Short title: *NbMORF8* negatively regulates plant immunity

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Cytidine-to-Uridine RNA editing factor NbMORF8 negatively regulates plant immunity to *Phytophthora* pathogens

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One-sentence summary: A mitochondrion- and chloroplast-targeted RNA editing factor negatively regulates plant immunity to *Phytophthora* pathogens by suppressing effector accumulation, ROS burst, and SA signaling.

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Abstract

Mitochondria and chloroplasts play key roles in plant-pathogen interactions. Cytidine-to-uridine (C-to-U) RNA editing is a critical post-transcriptional 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 (VIGS) and transient expression in *Nicotiana benthamiana*, we show that *NbMORF8* functions through regulation of ROS production, SA signaling, and accumulation of multiple RXLR 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 mitochondrial 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 mitochondrial and chloroplast localized NbMORF family members exert opposing effects on immune regulation.

Keywords

MORF proteins, plant immunity, RNA editing, oomycete, *Phytophthora*, plant susceptibility, RXLR effectors

Introduction

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 (Amirsadeghi et al., 2007; Colombatti et al., 2014). Production of several plant hormones involved in immunity, e.g. jasmonic acid (JA), 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; Rodriguez-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 nuclear-encoded RNA editing factors, is a critical post-transcriptional modification specific to flowering plants (Gray and Covello, 1993; Takenaka et al., 2013; Barkan and Small, 2014; Shikanai, 2015; Yan et al., 2017). C-to-U RNA editing usually changes the first or second positions 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 (ORRM) proteins, protoporphyrinogen IX oxidase 1 (PPO1), and organelle zinc finger 1 (OZ1) (Zhang et al., 2014; Sun et al., 2015; Sun et al., 2016; Yan et al., 2017). Increasing evidence supports the conclusion that this type of post-transcriptional modification plays important roles in plant metabolism, adaptations to the environment, and signal transduction (Zsigmond et al., 2008; Yan et al., 2017; Yang et al., 2017; He 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, e.g. Arabidopsis (*Arabidopsis thaliana*) contains more than 400 PPR proteins, whereas there are much few 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; Hartel 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, 9 MORFs, and 6 ORRMs in Arabidopsis (Yan et al., 2017)), 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* (Garcia-Andrade et al., 2013).

To detect and respond to invading pathogens, plants have evolved pattern recognition receptors (PRRs) to recognize conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (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 (NLR), 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 hypersensitive response (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 *R* gene pairs have been cloned from *Phytophthora* pathogens and their hosts, e.g. *P. infestans Avr* genes *PiAvr3a* (Armstrong et al., 2005), *PiAvrblb1* (Vleeshouwers et al., 2008), and *PiAvrVnt1* (Pel et al., 2009), and corresponding potato *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 recognition of pathogen effectors is genetically well known and molecularly well characterized, and widely used for 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 *P. parasitica*. We show 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 regulation of ROS burst and the SA signaling pathway, and 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 orthologue of AtMORF8 as revealed by rigorous phylogenetic 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-seq 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 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*, *NbMRF8a*, *NbMROF8b*, and *NbMORF9*. The genes were named according to the Arabidopsis orthologues. *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 approximately 100 amino acid residues, like their Arabidopsis orthologues (Fig. 1 and Supplemental Fig. S1). However, NbMORF2a lacks an N-terminal amino acid sequence preceding the MORF box. NbMORF8b is truncated from the C-terminal to within the MORF box when compared to NbMORF8a (Fig. 1 and 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 co-silenced *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. The results showed that the *TRV-NbMORF2a* and *TRV-NbMORF9* plants exhibited bleached leaves and were more susceptible to *P. parasitica* (Fig. 1 and Supplemental Fig. S2). *TRV-NbMORF2b/2c* plants were more susceptible but did not exhibit any bleaching phenotype

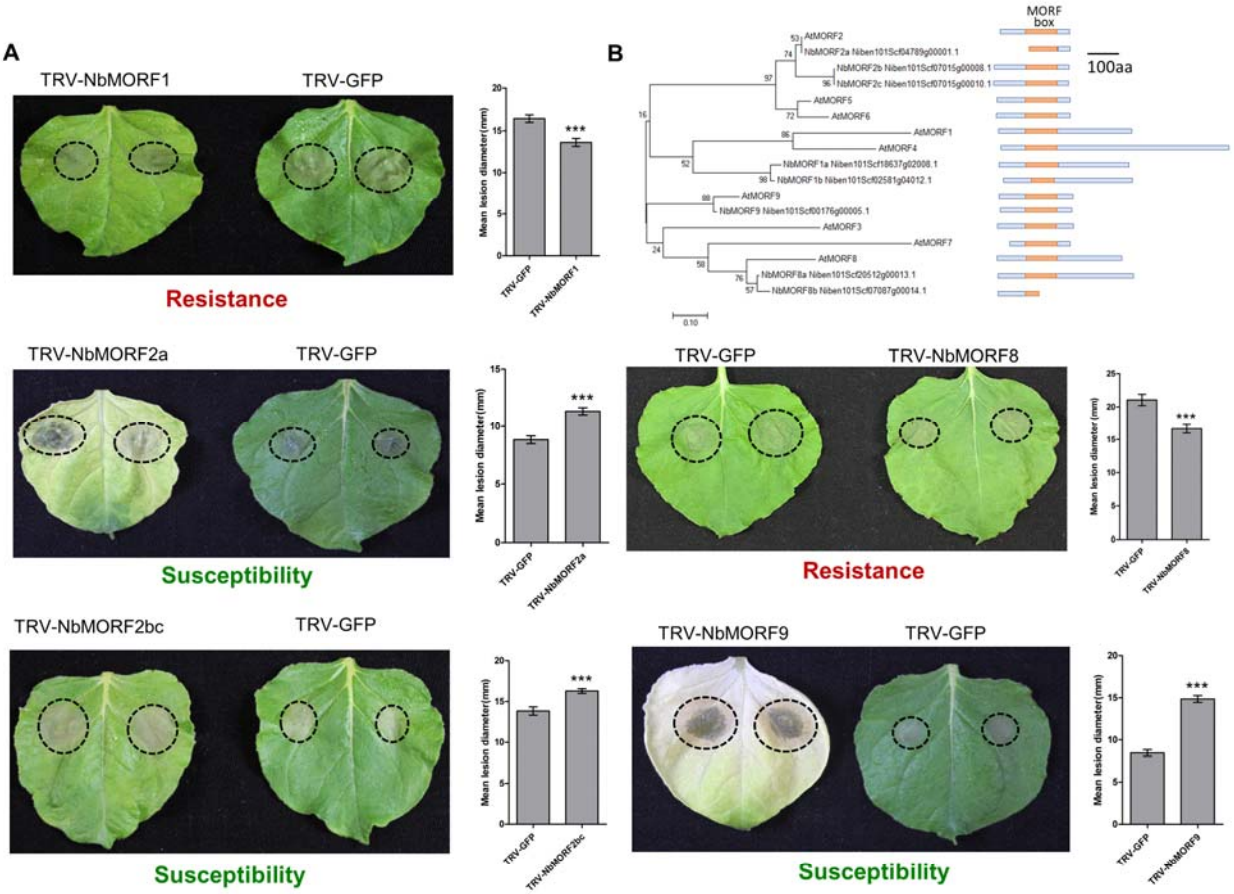


Figure 1. MORF proteins play different immune roles in *N. benthamiana* to *Phytophthora* pathogens. A, Silencing of *NbMORF1a/1b*, *NbMORF2a*, *NbMORF2b/c*, *NbMORF8* or *NbMORF9*, respectively, in *N. benthamiana* led to different responses to *P. parasitica*: silencing *NbMORF1a/1b* or *NbMORF8* enhanced resistance, while silencing of *NbMORF2a*, *NbMORF2b/2c* or *NbMORF9* promoted *P. parasitica* colonization. Images were taken at ~40 h after inoculation with *P. parasitica* zoospore. Results were the mean ±SE of 20 infections from at least 10 leaves. Statistical significance was assessed by *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 NJ method. All NbMORF proteins share a conserved MORF box.

(Fig. 1 and 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 Fig. S2, and Supplemental Fig. 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 PTI- or ETI-induced HR, we transiently expressed the *P. infestans* elicitin gene *INF1*, *Bax*, P. infestans RXLR effector genes, and cognate potato resistance 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*

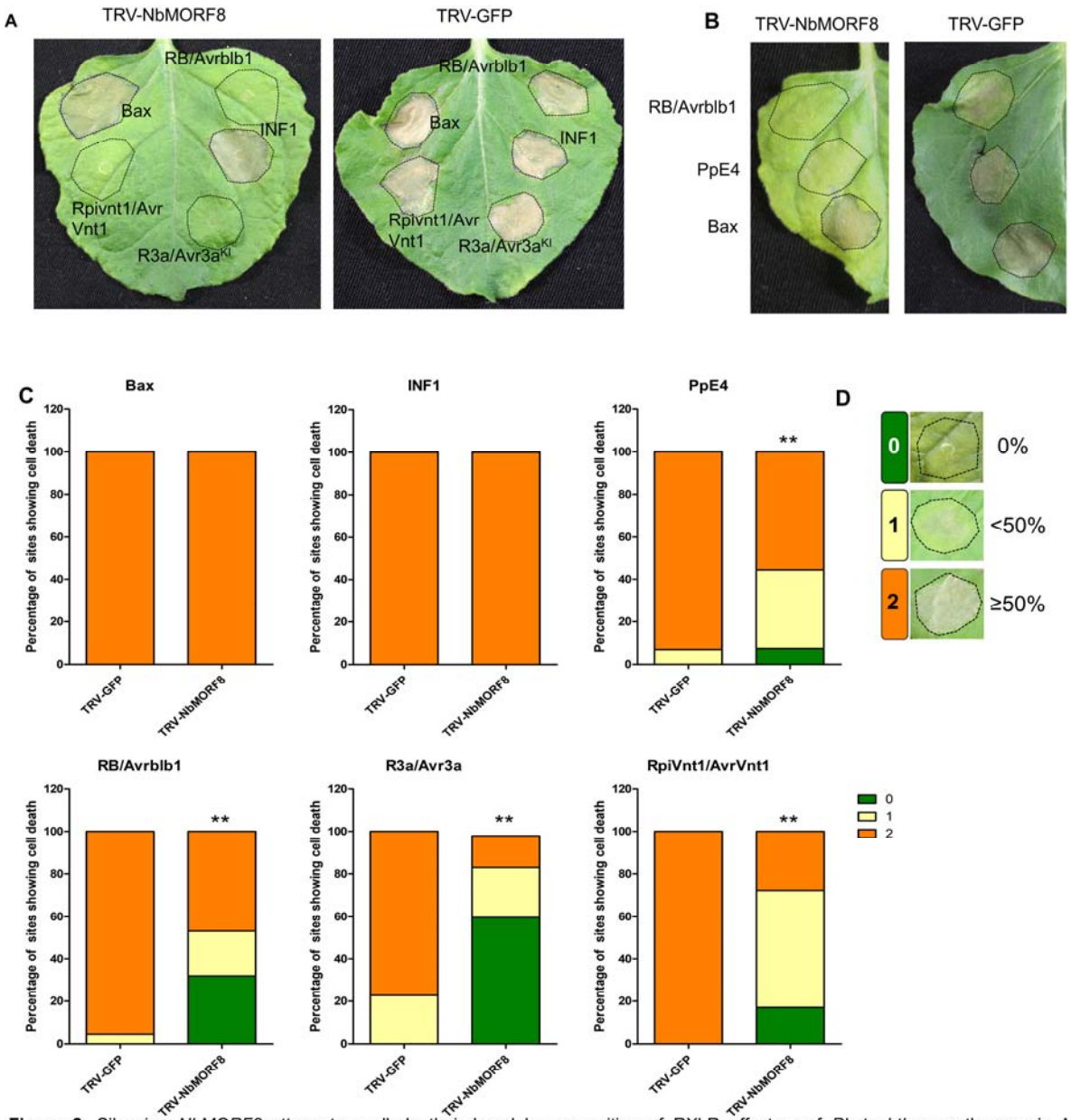
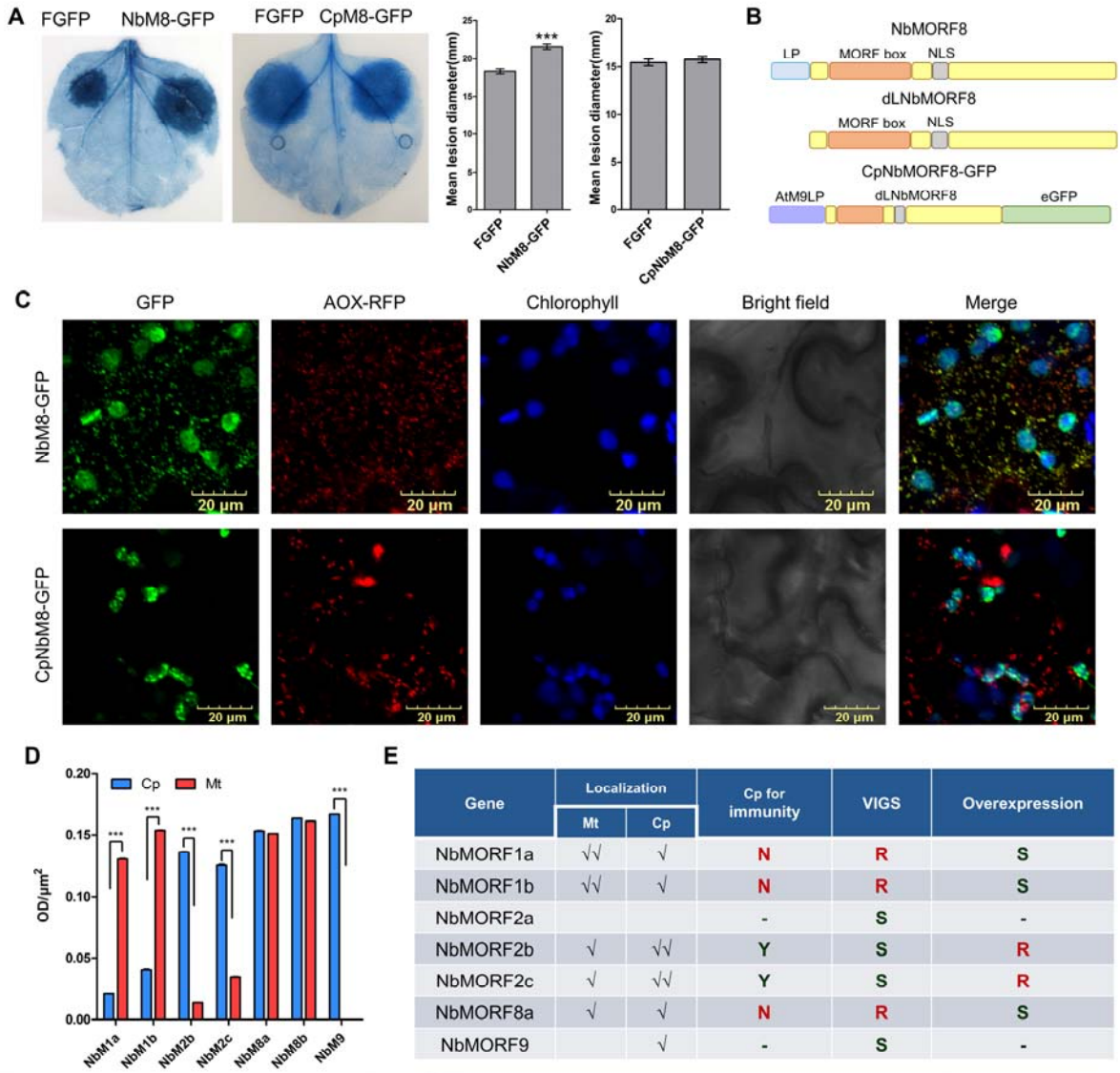


Figure 2. Silencing *NbMORF8* attenuates cell death induced by recognition of RXLR effectors of *Phytophthora* pathogens in *N. benthamiana*. A, Cell death observation of NbMORF8-silenced plants and control. Images were taken at five days after *A. tumefaciens*-mediated transient expression of *Avr*/*R*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

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 over-expressed in *N. benthamiana* leaves followed by inoculation with *P. parasitica*. The results showed that



E

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. Mitochondrial 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 were the mean±SE of 10 biological replicates. Similar results were observed in three independent experiments. Statistical significance was assessed by *t* test. *** P<0.001. B, Schematic view of NbMORF8, dLNbMORF8, and CpNbMORF8. LP, leading peptide. NLS, nuclear localization signal. dL, deleting leading peptide. The leading peptide of NbMORF8 was replaced with the AtMORF9 leading peptide to re-target the fusion proteins to the chloroplast. The NbMORFs were analyzed using the same method. C, Subcellular localization of NbMORF8 and CpNbMORF8. Confocal microscopy of *N. benthamiana* leaves expressing *NbMORF8-GFP* or *CpNbMORF8-GFP*. Subcellular localization was observed at 2 or 3 days post agroinfiltration (dpi). 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 were the mean ±SE of nine organelles from three images. Statistical significance was assessed by *t* test. *** P < 0.001. E, Summary of subcellular localization and immune function of NbMORF proteins. √, NbMORF protein localized. √√, NbMORF protein preference. -, not determined. N, chloroplast localization is not required for immune function. Y, chloroplast localization is required for immune function. R, resistant to *P. parasitica*. S, susceptible to *P. parasitica*.

188 overexpression of *NbMORF1a*, *NbMORF1b*, or *NbMORF8a* enhanced plant susceptibility to *P.*
189 *parasitica* (Fig. 3 and Supplemental Fig. S5), while overexpression of *NbMORF2b* or
190 *NbMORF2c* led to increased resistance. Overexpression of *NbMORF8b* had no effect on plant

191 immunity (Supplemental Fig. S5). We also found that the transcript levels of *NbMORF1a*,
192 *NbMORF1b*, *NbMORF2a*, *NbMORF2b*, *NbMORF2c*, *NbMORF8a*, and *NbMORF9* were all
193 induced during infection (Supplemental Fig. S6: primers to distinguish *NbMORF2b* and *2c* could
194 not be designed because of the high sequence similarity). These results implied that *NbMORF*
195 genes, with the exception of *NbMORF8b*, are involved in plant immune response to
196 *Phytophthora* infection.

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

210 To determine whether localization of NbMORF proteins (NbMORF1a, NbMORF1b,
211 NbMORF2b, NbMORF8a, and NbMORF2c) to either plastids or plastids and mitochondria is
212 required for their immune functions, we targeted GFP-tagged NbMORF proteins to chloroplasts
213 (Cp-NbMORF) by replacing the NbMORF leading peptides with AtMORF9's leading peptide,
214 which is reported to target the protein to the chloroplast (Takenaka et al., 2012). NbMORF2b and
215 NbMORF2c only differ in the leading peptide. The results showed that the GFP-derived
216 fluorescence was detected exclusively in the chloroplasts (Fig. 3 and Supplemental Fig. S8). The
217 pathogen inoculation assay showed that the targeted chloroplast localization of NbMORF1a,
218 NbMORF1b, and NbMORF8a abolished their ability to enhance plant susceptibility
219 (Supplemental Fig. S9). However, NbMORF2b/2c still enhanced the resistance (Supplemental
220 Fig. S9). These results demonstrate that mitochondrial-preferred NbMORF members
221 (NbMORF1a, 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 N*bMORF8* for further analysis. To examine whether *NbMORF8* silencing conferred resistance to different *Phytophthora* pathogens, we also inoculated *TRV-NbMORF8* leaves with *P. infestans* and *P. capsica* (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 to the *TRV-GFP* 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 defense-related genes _NbPR1_ and _NbPR2_

As mitochondria and chloroplasts are an important source 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 Fig. 4, upon PAMP flg22 treatment, silencing *NbMORF8* resulted in higher ROS levels compared to the *TRV-GFP* plants, suggesting that *NbMORF8* may regulate plant immunity through 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 PTI marker genes *NbWRKY7* and *NbWRKY8* (Yan et al., 2016), SA pathway markers *NbPR1* and *NbPR2* (Yan et al., 2016), and JA pathway

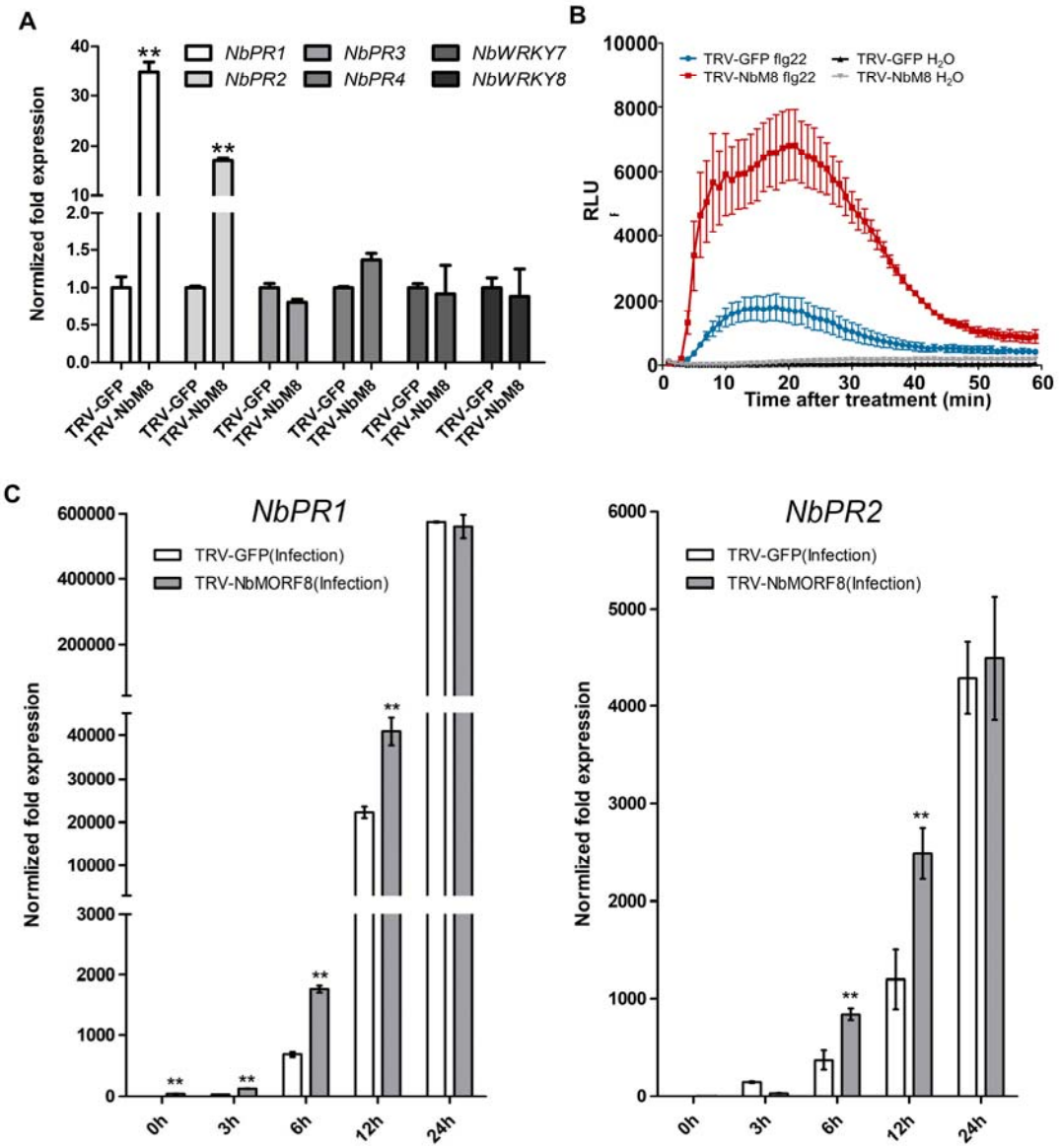


Figure 4. Silencing *NbMORF8* up-regulated expression of defense-related genes and enhanced ROS levels. A, Up-regulated expression of defense-related genes *NbPR1* and *NbPR2* in *TRV-NbMORF8* plants without pathogen treatment, as determined by RT-qPCR. B, ROS burst upon flg22 treatment of *NbMORF8*-silenced leaves. At least 12 leaves from six plants of each group were measured using a luminol-based chemiluminescence assay. C, The 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 (hpi). The *N. benthamiana EF1α* gene was used as an internal control. Results were the mean ±SE. Similar results were observed in at least three independent experiments.

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 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 measure editing levels in recent years (Zhu et al., 2012; Hartel 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 orthologues of target genes shown to be edited in *A. thaliana* by AtMORF8 (Bentolila et al., 2012; Bentolila et al., 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 mitochondrial genes *ccb206* (8 of 33 sites), which plays a role in cytochrome c synthesis; *cob* (1 of 8 sites), which encodes a subunit of complex III; and chloroplast gene *ndhB* (1 of 8 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 analysis (HRM) (Supplemental Fig. S11).

The ccb206 protein is involved in synthesis of cytochrome c, which 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 and 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 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, were 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.

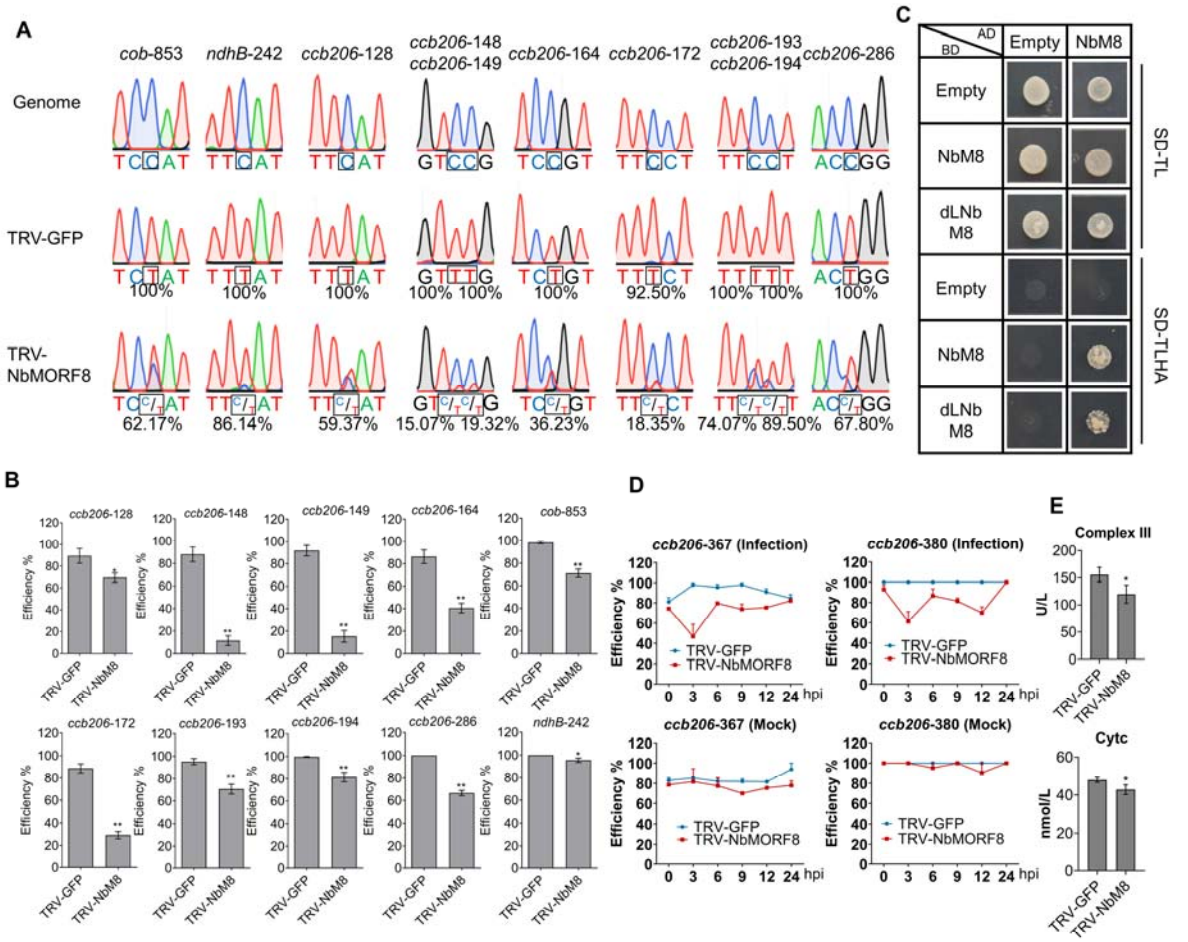


Figure 5. Silencing *NbMORF8* impaired RNA editing of *ccb206*, *cob*, and *ndhB*. A-B, The editing levels of mitochondrial *ccb206* transcripts at sites 128, 148, 149, 164, 172, 193, 194, and 286, and *cob* at site 853, and chloroplast *ndhb* at site 242. Results were the mean ±SE of three biological replicates. Statistical significance was assessed by *t* test. * P<0.05 ** P < 0.01. C, Yeast transformants were separately transferred onto SD/-Leu/-Trp (SD-LT) and SD-Leu/-Trp/-His/-Ade (SD-LTHA) medium. The growth of yeast transformants on SD-LT medium demonstrated successful transformations. The growth of yeast transformants on SD-LTHA indicates interactions. Image was taken three days after dropping the transformed yeast on SD/-LTHA 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 were the mean ±SE of three biological replicates. E, The cytochrome c levels and complex III activities were detected using ELISA. Results were the mean ±SE of three biological replicates. Statistical significance was assessed by *t* test. * P<0.05.

AtMORF8 was reported to form homomers (Zehrmann et al., 2015; Bayer-Csaszar et al., 2017). We used 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 indicated that NbMORF8 has a similar function to its *A. thaliana* orthologue *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 formation of heteromers or homomers. Moreover, it has distinct affinities to the PPR to regulate the RNA editing of different sites (Bayer-Csaszar et al., 2017; Haag et al., 2017). To analyze whether the immune function of NbMORF8 was dependent on its MORF box, we created a

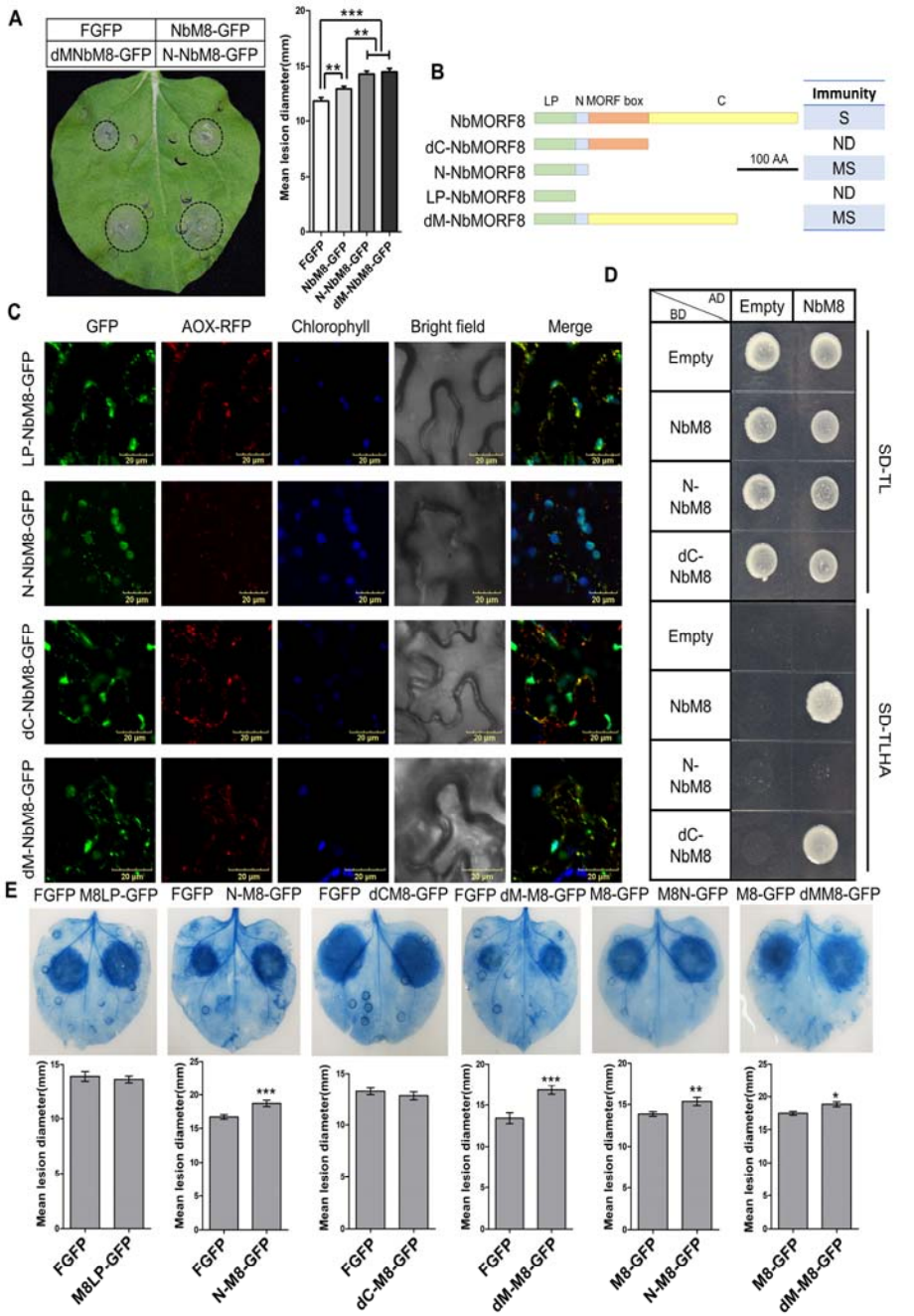


Figure 6. The MORF box domain suppresses the immune function of *NbMORF8*. A, Overexpression of *NbMORF8* mutants without the MORF box rendered plants more susceptible than the full-length *NbMORF8*. Transient expression of *N-NbM8-GFP*, *dM-NbM8-GFP*, *NbM8-GFP*, and *GFP* in single *N. benthamiana* leaves was done by agroinfiltration, followed by inoculation with *P. parasitica* zoospores. Images were taken at 40 h after zoospore inoculation. Results were the mean ±SE of nine biological replicates. Similar results were obtained in at least three independent experiments. Statistical significance was assessed by *t* test. B, Schematic view of NbMORF8 deletion mutant constructs. LP, leading peptide. N, N terminal ahead of MORF box. C, C terminal behind MORF box. dC, deleting C terminal behind MORF box. dM, deleting MORF box. S, susceptibility. ND, no difference compared with GFP. MS, more susceptible than the full length NbMORF8. C, Subcellular localization of LP-NbMORF8, N-NbMORF8, dC-NbMORF8, and dM-NbMORF8 was determined using confocal microscopy in *N. benthamiana* leaves three days after agroinfiltration (dpi). AOX-RFP was used as a mitochondrial marker. Chlorophyll florescence was used as chloroplast marker, shown in blue. D, The yeast-two-hybrid assay of NbMORF8 mutations. Yeast transformants were separately transferred onto SD/-Leu/-Trp (SD-LT) and SD-Leu/-Trp/-His/-Ade (SD-LTHA) medium. The growth of yeast transformants on SD-LT medium demonstrated successful transformations, and the growth of yeast transformants on SD-LTHA medium indicated interactions. Images were taken five days after dropping the transformed yeast cells on SD/-LTHA 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 the mean ±SE of six biological replicates. Statistical significance was assessed by *t* test. * P<0.05 ** P < 0.01 *** 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.

301 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 were 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 to 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 (NLS) 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 overexpression of all deletion mutants in *N. benthamiana* leaves (Supplemental Fig. S14 and Fig. 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 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 which still had immune function, using yeast-two-hybrid. The results showed that NbMORF8-N lost interaction with full-length NbMORF8 though it retained its immune function (Fig. 6), suggesting that the immune function of *NbMORF8* does not require the interaction activity.

Silencing *NbMORF8* suppresses the accumulation of RXLR effectors

We found that silencing *MORF8* attenuated HR induced by *R3a*/*Avr3a*^{KI}, *RB*/*Avrblb1*, and

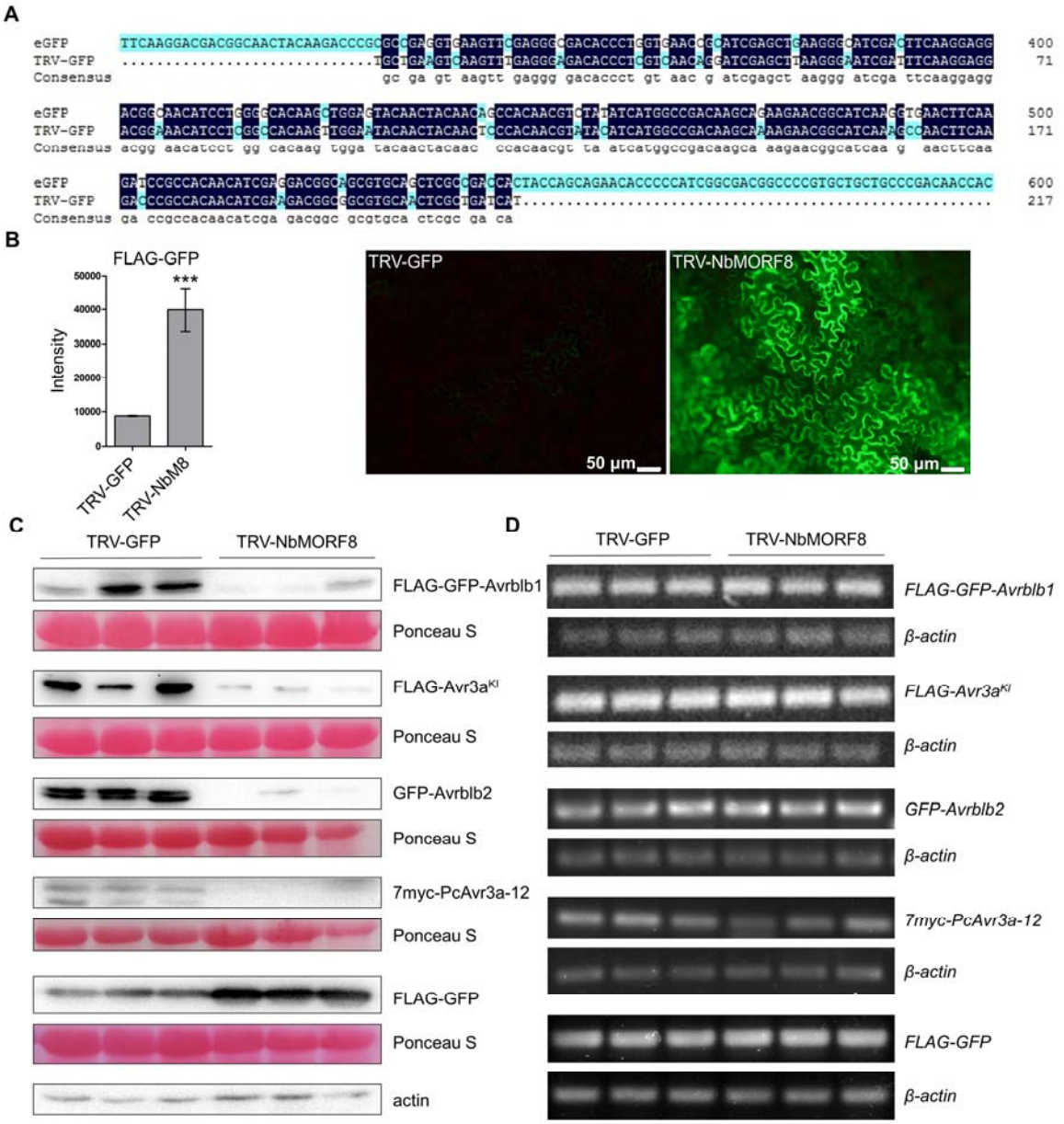


Figure 7. Silencing *NbMORF8* suppresses accumulation of *Phytophthora* effectors in *N. benthamiana*. A, Sequence alignment of eGFP and TRV-GFP fragments designed for silencing. B, Fluorescence intensity and eGFP accumulation in *NbMORF8*-silenced leaves. The eGFP fluorescence was detected at 4 dpi. For the fluorescence intensity analysis, six pictures from each group were analyzed using ImageJ. Results were the mean ±SE of 6 pictures. Statistical significance was assessed by *t* test. *** $P < 0.001$. C, Western blot 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, Semi-quantitative 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.

RpiVnt1/*AvrVnt1* recognition, but not HR induced by INF1 or Bax (Fig. 2). We also tested *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 efficiency of *Agrobacterium 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 do 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 *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 (Hartel et al., 2013; Glass et al., 2015; Sun et al., 2015; Zehrmann et al., 2015; Bayer-Csaszar et al., 2017; Hackett et al., 2017; Sandoval et al., 2019), or on their RNA editing sites (Bentolila et al., 2012; Bentolila et al., 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 it functioned in mitochondria (Fig. 1, and Fig. 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; Bentolila et al., 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 silencing *NbMORF8* may significantly affect *ccb206* function (Fig. 5 and Supplemental Fig. S12).

The ccb206 protein is involved in the synthesis of cytochrome c, which 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, and 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 orthologue of which was reported to be involved

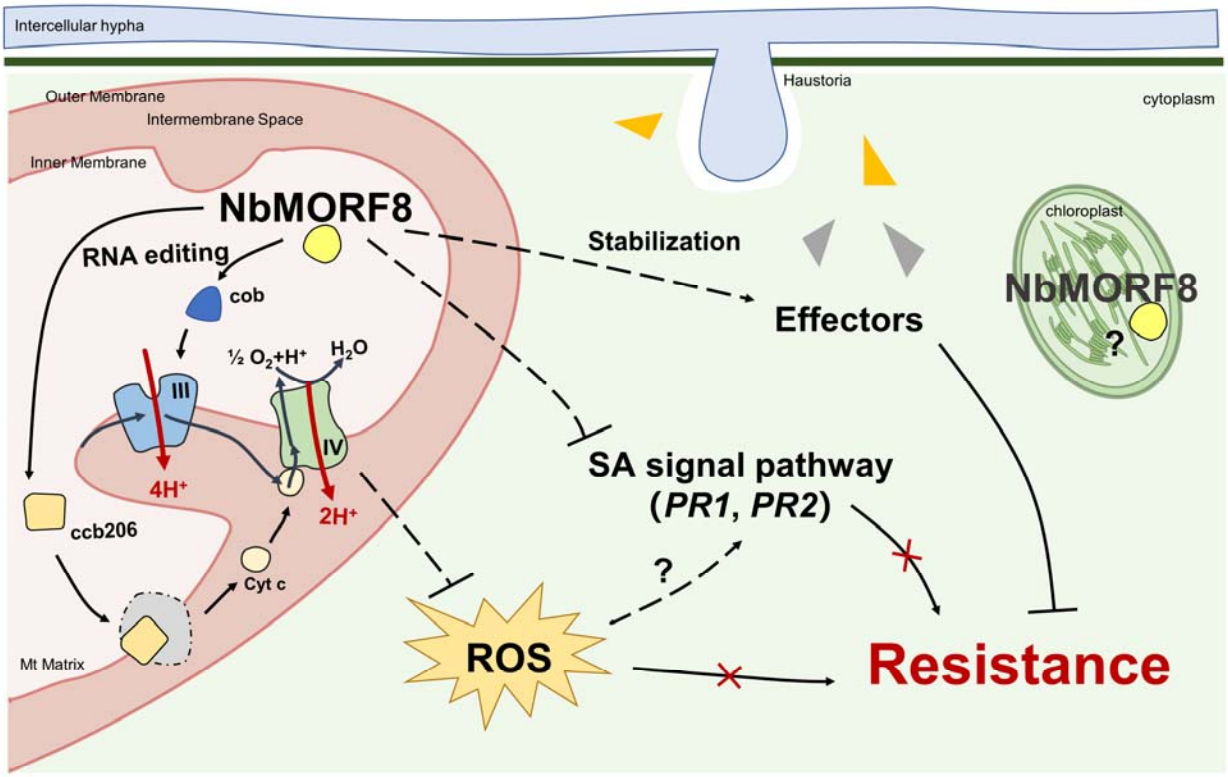


Figure 8. Schematic model for the role of NbMORF8 in plant immunity. NbMORF8 takes part in the RNA editing of mitochondrial *cob* and *ccb206* genes and further influent the levels of cytochrome c and complex III activity. Silencing of *NbMORF8* up-regulated the expression of SA signal pathway markers (*NbPR1* and *NbPR2*) and ROS level, which enhanced the immunity to *Phytophthora* pathogens. *NbMORF8* regulated ROS burst is likely achieved through its effect in functionality of respiratory chain components. But the ROS production sites in *NbMORF8* silenced leaves should be further analyzed to confirm whether the high-level ROS was produced in mitochondria or chloroplasts. *NbMORF8* are also required for the accumulation of *Phytophthora* RXLR effectors which will suppress the plant immunity. We have found that the mitochondrial localization of NbMORF8 is sufficient for its immune function. Whether the chloroplast localization takes part in plant immunity or whether it have influence in the cross-talk between mitochondria and chloroplasts need to be analyzed in the future.

in immunity (Garcia-Andrade et al., 2013).

The MORF box of MORF proteins has been revealed to mainly interact with PPR proteins (Bayer-Csaszar 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 or silencing of *NbMORF8* resulted in 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

391 *NbMORF8* deletion mutants decreased or showed no changes compared to the control, like that
392 of full-length *NbMORF8*, though the MORF box is known to be essential for the interaction
393 between MORF proteins and other RNA editing factors (Supplemental Fig. S14). It is possible
394 that the overexpression of *NbMORF8* mutants interfered with the endogenous *NbMORF8*
395 function.

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

409 Our results showed that silencing *NbMORF8* suppresses the HR triggered by avirulence
410 RXLR effectors, but has no influence on INF- or Bax-induced cell death (Fig. 2). In addition,
411 silencing *NbMORF8* enhanced disease resistance, possibly by activating the SA signaling
412 pathway (Fig. 4). We further showed that silencing *NbMROF8* specifically reduced
413 accumulation of multiple RXLR effectors of *Phytophthora* pathogens, but not their transcript
414 accumulation, since the accumulation of the control protein GFP was increased in
415 *NbMORF8*-silenced plants, which indicates that *NbMORF8* did not suppress
416 *Agrobacterium*-mediated transient expression (Fig. 7). Silencing *NbMORF8* and *NbMORF1a/1b*
417 showed some similar phenotypes: enhanced resistance to *P. parasitica*, higher ROS burst after
418 flg22 treatment, functioning in mitochondria, reduced plant height, curly leaves, and infertility
419 (Fig. 1, Fig. 4, Supplemental Fig. S2, Supplemental Fig. S3, and Supplemental Fig. S17). Our
420 further testing on PTI- and ETI-induced cell death showed that silencing *NbMORF1a/1b* had no
421 influence on cell death induced by recognition of RXLR effectors $Avr3a^{KI}$, Avrblb1, and

AvrVnt1 (Supplemental Fig. S4), suggesting that *NbMORF8* is the NbMORF member that is specifically involved in accumulation of *Phytophthora* RXLR effectors.

We identified eight MORF family members in *N. benthamiana* and used 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 and Supplemental Fig. S2). Sequence alignment showed that the predicted translated 5' UTR of *NbMORF2a* was identical to the N-terminal MORF box of NbMORF2b and 2c (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 heights, malformed leaves and flowers, and infertility (*NbMORF1a/1b* and *NbMORF8*), which are similar with their *A. thaliana* orthologues (Takenaka et al., 2012). We further examined the immune signaling pathway that *NbMORF* genes participate in using RT-qPCR assay. 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 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 source 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 to 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 this function is independent of its MORF box. The enhanced disease resistance of *NbMORF8*-silenced plants resulted from reduced

accumulation of effector proteins, activated SA signaling pathway, and enhanced ROS burst (Fig. 8). Our work showed that the NbMORF family genes are involved in regulating plant immune responses to *Phytophthora* pathogens, and 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.

Material and Methods

Plasmid constructs

For VIGS, ~300bp 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 Xba1 and BamH1 sites while other NbMORF family genes were between EcoR1 and Xho1 sites. For yeast-two-hybrid the resultant products were cloned into pGBKT7 using the Nde1 and Xho1 sites and cloned into pGADT7 using EcoR1 and Xho1. *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 NbMORFs-eGFP fusion constructs, the fusion fragments were amplified using overlap PCR and cloned into the pKannibal (Wesley et al., 2001) vector using Xho1 and Xba1 sites. Then, the constructs were digested by Not1 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 hours in LB medium with appropriate antibiotics at 28°C. The medium containing bacteria was gathered and resuspended in infiltration buffer (10mM MES, 10mM $MgCl_2$ 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,

Agrobacterium 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 co-cultures were then infiltrated into the two largest leaves of 4-week old plants. Plants were grown for 2 more weeks before using for *Phytophthora* 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. tumefacien*s strain AGL1 was used for *RpiVnt1* and *AvrVnt1* expression. The extent of cell death or HR was monitored daily up to 5 days post-agroinfiltration. The extent of cell death or HR was divided into 3 categories: grade 0 - no cell death of the agroinfiltrated area; grade 1 - clear necrosis occupying <50% of the agroinfiltrated area; and grade 2 - necrosis area occupying >50% of the agroinfiltrated area.

Confocal microscopy

N. benthamiana cells expressing fusion proteins were observed two or three days after infiltration using an Olympus FV3000 confocal microscope (Japan). RFP (GenBank: ABC69141) was imaged using an excitation wavelength of 559 nm with emissions collected at 600-680 nm. GFP was excited at 488 nm with emissions collected at 500-540 nm. AOX-RFP (Narsai et al., 2011) was used as the mitochondria fluorescent marker. Chloroplasts were identified by their chlorophyll autofluorescence.

***Phytophthora* infection assay**

P. parasitica strain Pp016, *P. capsici* strain LT263, and *P. 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; Wang et al., 2013; Li et al., 2019). Zoospore inoculation was performed by inoculating 2000 zoospores for *P. parasitica*, 800 for *P. capsici* and 1200 for *P. infestans*. *P. infestans* sporangia counts were performed as described previously on 10 days post inoculation leaves (McLellan et al., 2013; Boevink et al., 2016).

Gene expression assay

Total RNA was extracted using TRIzol reagent (Invitrogen). 800 ng total RNA was reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa). Reverse transcription quantitative PCR (RT-qPCR) was performed using FastStart Universal SYBR Green Master (ROX) (Roche) with specific primers in an iQ7 Real-Time Cycler (Life Technologies, USA). 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*. Semi-quantitative PCR was performed using EasyTaq DNA polymerase (TransGen Biotech) and amplified for 27 cycles. All primers are listed in Supplemental Table S2.

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 co-transformed 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, 25mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 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 sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE). Gels were blotted onto PVDF membranes (Roche) for 1.75 h at 250 mA in transfer buffer (25 mM Tris, 200 mM glycine, 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, 0.05% v/v Tween 20, pH 7.2) for 3-5 hours. The blocked membranes were incubated with primary antibodies at 1:2000 dilution, either a monoclonal GFP antibody raised in mouse (ABclonal, #AE012) or a monoclonal anti-FLAG antibody raised in mouse (ABclonal, #AE005). The membranes were washed with TBST three times before addition of the secondary antibody at 1:2000 dilution: HRP Goat Anti-Mouse IgG (H+L) Antibody (ABclonal, #AS003). Before ECL (ComWin, #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, 0.15 M NaCl, pH 7.2).

Bioinformatics

For phylogenetic analysis, protein sequences of the AtMORF/RIP family were downloaded from the Arabidopsis Information Resource (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 Genomic 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 1000 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.

High-resolution melting (HRM) analysis

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

Genes described here in 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), *NbMORF9* (Niben101Scf00176g00005.1)

Supplemental Data

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 high-resolution melting (HRM) results of *cob*-853 and *ndhB*-217, 242.

Supplemental Figure S12. Prediction of 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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Figure legends

Figure 1. MORF proteins play different immune roles in response to *Phytophthora* pathogens in *Nicotiana benthamiana*. A, Silencing *NbMORF1a/1b*, *NbMORF2a*, *NbMORF2b/c*, *NbMORF8*, or *NbMORF9*, respectively, in *N. benthamiana* led to different responses to *P. parasitica*. Images were taken at ~40 h after inoculation with *P. parasitica* zoospores. Results were the mean ±SE of 20 infections from at least 10 leaves. Statistical significance was assessed by *t* test. *** $P < 0.001$. Similar results were observed in three independent experiments. B, Cladogram of similarities between the AtMORF (*Arabidopsis thaliana*) and NbMORF proteins. The phylogenetic tree was constructed by using the neighbor-joining method. All NbMORF proteins share a conserved MORF box.

Figure 2. Silencing *NbMORF8* attenuates cell death induced by recognition of RXLR effectors of *Phytophthora* pathogens in *N. benthamiana*. A, Cell death observation of NbMORF8-silenced plants and control. Images were taken at five days 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 *P. parasitica* effector PpE4. C, The cell death severity assessment of the *NbMORF8*-silenced leaves and control leaves. Results were the mean ±SE of at least 25 leaves from 10 plants for each group. Statistical significance was assessed by Wilcoxon-Mann-Whitney test. ** $P < 0.01$. Similar results were observed in at least six independent experiments. 0, no necrosis (green); 1, necrosis area <50% of the agroinfiltrated area (yellow); 2, necrosis area >50% of the agroinfiltrated area (orange). D, Quantitation of cell death.

0, no necrosis (green); 1, necrosis area <50% of the agroinfiltrated area (yellow); 2, necrosis area >50% of the agroinfiltrated area (orange).

Figure 3. Mitochondrial 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 were the mean±SE of 10 biological replicates. Similar results were observed in three independent experiments. Statistical significance was assessed by *t* test. *** P<0.001. B, Schematic view of NbMORF8, dLNbMORF8, and CpNbMORF8. LP, leading peptide. NLS, nuclear localization signal. dL, deleting leading peptide. The leading peptide of NbMORF8 was replaced with the AtMORF9 leading peptide to re-target the fusion proteins to the chloroplast. The NbMORFs were analyzed using the same method. C, Subcellular localization of NbMORF8 and CpNbMORF8. Confocal microscopy of *N. benthamiana* leaves expressing *NbMORF8-GFP* or *CpNbMORF8-GFP*. Subcellular localization was observed at 2 or 3 days post agroinfiltration (dpi). 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 were the mean ±SE of nine organelles from three images. Statistical significance was assessed by *t* test. *** P < 0.001. E, Summary of subcellular localization and immune function of NbMORF proteins. √, NbMORF protein localized. √√, NbMORF protein preference. -, not determined. N, chloroplast localization is not required for immune function. Y, chloroplast localization is required for immune function. R, resistant to *P. parasitica*. S, susceptible to *P. parasitica*.

Figure 4. Silencing *NbMORF8* up-regulated 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 qPCR. 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. C, The 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 (hpi). The *N. benthamiana EF1α* gene was used as an internal control. Results were the mean ±SE. Similar results were observed in at least three independent experiments.

Figure 5. Silencing *NbMORF8* impaired RNA editing of *ccb206*, *cob*, and *ndhB*. A-B, The editing levels of mitochondrial *ccb206* transcripts at sites 128, 148, 149, 164, 172, 193, 194, and 286, and *cob* at site 853, and chloroplast *ndhb* at site 242. Results were the mean ±SE of three biological replicates. Statistical significance was assessed by *t* test. * $P<0.05$ ** $P < 0.01$. C, Yeast transformants were separately transferred onto SD/-Leu/-Trp (SD-LT) and SD-Leu/-Trp/-His/-Ade (SD-LTHA) medium. The growth of yeast transformants on SD-LT medium demonstrated successful transformations. The growth of yeast transformants on SD-LTHA indicates interactions. Image was taken three days after dropping the transformed yeast on SD/-LTHA 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 were the mean ±SE of three biological replicates. E, The cytochrome c levels and complex III activities were detected using ELISA. Results were the mean ±SE of three biological replicates. Statistical significance was assessed by *t* test. * $P<0.05$.

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 were the mean ±SE of nine biological replicates. Similar results were obtained in at least three independent experiments. Statistical significance was assessed by *t* test. B, Schematic view of NbMORF8 deletion mutant constructs. LP, leading peptide. N, N terminal ahead of MORF box. C, C terminal behind MORF box. dC, deleting C terminal behind MORF box. dM, deleting

MORF box. S, susceptibility. ND, no difference compared with GFP. MS, more susceptible than the full length NbMORF8. C, Subcellular localization of LP-NbMORF8, N-NbMORF8, dC-NbMORF8, and dM-NbMORF8 was determined using confocal microscopy in *N. benthamiana* leaves three days after agroinfiltration (dpi). AOX-RFP was used as a mitochondrial marker. Chlorophyll florescence was used as chloroplast marker, shown in blue. D, The yeast-two-hybrid assay of NbMORF8 mutations. Yeast transformants were separately transferred onto SD/-Leu/-Trp (SD-LT) and SD-Leu/-Trp/-His/-Ade (SD-LTHA) medium. The growth of yeast transformants on SD-LT medium demonstrated successful transformations, and the growth of yeast transformants on SD-LTHA medium indicated interactions. Images were taken five days after dropping the transformed yeast cells on SD/-LTHA 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 the mean ±SE of six biological replicates. Statistical significance was assessed by *t* test. * $P<0.05$ ** $P < 0.01$ *** $P < 0.001$. 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.

Figure 7. Silencing *NbMORF8* suppresses 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 dpi. For the fluorescence intensity analysis, six pictures from each group were analyzed using ImageJ. Results were the mean ±SE of 6 pictures. Statistical significance was assessed by *t* test. *** $P < 0.001$. C, Western blot 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, Semi-quantitative 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.

Figure 8. Schematic model for the role of *NbMORF8* in plant immunity. NbMORF8 participates 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

731 expression of SA signal pathway markers (*NbPR1* and *NbPR2*) and ROS levels, which enhances
732 the immunity to *Phytophthora* pathogens. The *NbMORF8*-regulated ROS burst is likely achieved
733 through its effect on functionality of respiratory chain components. However, the exact ROS
734 production sites in *NbMORF8*-silenced plants remain to be revealed. *NbMORF8* is required for
735 the accumulation of multiple *Phytophthora* RXLR effectors that suppress plant immunity.
736 Mitochondrial localization of NbMORF8 is sufficient for its immune function. It remains to be
737 determined whether the potential chloroplast localization of NbMORF8 is required for plant
738 immunity or if cross-talk exists between mitochondria and chloroplasts.
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741 **Literature Cited**
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