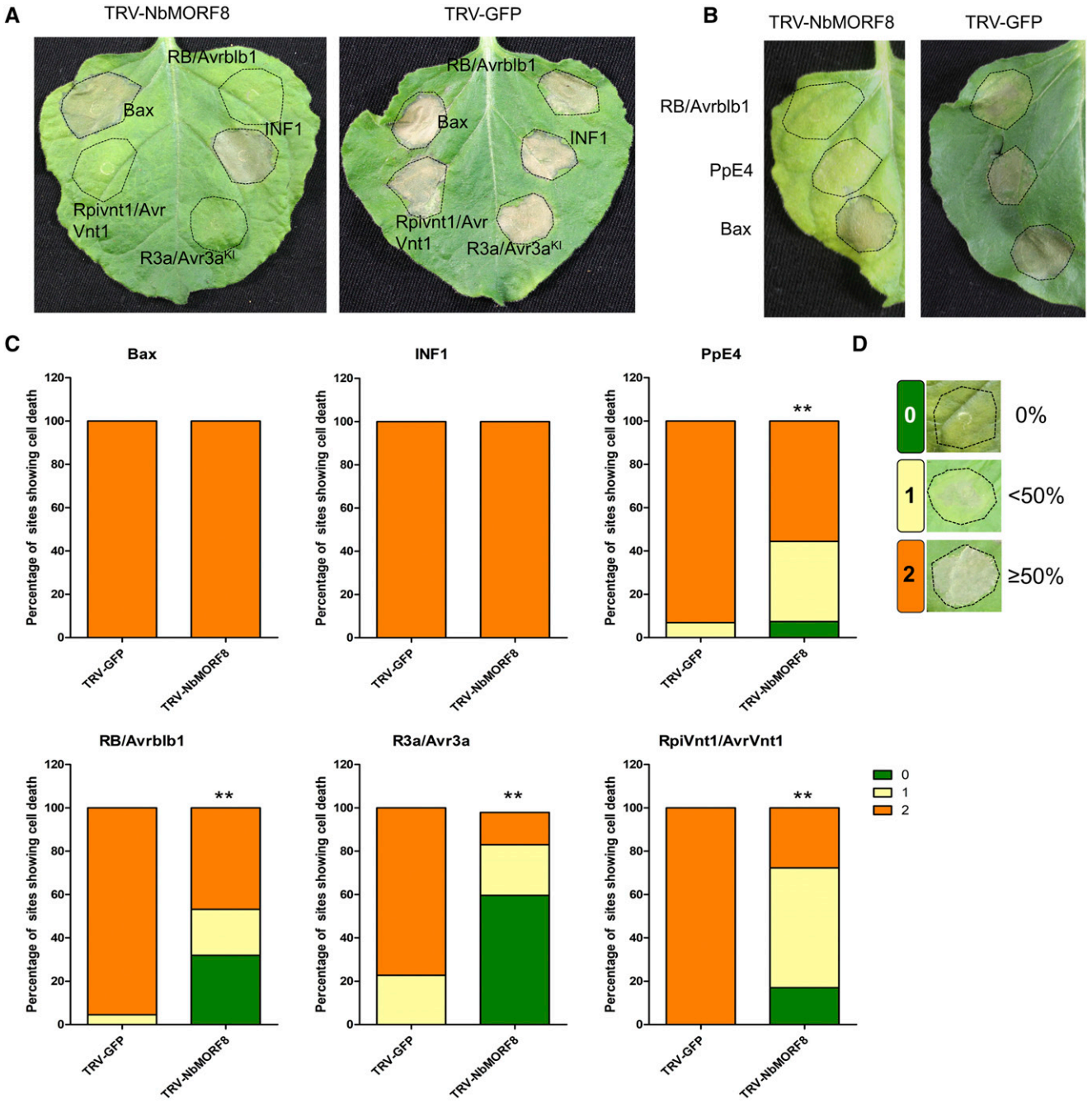

Figure 2. Silencing *NbMORF8* attenuates cell death induced by the recognition of RXLR effectors of *Phytophthora* pathogens in *N. benthamiana*. A, Cell death observation of NbMORF8-silenced and control plants. Images were taken at 5 d after *A. tumefaciens*-mediated transient expression of *Avr*/*R* gene pairs, *INF1*, and *Bax* on VIGS plants. B, *NbMORF8*-silenced plants attenuated cell death induced by the *P. parasitica* effector PpE4. C, Cell death severity assessment of *NbMORF8*-silenced leaves and control leaves. Results are means ± SE of at least 25 leaves from 10 plants for each group. Statistical significance was assessed by the Wilcoxon-Mann-Whitney test (**$P < 0.01$). Similar results were observed in at least six independent experiments. 0, No necrosis (green); 1, necrosis area less than 50% of the agroinfiltrated area (yellow); 2, necrosis area greater than 50% of the agroinfiltrated area (orange). D, Quantitation of cell death. Numbers and colors are as in C.

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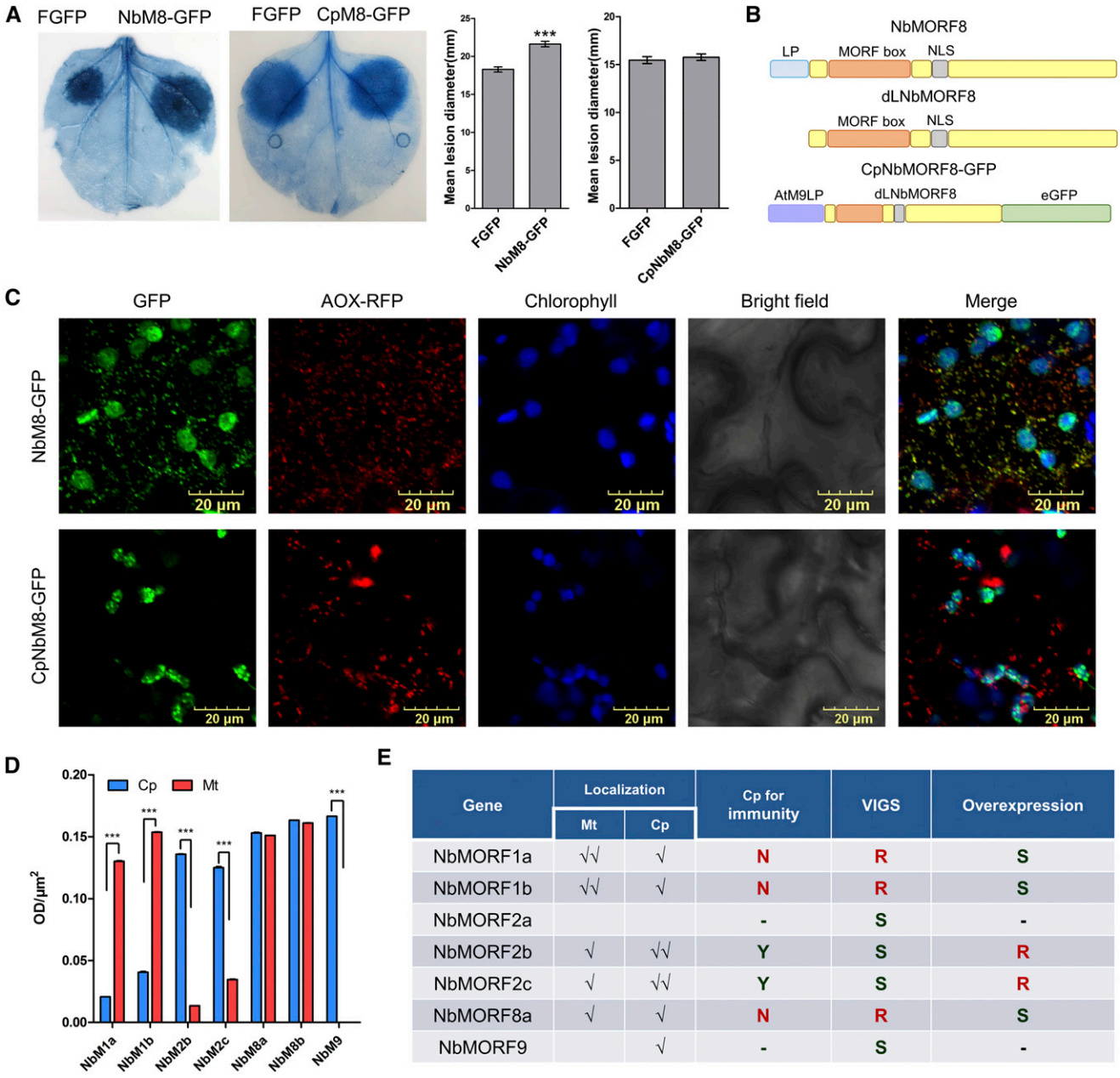

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Gene	Localization		Cp for immunity	VIGS	Overexpression
	Mt	Cp			
NbMORF1a	√√	√	N	R	S
NbMORF1b	√√	√	N	R	S
NbMORF2a			-	S	-
NbMORF2b	√	√√	Y	S	R
NbMORF2c	√	√√	Y	S	R
NbMORF8a	√	√	N	R	S
NbMORF9		√	-	S	-

Figure 3. Mitochondrion- and chloroplast-localized MORF proteins exert opposing roles in the immune response to *Phytophthora* pathogens in *N. benthamiana*. A, *P. parasitica* inoculation assay on *CpNbMORF8* or *NbMORF8* overexpression leaves. Images were taken at 36 h after zoospore inoculation, with *GFP* plants used as a control. The inoculated leaves were stained with Trypan Blue to indicate the lesion area. Lesion diameter results are means ± SE of 10 biological replicates. Similar results were observed in three independent experiments. Statistical significance was assessed by Student's *t* test (***$P < 0.001$). B, Schematic view of NbMORF8, dLNbMORF8, and CpNbMORF8. dL, Deleting leading peptide; LP, leading peptide; NLS, nuclear localization signal. The leading peptide of NbMORF8 was replaced with the AtMORF9 leading peptide to retarget the fusion proteins to the chloroplast. The NbMORFs were analyzed using the same method. C, Subcellular localization of NbMORF8 and CpNbMORF8. Confocal microscopy shows *N. benthamiana* leaves expressing *NbMORF8-GFP* or *CpNbMORF8-GFP*. Subcellular localization was observed at 2 or 3 d post agroinfiltration. AOX-RFP was used as a mitochondrial marker. Chloroplasts of *N. benthamiana* leaf cells were identified by their chlorophyll autofluorescence, shown in blue. D, Mean density analysis of mitochondria and chloroplasts in the subcellular localization images of NbMORF proteins. The three mitochondria or chloroplasts showing the strongest fluorescence were analyzed from each image of NbMORF proteins using ImageJ. Three images of each NbMORF were analyzed. Results are means ± SE of nine organelles from three images. Statistical significance was assessed by Student's *t* test (***$P < 0.001$). E, Summary of subcellular localization and immune function of NbMORF proteins. Cp, Chloroplast; Mt, mitochondrion; N, chloroplast localization is not required for immune function; R, resistant to *P. parasitica*;

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control plants, and most of these flowers had deformed petals, with very few pollen particles and a shortened stigma, leading to sterility (Supplemental Fig. S3).

Silencing *NbMORF8* Leads to Enhanced ROS Levels and Up-Regulated Expression of the Defense-Related Genes *NbPR1* and *NbPR2*

As mitochondria and chloroplasts are important sources of ROS (Amirsadeghi et al., 2007; Colombatti et al., 2014) and NbMORF8 targets these two organelles, we examined ROS levels in the *TRV-NbMORF8* plants using a luminol-based chemiluminescence assay. As shown in Figure 4, upon PAMP flg22 treatment, silencing *NbMORF8* resulted in higher ROS levels compared with the *TRV-GFP* plants, suggesting that *NbMORF8* may regulate plant immunity through the regulation of ROS bursts. Interestingly, water treatment also induced ROS bursts in *NbMORF8*-silenced plants after 10 min of treatment.

To further examine whether *NbMORF8* participates in the regulation of immune signaling pathways, we tested the expression levels of the PTI marker genes *NbWRKY7* and *NbWRKY8* (Yan et al., 2016), the SA pathway markers *NbPR1* and *NbPR2* (Yan et al., 2016), and the jasmonic acid pathway markers *NbPR3* and *NbPR4* (Yang et al., 2016) in *NbMORF8*-silenced plants. The results showed that silencing *NbMORF8* up-regulated *NbPR1* and *NbPR2* expression even without inoculation of *P. parasitica* (Fig. 4). We further detected the expression of *NbPR1* and *NbPR2* during the early infection stage of *P. parasitica* on *TRV-NbMORF8* leaves. These results indicate that *NbMORF8* suppresses plant immunity by negatively regulating *NbPR1* and *NbPR2* expression and the SA signaling pathway (Fig. 4).

NbMORF8 Is Involved in C-to-U RNA Editing of Mitochondria and Chloroplast Genes

As NbMORF proteins and their RNA-editing sites in *N. benthamiana* are not certain, we examined whether NbMORF8 functions in C-to-U RNA editing. DNA sequencing has been widely used to identify RNA-editing sites and to measure editing levels in recent years (Zhu et al., 2012; Härtel et al., 2013; Brehme et al., 2015; Shi et al., 2015; Yang et al., 2017; He et al., 2018; Zhao et al., 2019). We amplified and sequenced orthologs of target genes shown to be edited in Arabidopsis by AtMORF8 (Bentolila et al., 2012, 2013; Glass et al., 2015) from cDNA isolated from *TRV-GFP* and *TRV-NbMORF8* plants, respectively, and *N. benthamiana* genomic DNA. The *NbMORF8*-silenced plants showed significant reductions in the level of editing of the mitochondrial genes *ccb206* (eight of 33 sites), which plays a role in cytochrome *c* synthesis; *cob* (one of eight sites), which encodes a subunit of complex III; and the chloroplast gene *ndhB* (one of eight sites), which encodes a subunit of NADH dehydrogenase (Fig. 5). However, editing of *ndhB*-242 was only slightly reduced. We further confirmed the defects in editing of *cob*-853 and *ndhB*-242 using high-resolution melting (HRM) analysis (Supplemental Fig. S11).

The ccb206 protein is involved in the synthesis of cytochrome *c* that participates in electron transport (Itani and Handa, 1998). Editing of *ccb206* transcripts in *TRV-NbMORF8* plants was the most affected, and loss of editing was predicted to substantially change the ccb206 transmembrane structure (Fig. 5; Supplemental Fig. S12), which suggests that silencing *NbMORF8* may reduce cytochrome *c* levels. Furthermore, since *cob* encodes a subunit of complex III in the mitochondrial electron transport chain (Weiss, 1987), loss of RNA editing may cause defects in complex III function. We further examined the levels of cytochrome *c* and complex III activities in both *TRV-NbMORF8* and *TRV-GFP* plants using ELISA. The results showed that the cytochrome *c* level and complex III activities were significantly reduced in *NbMORF8*-silenced plants (Fig. 5).

Editing of *ccb206* transcripts in *TRV-NbMORF8* plants was the most affected. Hence, we also examined the editing of *ccb206* transcripts during the early infection stage by *P. parasitica*, during which the editing of two more sites, *ccb206*-367 and *ccb206*-380, was significantly reduced in the *NbMORF8*-silenced plants (Fig. 5). In addition, the level of RNA editing of *ccb206*-367 was about 80% without inoculation but was further up-regulated in the *TRV-GFP* plants when the plants were inoculated with *P. parasitica* (Fig. 5), suggesting that the RNA editing of *ccb206* may also be regulated by *P. parasitica* infection.

AtMORF8 was reported to form homomers (Zehrmann et al., 2015; Bayer-Császár et al., 2017). We used a yeast two-hybrid assay to examine whether NbMORF8 had a similar function and confirmed that NbMORF8 could form homomers in yeast cells (Fig. 5), which indicates that NbMORF8 has a similar function to its Arabidopsis ortholog *AtMORF8* and plays a role in C-to-U RNA editing.

The MORF Box of NbMORF8 Is Not Required for Its Immune Function

The conserved MORF box is crucial for MORF protein interaction with PPRs and the formation of heteromers or homomers. Moreover, it has distinct affinities to the PPR to regulate the RNA editing of different sites (Bayer-Császár et al., 2017; Haag et al.,

Figure 3. (*Continued.*)
S, susceptible to *P. parasitica*; Y, chloroplast localization is required for immune function; √, NbMORF protein localized; √√, NbMORF protein preference; –, not determined.

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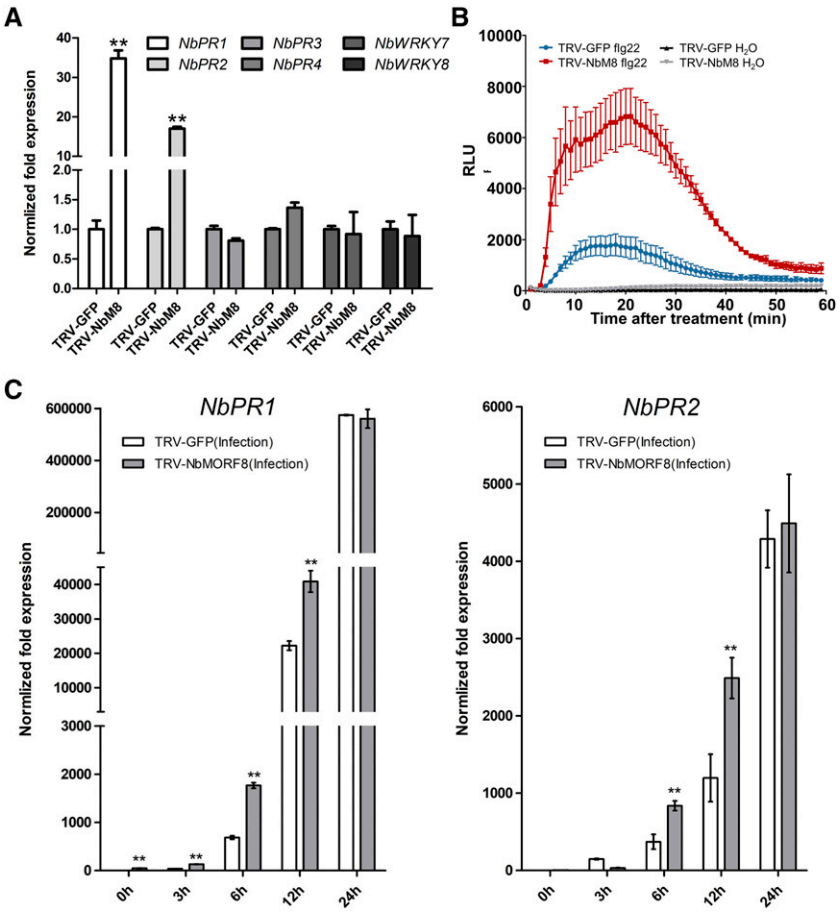

Figure 4. Silencing *NbMORF8* up-regulated the expression of defense-related genes and enhanced ROS levels. A, Up-regulated expression of defense-related genes *NbPR1* and *NbPR2* in *TRV-NbMORF8* plants without pathogen treatment, as determined by quantitative PCR (qPCR). Results are means ± SE. Statistical significance was assessed by Student's *t* test (**$P < 0.01$). B, ROS burst upon flg22 treatment of *NbMORF8*-silenced leaves. At least 12 leaves from six plants of each group were measured using a luminol-based chemiluminescence assay. RLU, Relative light units. C, Expression levels of *NbPR1* and *NbPR2* in *TRV-NbMORF8* plants in the early *P. parasitica* infection stage. Total RNA was extracted from *P. parasitica* zoospore-infected leaves at 3, 6, 12, and 24 h post inoculation. The *N. benthamiana EF1α* gene was used as an internal control. Results are means ± SE. Statistical significance was assessed by Student's *t* test (**$P < 0.01$). Similar results were observed in at least three independent experiments.

2017). To analyze whether the immune function of NbMORF8 was dependent on its MORF box, we created a series of deletion mutant constructs (Fig. 6). All the mutant constructs preserved the leading peptide to avoid altered localization. The localization of NbMORF8 deletion mutants was monitored by transient expression of GFP-tagged versions in *N. benthamiana* followed by confocal microscopy observation. All NbMORF8 mutant proteins were localized in mitochondria and chloroplasts, like NbMORF8 (Fig. 6). Mutants were overexpressed in *N. benthamiana* leaves, with *GFP* as a control, followed by inoculation with *P. parasitica* zoospores. The results showed that overexpression of NbMORF8 mutants with the MORF box deleted displayed higher levels of susceptibility to *P. parasitica* compared with the full-length NbMORF8 (Fig. 6). Overexpression of the leading peptide of NbMORF8 and the mutant dCNbMORF8 did not promote susceptibility to *P. parasitica* (Fig. 6).

We also found that NbMORF8 contains a nuclear localization sequence, as was predicted by LOCALIZER (Sperschneider et al., 2017; Fig. 1). To determine whether NbMORF8 has an immune function outside mitochondria and chloroplasts, we generated a construct with the leading peptide (dLNbMORF8) deleted and overexpressed it in *N. benthamiana* using GFP as a control. This was followed by *P. parasitica* inoculation. The results showed that GFP-tagged dLNbMORF8 was localized in the nucleus and cytoplasm and its ability to increase susceptibility was abolished (Supplemental Fig. S13). These results suggest that NbMORF8 exerts its immune function in mitochondria but not in the nucleus or cytoplasm. We confirmed the overexpression of all deletion mutants in *N. benthamiana* leaves (Supplemental Figs. S14 and S15). These results suggest that the MORF box is not required for the immune function of NbMORF8 and may instead suppress its immune function.

To examine the RNA-editing activity of the NbMORF8 deletion mutants, we performed sequencing to identify RNA editing in the overexpression plant leaves. The results showed that overexpression of *NbMORF8* or full-length *NbMORF8* led to slightly decreased or no changing of editing (Supplemental Fig. S14). As MORF proteins can form homomers to function as RNA-editing factors, we analyzed the interaction between full-length NbMORF8 and the NbMORF8-N deletion mutant, the shortest mutant that still had immune function, using yeast two-hybrid analysis. The results showed that NbMORF8-N lost interaction with full-length NbMORF8 although it retained its immune function (Fig. 6), suggesting that the immune function of *NbMORF8* does not require the interaction activity.

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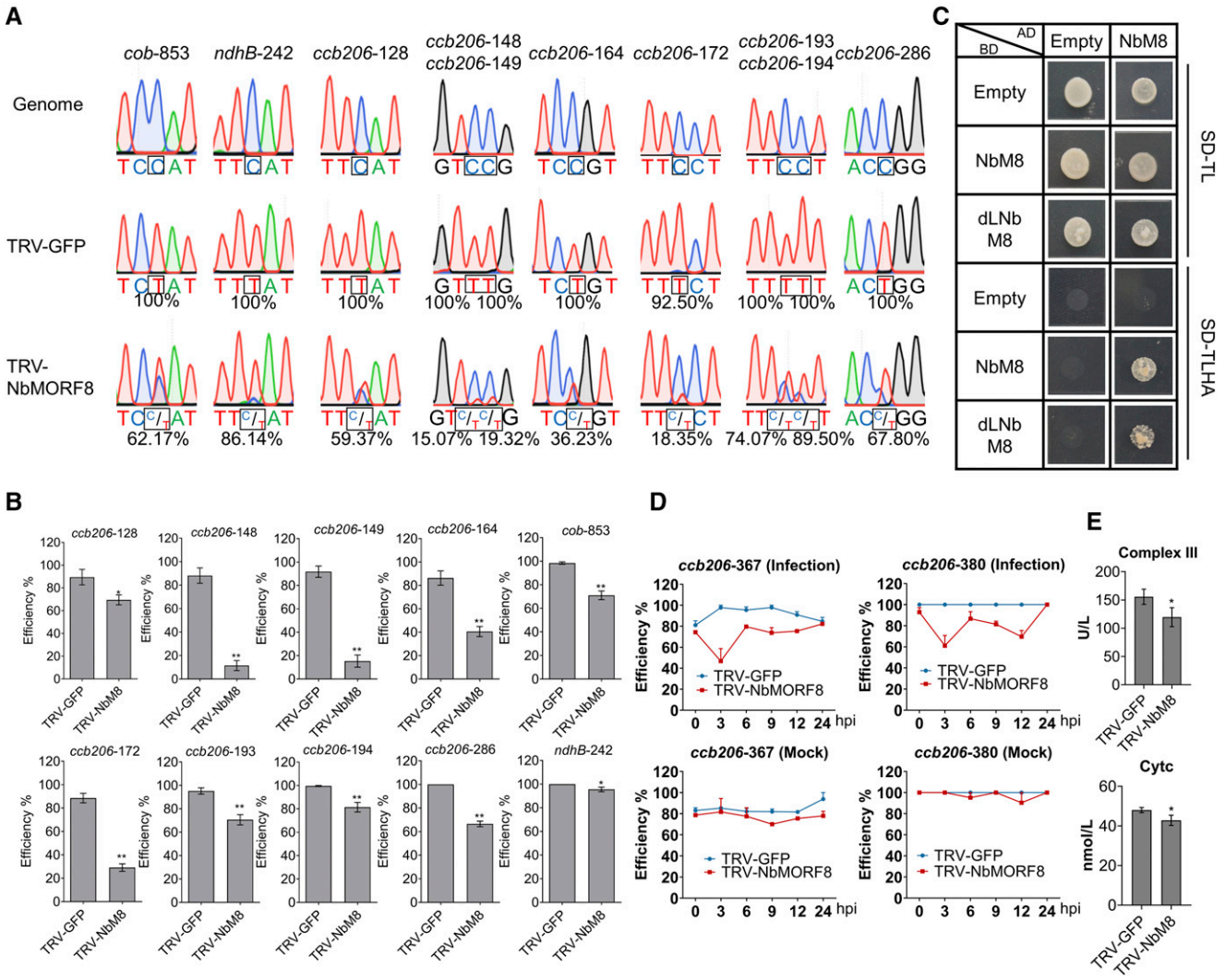

Figure 5. Silencing *NbMORF8* impaired RNA editing of *ccb206*, *cob*, and *ndhB*. A and B, Editing levels of mitochondrial *ccb206* transcripts at sites 128, 148, 149, 164, 172, 193, 194, and 286, *cob* at site 853, and chloroplast *ndhb* at site 242. Results are means ± SE of three biological replicates. Statistical significance was assessed by Student's *t* test (*$P < 0.05$ and **$P < 0.01$). C, Yeast transformants were separately transferred onto synthetic dextrose (SD)/-Leu/-Trp (SD-TL) and SD-Leu/-Trp/-His/-Ade (SD-TLHA) medium. The growth of yeast transformants on SD-TL medium demonstrated successful transformations. The growth of yeast transformants on SD-TLHA indicates interactions. The image was taken 3 d after dropping the transformed yeast on SD-TLHA medium. D, The RNA editing of *ccb206* transcripts at sites 367 and 380 was down-regulated in *NbMORF8*-silenced plants during *P. parasitica* infection. Total RNA was extracted from *P. parasitica* zoospore-infected leaves of *TRV-NbMORF8* and *TRV-GFP* leaves at 3, 6, 12, 24, and 48 h post inoculation (hpi). Water was used as a control at each time point. Results are means ± SE of three biological replicates. E, The cytochrome *c* levels and complex III activities were detected using ELISA. Results are means ± SE of three biological replicates. Statistical significance was assessed by Student's *t* test (*$P < 0.05$).

Silencing *NbMORF8* Suppresses the Accumulation of RXLR Effectors

We found that silencing *MORF8* attenuated HR induced by *R3a/Avr3a*^{KI}, *RB/Avrblb1*, and *RpiVnt1/AvrVnt1* recognition but not cell death induced by INF1 or Bax (Fig. 2). We also tested the *P. parasitica* RXLR effector PpE4 (Huang et al., 2019) in *TRV-NbMORF8* plants. Silencing *NbMORF8* consistently attenuated cell death induced by PpE4, which triggers cell death in *N. benthamiana* (Fig. 2). To test whether silencing *NbMORF8* affects the efficiency of *A. tumefaciens*-mediated transient expression, we examined protein accumulation of AVR3a^{KI} and AVRblb1, whose HR was attenuated, and two more effectors (AVRblb2 and PcAVR3a12) that did not trigger cell death on *NbMORF8*-silenced plant leaves, using GFP as a control. For all the effectors examined, no differences were notable between transcript levels in *NbMORF8*-silenced plants and the control (Fig. 7). However, the accumulation of RXLR effector proteins was substantially decreased in

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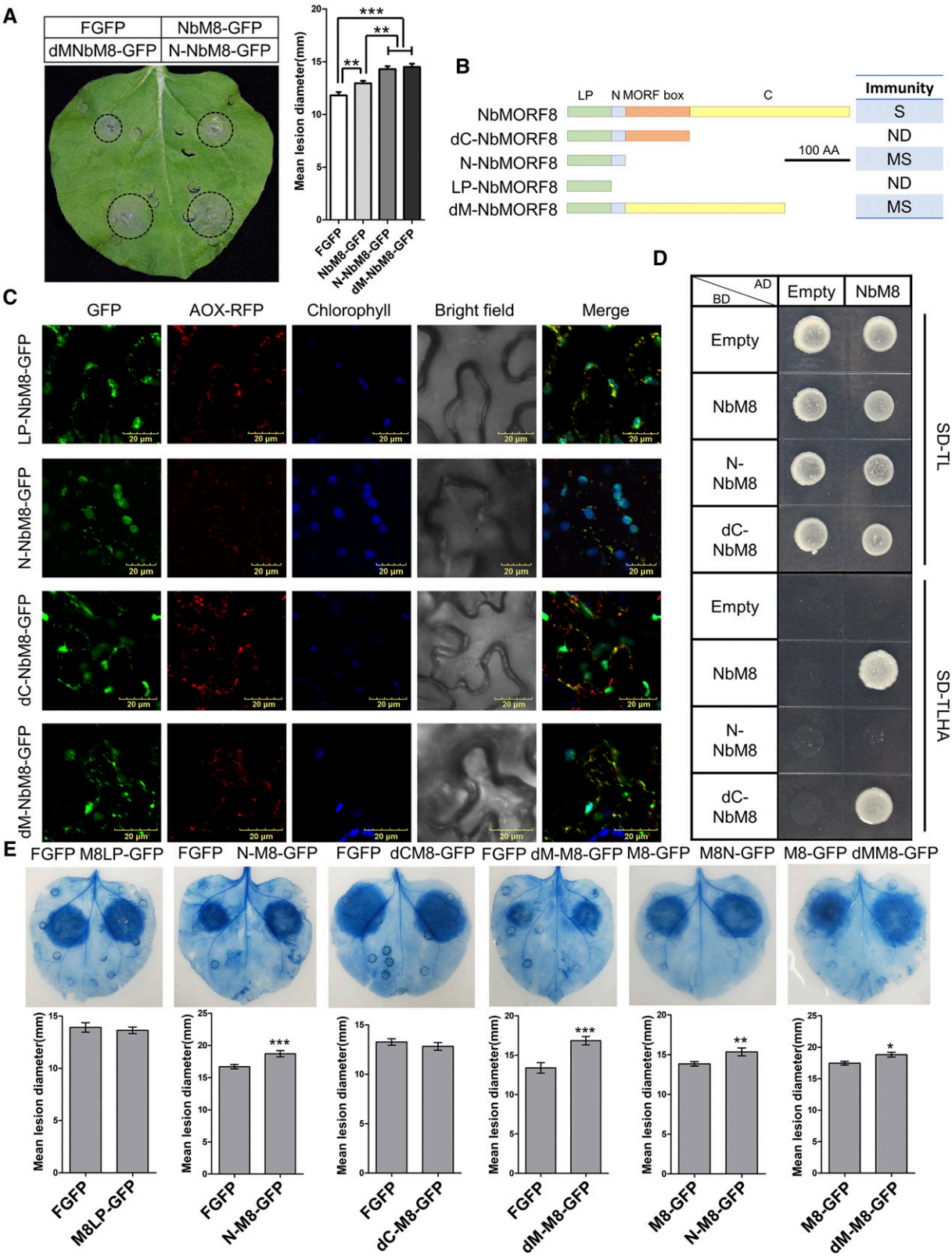

Figure 6. The MORF box domain suppresses the immune function of *NbMORF8*. A, Overexpression of *NbMORF8* mutants without the MORF box rendered plants more susceptible than the full-length *NbMORF8*. Transient expression of *N-NbM8-GFP*, *dM-NbM8-GFP*, *NbM8-GFP*, and *GFP* in single *N. benthamiana* leaves was done by agroinfiltration, followed by inoculation with *P. parasitica* zoospores. Images were taken at 40 h after zoospore inoculation. Results are means ± SE of nine biological

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NbMORF8-silenced plants (Fig. 7). Fluorescence intensities and GFP accumulation levels were increased in *NbMORF8*-silenced leaves, which indicates that silencing *NbMORF8* did not suppress *A. tumefaciens*-mediated transient expression (Fig. 7). Our results suggest that *NbMORF8* is specifically required for the accumulation of *Phytophthora* RXLR effectors.

DISCUSSION

MORF family proteins are important RNA-editing factors unique to land plants. To date, most research on the MORF proteins has mainly focused on their interactions with RNA-editing factors (Härtel et al., 2013; Glass et al., 2015; Sun et al., 2015; Zehrmann et al., 2015; Bayer-Császár et al., 2017; Hackett et al., 2017; Sandoval et al., 2019) or on their RNA-editing sites (Bentolila et al., 2012, 2013). However, the physiological processes regulated by *NbMORF* genes are not well understood.

In this study, we identified that *NbMORF8* is a negative regulator of plant immunity to *P. parasitica* and that it functioned in mitochondria (Figs. 1 and 3). We confirmed that it is an RNA-editing factor in *N. benthamiana* (Fig. 5) by testing the editing extent of cytochrome *c* synthesis-related genes and the editing sites of AtMORF8-interacting PPR proteins. AtMORF8 is a crucial editing factor in Arabidopsis and is involved in the RNA editing of 20% of chloroplast sites and 75% of mitochondrial sites, mainly in the editing of cytochrome *c* synthesis-related genes (Bentolila et al., 2012, 2013). RNA editing of *ccb206* in *TRV-NbMORF8* plants was the most affected, and loss of editing was predicted to change the transmembrane structure of the ccb206 protein, which indicates that silencing *NbMORF8* may significantly affect *ccb206* function (Fig. 5; Supplemental Fig. S12).

The ccb206 protein is involved in the synthesis of cytochrome *c* that participates in electron transport (Itani and Handa, 1998). We further confirmed that cytochrome *c* levels were decreased in *NbMORF8*-silenced plants (Fig. 5). We also found that editing of *ccb206* could be regulated by *P. parasitica* in the early infection stage, during which the editing of two more sites, *ccb206*-367 and *ccb206*-380, was significantly reduced in the *NbMORF8*-silenced plants (Fig. 5). *NbMORF8* also participates in RNA editing of the *cob* gene, which encodes a component of complex III in the respiratory electron transport chain (Weiss, 1987). These results suggest that silencing *NbMORF8* may affect the respiratory electron transport chain, an important source of ROS (Møller, 2001). Therefore, the *NbMORF8*-regulated ROS burst (Fig. 4) is likely achieved through its effect on the functionality of respiratory chain components (Fig. 8). However, there have been no reports on the role of *ccb206* or *cob* in the regulation of ROS/SA or whether their RNA editing will have influence on ROS/SA. Future studies should focus on the sites of ROS production in *NbMORF8*-silenced leaves to further analyze whether the high-level ROS was produced in mitochondria or chloroplasts. *NbMORF8* also has a slight influence on RNA editing of the *ndhB* gene, the Arabidopsis ortholog of which was reported to be involved in immunity (García-Andrade et al., 2013).

The MORF box of MORF proteins has been revealed to mainly interact with PPR proteins (Bayer-Császár et al., 2017). The crystal structures of AtMORF1/AtRIP8 and AtMORF9/AtRIP9 indicate that the interaction between MORF proteins occurs within the MORF box (Haag et al., 2017). Our results showed that both overexpression and silencing of *NbMORF8* resulted in the suppression of RNA editing in some sites (Fig. 5; Supplemental Fig. S14), consistent with reports that both *AtMORF8* overexpression and silenced plants showed a negative effect on C-to-U RNA editing (Bentolila et al., 2012). These results suggest that NbMORF8 interacts with different RNA-editing factors and alters the NbMORF8-dependent editosome, whether *NbMORF8* is silenced or overexpressed. Unexpectedly, the RNA-editing level of all the overexpressed *NbMORF8* deletion mutants decreased or showed no changes compared with the control, like that of full-length *NbMORF8*, although the MORF box is known to be essential for the interaction between MORF proteins and other RNA-editing factors

Figure 6. (*Continued.*)
replicates. Similar results were obtained in at least three independent experiments. Statistical significance was assessed by Student's *t* test (**$P < 0.01$ and ***$P < 0.001$). B, Schematic view of NbMORF8 deletion mutant constructs. AA, Amino acids; C, C-terminal end behind the MORF box; dC, deleting C-terminal end behind the MORF box; dM, deleting the MORF box; LP, leading peptide; MS, more susceptible than the full-length NbMORF8; N, N-terminal end ahead of the MORF box; ND, no difference compared with GFP; S, susceptibility. C, Subcellular localization of LP-NbMORF8, N-NbMORF8, dC-NbMORF8, and dM-NbMORF8 was determined using confocal microscopy in *N. benthamiana* leaves 3 d after agroinfiltration. AOX-RFP was used as a mitochondrial marker. Chlorophyll florescence was used as a chloroplast marker, shown in blue. D, Yeast two-hybrid assay of NbMORF8 mutations. Yeast transformants were separately transferred onto SD/-Leu/-Trp (SD-TL) and SD-Leu/-Trp/-His/-Ade (SD-TLHA) medium. The growth of yeast transformants on SD-TL medium demonstrated successful transformations, and the growth of yeast transformants on SD-TLHA medium indicated interactions. Images were taken 5 d after dropping the transformed yeast cells on SD-TLHA medium. E, The immune function of *NbMORF8* mutants was determined by transient overexpression in *N. benthamiana* followed by inoculation with *P. parasitica* zoospores. *GFP* was used as a control. The results of lesion diameter were means ± SE of six biological replicates. Statistical significance was assessed by Student's *t* test (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). Similar results were obtained in at least three independent experiments. Images were taken at 36 h after zoospore inoculation for *P. parasitica*. The inoculated leaves were stained with Trypan Blue to show the lesion area.

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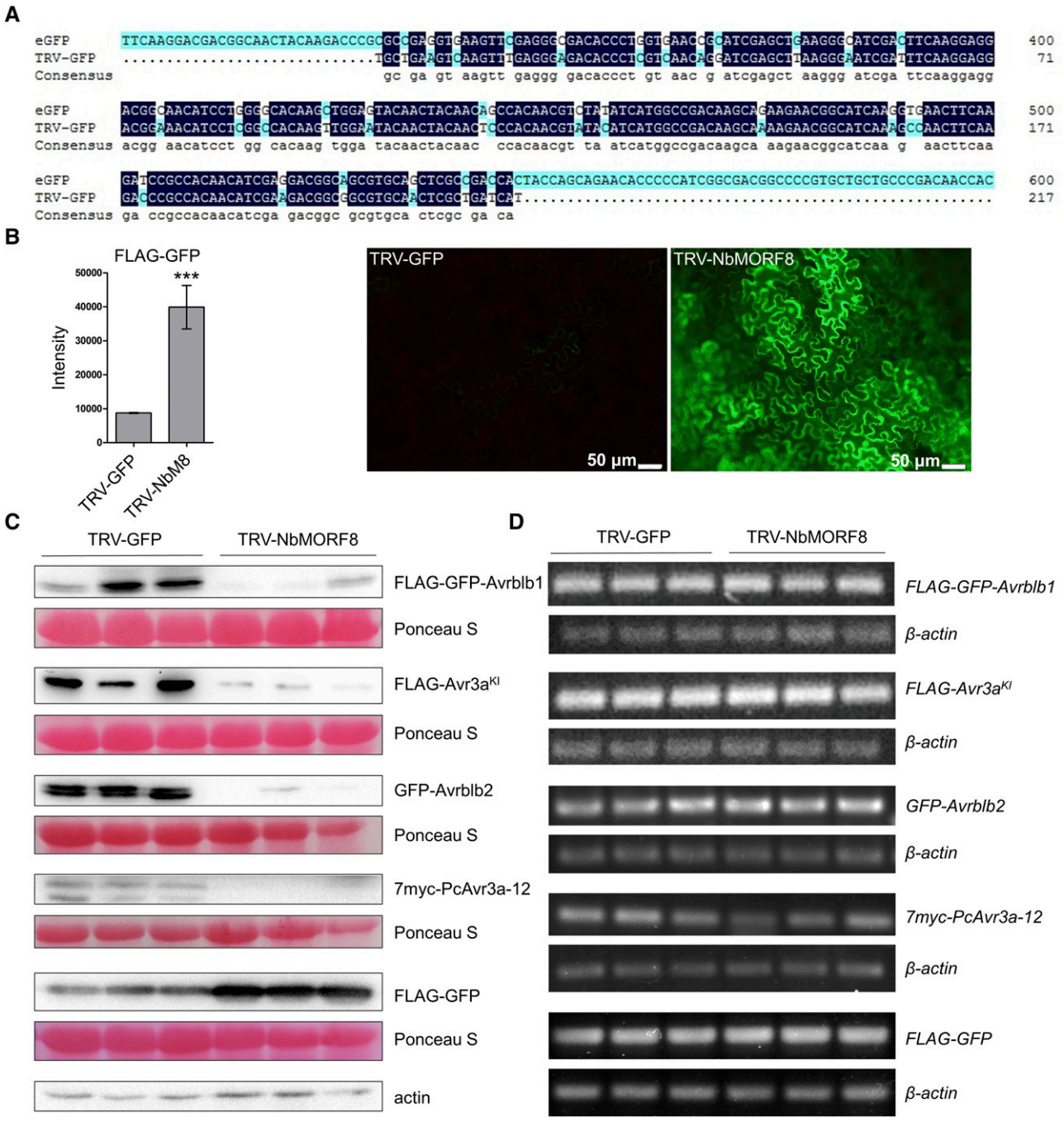

Figure 7. Silencing *NbMORF8* suppresses the accumulation of *Phytophthora* effectors in *N. benthamiana*. A, Sequence alignment of eGFP and TRV-GFP fragments designed for silencing. B, Fluorescence intensity and eGFP accumulation in *NbMORF8*-silenced leaves. The eGFP fluorescence was detected at 4 d post agroinfiltration. For the fluorescence intensity analysis, six images from each group were analyzed using ImageJ. Results are means ± SE of six images. Statistical significance was assessed by Student's *t* test (***$P < 0.001$). C, Western blots of the accumulation of RXLR effector proteins. Three lanes of each group indicate three biological replicates. Ponceau S staining shows equal loading of protein samples. D, Semiquantitative PCR results of effector transcripts. Three lanes of each group show three biological replicates. *N. benthamiana* gene *β-actin* was used to normalize equal loadings.

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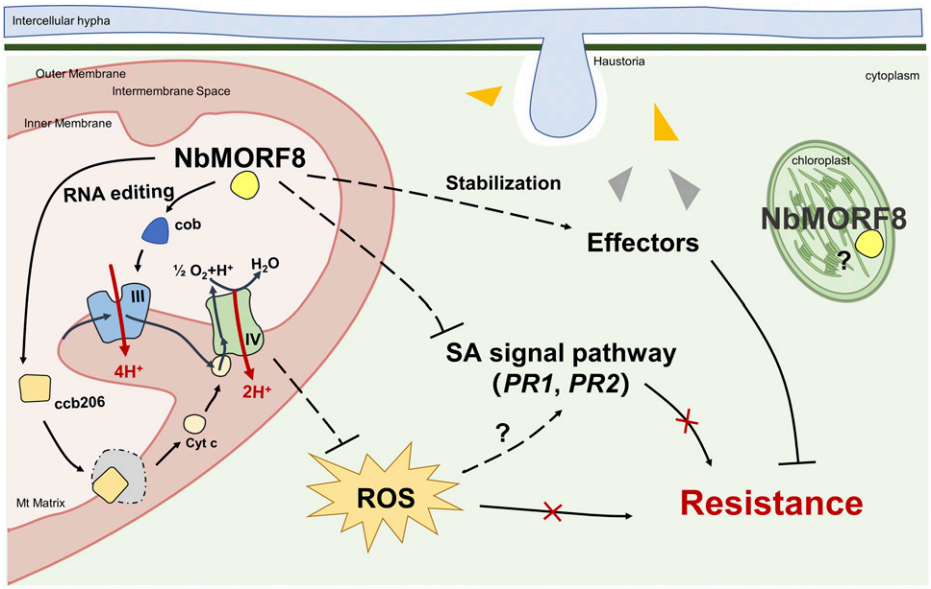

Figure 8. Schematic model for the role of *NbMORF8* in plant immunity. NbMORF8 participates in the RNA editing of mitochondrial genes *cob* and *ccb206* and subsequently further affects the level of cytochrome *c* and complex III activities. Silencing *NbMORF8* up-regulates the expression of SA signal pathway markers (*NbPR1* and *NbPR2*) and ROS levels, which enhances the immunity to *Phytophthora* pathogens. The *NbMORF8*-regulated ROS burst is likely achieved through its effect on the functionality of respiratory chain components. However, the exact ROS production sites in *NbMORF8*-silenced plants remain to be revealed. *NbMORF8* is required for the accumulation of multiple *Phytophthora* RXLR effectors that suppress plant immunity. Mitochondrial localization of NbMORF8 is sufficient for its immune function. It remains to be determined whether the potential chloroplast localization of NbMORF8 is required for plant immunity or if cross talk exists between mitochondria and chloroplasts.

(Supplemental Fig. S14). It is possible that the overexpression of *NbMORF8* mutants interfered with the endogenous *NbMORF8* function.

Our results showed that the mutant NbMORF8-N lost its ability to interact with full-length NbMORF8 but retained its immune function, even rendering plants more susceptible than *NbMORF8* (Fig. 6), suggesting that the immune function of *NbMORF8* does not require the interaction activity and that the MORF box is likely suppressive to its immune function. Furthermore, our results on the deletion mutant analysis of NbMORF1a, NbMORF2b, NbMORF2c, and NbMORF8b showed that the N-terminal region prior to the MORF box of these NbMORF proteins was sufficient for their immune function (Supplemental Fig. S16), suggesting that *NbMORF* genes may regulate plant immunity in a similar way. Considering that full-length NbMORF8b is longer than NbMORF8b-N (containing ~40 amino acid residues of the N-terminal MORF box) and NbMORF8b exhibited no immune function, it is likely that the N-terminal MORF box suppresses the immune function of NbMORF8 (Supplemental Fig. S16). Future studies should focus on identifying the interacting proteins of NbMORF8-N to investigate how such a short region can function as an immune regulator.

Our results showed that silencing *NbMORF8* suppresses the HR triggered by avirulence RXLR effectors but has no influence on INF1- or Bax-induced cell death (Fig. 2). In addition, silencing *NbMORF8* enhanced disease resistance, possibly by activating the SA signaling pathway (Fig. 4). We further showed that silencing *NbMORF8* specifically reduced the accumulation of multiple RXLR effectors of *Phytophthora* pathogens but not their transcript accumulation, since the accumulation of the control protein GFP was increased in *NbMORF8*-silenced plants, which indicates that *NbMORF8* did not suppress *A. tumefaciens*-mediated transient expression (Fig. 7). Silencing *NbMORF8* and *NbMORF1a/1b* showed some similar phenotypes: enhanced resistance to *P. parasitica*, higher ROS burst after flg22 treatment, functioning in mitochondria, reduced plant height, curly leaves, and infertility (Figs. 1 and 4; Supplemental Figs. S2, S3, and S17). Our further testing on PTI- and ETI-induced cell death showed that silencing *NbMORF1a/1b* had no influence on cell death induced by the recognition of RXLR effectors Avr3a^{KI}, Avrblb1, and AvrVnt1 (Supplemental Fig. S4), suggesting that *NbMORF8* is the NbMORF member that is specifically involved in the accumulation of *Phytophthora* RXLR effectors.

We identified eight MORF family members in *N. benthamiana* and used a VIGS assay followed by *P. parasitica* inoculation to investigate their roles in plant immunity. Although NbMORF2a has no leading peptide, the *NbMORF2a*-silenced plants exhibited bleached leaves and were more susceptible to *P. parasitica* (Fig. 1; Supplemental Fig. S2). Sequence alignment showed that the predicted translated 5′ untranslated region of *NbMORF2a* was identical to the N-terminal MORF box

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of NbMORF2b and NbMORF2c (Supplemental Fig. S18), which indicates that the predicted open reading frame of NbMORF2a may have lost its 5′ sequence. Loss of C-to-U RNA editing in mitochondria or chloroplasts usually leads to a defective phenotype, which is usually manifested through bleached leaves, infertility, etc. (Takenaka et al., 2013; Barkan and Small, 2014). Most *NbMORF*-silenced plants showed significant phenotypic changes such as bleached leaves (*NbMORF2a* and *NbMORF9*), reduced plant height, malformed leaves and flowers, and infertility (*NbMORF1a/1b* and *NbMORF8*), which are similar to their Arabidopsis orthologs (Takenaka et al., 2012). We further examined the immune signaling pathway that *NbMORF* genes participate in using reverse transcription qPCR. The results showed that *NbMORF1a/1b*-silenced plants displayed up-regulated expression of *NbPR1*, *NbPR4*, and *NbWRKY7* (Supplemental Fig. S17), while silencing *NbMORF2b/2c* down-regulated the expression of *NbPR1*, *NbPR2*, *NbPR3*, and *NbPR4* (Supplemental Fig. S17). Silencing *NbMORF8* up-regulated *NbPR1* and *NbPR2* (Fig. 4). These results suggest that different NbMORF proteins are involved in regulating different signal pathways.

Since mitochondria and chloroplasts are important sources of ROS (Amirsadeghi et al., 2007; Colombatti et al., 2014) and NbMORF proteins are targeted to these two organelles, we examined ROS levels in the *TRV-NbMORF* plants. Silencing *NbMORF1a/b* or *NbMORF8* resulted in higher ROS levels compared with the *TRV-GFP* plants, while silencing *NbMORF2b/c* did not produce significant changes in maximum ROS burst. However, the ROS decreased faster in *NbMORF2b/2c*-silenced plants (Supplemental Fig. S17). These results suggest that ROS may play important roles in *NbMORF*-regulated immunity.

In summary, we found that *NbMORF8* negatively regulates plant immunity to *P. parasitica* via mitochondrial targeting of its encoded protein and that this function is independent of its MORF box. The enhanced disease resistance of *NbMORF8*-silenced plants resulted from the reduced accumulation of effector proteins, activated SA signaling pathway, and enhanced ROS burst (Fig. 8). Our work shows that the NbMORF family genes are involved in regulating plant immune responses to *Phytophthora* pathogens and that the NbMORF members that are preferentially targeted to mitochondria negatively regulate plant resistance against *Phytophthora*, whereas NbMORF members that are preferentially targeted to chloroplasts are positive immune regulators.

MATERIALS AND METHODS

Plasmid Constructs

For VIGS, ~300-bp specific fragments of *NbMORF* genes were chosen by the VIGS tool (http://vigs.solgenomics.net/) and amplified from *Nicotiana benthamiana* cDNA. The fragment of *NbMORF8* was cloned into pTRV2 vector between *Xba*I and *Bam*HI sites while other NbMORF family genes were between *Eco*RI and *Xho*I sites. For yeast two-hybrid assays, the resultant products were cloned into pGBKT7 using the *Nde*I and *Xho*I sites and cloned into pGADT7 using *Eco*RI and *Xho*I. *NbMORF8* and deletion mutants were amplified from *N. benthamiana* cDNA, and the leading peptide of *NbMORF8* was predicted using Mitoprot (Claros and Vincens, 1996; https://ihg.gsf.de/ihg/mitoprot.html). The signal defining mitochondria or chloroplast subcellular localization is contained within the first 100 amino acids (leading sequence) of a protein's N terminus (Clark et al., 2009; Koprivova et al., 2010; Narsai et al., 2011). Hence, we fused the first ~100 amino acids of NbMORFs to the N terminus of GFP (Bottin et al., 1999) to detect the subcellular localization. To generate NbMORF-eGFP fusion constructs, the fusion fragments were amplified using overlap PCR and cloned into the pKannibal (Wesley et al., 2001) vector using *Xho*I and *Xba*I sites. Then, the constructs were digested by *Not*I and inserted into pART27 (Gleave, 1992). All primers used are listed in Supplemental Table S2.

Agroinfiltration and VIGS

Agrobacterium tumefaciens strain GV3101 containing plasmid constructs was grown for 36 h in Luria-Bertani medium with appropriate antibiotics at 28°C. The medium containing bacteria was gathered and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 200 mM acetosyringone) and adjusted to the required OD_{600} before infiltration into *N. benthamiana* leaves (the OD_{600} was generally 0.3 for transient expression).

VIGS was performed as described previously (Senthil-Kumar and Mysore, 2014). Briefly, *A. tumefaciens* strains harboring the pTRV1 vector and pTRV2-GFP or pTRV2-NbMORFs were mixed in a 1:1 ratio, and the final OD_{600} for each strain was 0.25. The cocultures were then infiltrated into the two largest leaves of 4-week-old plants. Plants were grown for 2 more weeks before using for *Phytophthora* spp. infection or cell death assay. Plant growing conditions for *N. benthamiana* were the same as previously described (Pan et al., 2016).

In the cell death assay, *A. tumefaciens* strain AGL1 was used for *RpiVnt1* and *AvrVnt1* expression. The extent of cell death or HR was monitored daily up to 5 d post agroinfiltration. The extent of cell death or HR was divided into three categories: grade 0, no cell death of the agroinfiltrated area; grade 1, clear necrosis occupying less than 50% of the agroinfiltrated area; and grade 2, necrosis area occupying more than 50% of the agroinfiltrated area.

Confocal Microscopy

N. benthamiana cells expressing fusion proteins were observed 2 or 3 d after infiltration using an Olympus FV3000 confocal microscope. RFP (GenBank accession no. ABC69141) was imaged using an excitation wavelength of 559 nm with emissions collected at 600 to 680 nm. GFP was excited at 488 nm with emissions collected at 500 to 540 nm. AOX-RFP (Narsai et al., 2011) was used as the mitochondria fluorescent marker. Chloroplasts were identified by their chlorophyll autofluorescence.

Phytophthora Infection Assay

Phytophthora parasitica strain Pp016, *Phytophthora capsici* strain LT263, and *Phytophthora infestans* strain 88069 were used for plant infection. *P. parasitica*, *P. capsici*, and *P. infestans* culture and inoculation were performed as in previous reports (Wang et al., 2011, 2013; Li et al., 2019). Zoospore inoculation was performed by inoculating 2,000 zoospores for *P. parasitica*, 800 for *P. capsici*, and 1,200 for *P. infestans*. *P. infestans* sporangia counts were performed as described previously on leaves at 10 d post inoculation (McLellan et al., 2013; Boevink et al., 2016).

Gene Expression Assay

Total RNA was extracted using TRIzol reagent (Invitrogen). Eight hundred nanograms of total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa). Reverse transcription qPCR was performed using FastStart Universal SYBR Green Master (ROX; Roche) with specific primers in an iQ7 Real-Time Cycler (Life Technologies). The relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method with the housekeeping gene *Ppactin* as the reference for *P. parasitica* and *EF1α* or *β-actin* for *N. benthamiana*. Semiquantitative PCR was performed using EasyTaq DNA polymerase (TransGen Biotech) and amplified for 27 cycles. All primers are listed in Supplemental Table S2.

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Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed as described in the Matchmaker Two-Hybrid System 3 protocol (Clontech). The constructs of *NbMORF8* and its deletion mutants were cotransformed into *Saccharomyces cerevisiae* strain AH109. The transformations were confirmed by selection on SD/-Trp-Leu medium, and the interaction was tested by selection on SD/-Trp-Leu-His-Ade medium.

RNA-Editing Assay

To analyze the extent of RNA editing, RNA was isolated from VIGS-treated plant leaves and reverse transcribed into cDNA. The mitochondria and chloroplast genes were amplified with specific primers and then sequenced. At the RNA-editing sites, cDNA sequences were evaluated for their respective C-to-T differences. The extent of RNA editing was estimated by the relative height of the respective nucleotide peaks in the sequence analysis.

Protein Extraction and Immunoblotting

All the protein samples were extracted using GTEN buffer (10% [v/v] glycerol, 25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, and 0.1% [v/v] Tween 20) with 10 mM DTT, protease inhibitor cocktail, and 0.1% (v/v) Nonidet P-40. Proteins were separated by SDS-PAGE. Gels were blotted onto polyvinylidene difluoride membranes (Roche) for 1.75 h at 250 mA in transfer buffer (25 mM Tris, 200 mM Gly, and 20% [v/v] methanol), and the membranes were blocked in 10% (w/v) skim milk in TBST buffer (1 mM Tris, 0.15 M NaCl, and 0.05% [v/v] Tween 20, pH 7.2) for 3 to 5 h. The blocked membranes were incubated with primary antibodies at 1:2,000 dilution, either a monoclonal GFP antibody raised in mouse (ABclonal; no. AE012) or a monoclonal anti-FLAG antibody raised in mouse (ABclonal; no. AE005). The membranes were washed with TBST buffer three times before addition of the secondary antibody at 1:2,000 dilution: HRP goat anti-mouse IgG (H+L) antibody (ABclonal; no. AS003). Before enhanced chemiluminescence (ComWin; no. CW0049S) photographing using a molecular imager (Bio-Rad; ChemiDocTM XRS+), the membranes were washed twice with TBST buffer and once in TBS buffer (1 mM Tris and 0.15 M NaCl, pH 7.2).

Bioinformatics

For phylogenetic analysis, protein sequences of the AtMORF/RIP family were downloaded from TAIR (http://www.arabidopsis.org/). BLASTP searches were then performed using AtMORF/RIP family protein sequences as queries with an expected value (e-value) cutoff of e^{-10} using the Sol Genomics Network (https://solgenomics.net/tools/blast/) to identify the potential NbMORF/RIP family member protein sequences. Alignment and phylogenetic analysis were performed using MEGA7 with default parameters (Saitou and Nei, 1987; Kumar et al., 2016). The neighbor-joining method with 1,000 bootstrap replicates was used. The subcellular location of NbMORFs was predicted using TargetP (Emanuelsson et al., 2000) and LOCALIZER (Sperschneider et al., 2017).

ROS Burst Detection

ROS production was measured with a previously reported luminol-based assay (Sang and Macho, 2017). Two-week-silenced *N. benthamiana* leaves were sliced into 0.785-cm^2 leaf discs and floated in water overnight. Water was replaced with reagent containing luminol, peroxidase, and 1 μM flg22. ROS released by leaf discs was detected by luminescence of luminol.

HRM Analysis

The HRM assay was performed according to the method previously reported (Chateigner-Boutin and Small, 2007). The PCR cycling and HRM were performed on a LightCycler 480 II machine (Roche), and the HRM analysis was performed using gene scanning software (Roche).

Accession Numbers

The genes described here have the following Sol Genomics Network (https://solgenomics.net/) gene accession numbers: *NbMORF1a* (Niben101Scf18637g02008.1), *NbMORF1b* (Niben101Scf02581g04012.1), *NbMORF2a* (Niben101Scf04789g00001.1), *NbMORF2b* (Niben101Scf07015g00008.1), *NbMORF2c* (Niben101Scf07015g00010.1), *NbMORF8a* (Niben101Scf20512g00013.1), *NbMORF8b* (Niben101Scf07087g00014.1), and *NbMORF9* (Niben101Scf00176g00005.1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequence alignment of all NbMORF and AtMORF proteins.

Supplemental Figure S2. Efficient silencing of *NbMORF* genes in *N. benthamiana*.

Supplemental Figure S3. *NbMORF8* is required for flower development in *N. benthamiana*.

Supplemental Figure S4. Silencing *NbMORF1a/1b* or *NbMORF2b/2c* had no influence on PTI- and ETI-induced cell death.

Supplemental Figure S5. Multiple *NbMORF* genes are involved in immunity in *N. benthamiana* to *P. parasitica*.

Supplemental Figure S6. The expression of *NbMORF* genes during *P. parasitica* infection.

Supplemental Figure S7. Subcellular localization of NbMORF proteins.

Supplemental Figure S8. Confocal microscopy of *N. benthamiana* leaf pavement cells expressing CpNbMORF proteins.

Supplemental Figure S9. The effect of targeted expression of NbMORF proteins on their immune functions.

Supplemental Figure S10. *NbMORF8*-silenced plants exhibited enhanced resistance to *P. infestans* and *P. capsici*.

Supplemental Figure S11. The HRM results of *cob*-853, *ndhB*-217, and *ndhB*-242.

Supplemental Figure S12. Prediction of the transmembrane structure of the *ccb206*-encoded protein.

Supplemental Figure S13. Overexpression of dLNbMORF8 did not promote *P. parasitica* infection.

Supplemental Figure S14. RNA-editing levels of different *NbMORF8* deletion mutants in *N. benthamiana*.

Supplemental Figure S15. Detection of transiently expressed NbMORF8 proteins and NbMORF8 deletion mutant proteins in *N. benthamiana*.

Supplemental Figure S16. The role of the conserved MORF box of NbMORF proteins in their immune function in *N. benthamiana* to *P. parasitica*.

Supplemental Figure S17. Silencing of *NbMORF1a/1b* and *NbMORF2b/2c* affected different immune signaling pathways.

Supplemental Figure S18. Sequence alignment of NbMORF2a, NbMORF2b, and NbMORF2c.

Supplemental Table S1. List of NbMORF family members.

Supplemental Table S2. Primers used in this study.

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