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The GHKL ATPase MORC1 Modulates Species-Specific Plant Immunity in Solanaceae

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The microrchidia (MORC) proteins, a subset of the GHKL ATPase superfamily, were recently described as components involved in transcriptional gene silencing and plant immunity in *Arabidopsis*. To assess the role of MORC1 during resistance to *Phytophthora infestans* in solanaceous species, we altered the expression of the corresponding MORC1 homologs in potato, tomato, and *Nicotiana benthamiana*. Basal resistance to *P. infestans* was compromised in *StMORC1*-silenced potato and enhanced in overexpressing lines, indicating that StMORC1 positively affects immunity. By contrast, silencing *SlMORC1* expression in tomato or *NbMORC1* expression in *N. benthamiana* enhanced basal resistance to this oomycete pathogen. In addition, silencing *SlMORC1* further enhanced resistance conferred by two resistance genes in tomato. Transient expression of StMORC1 in *N. benthamiana* accelerated cell death induced by infestin1 (INF1), whereas SlMORC1 or NbMORC1 suppressed it. Domain-swapping and mutational analyses indicated that the C-terminal region dictates the species-specific effects of the solanaceous MORC1 proteins on INF1-induced cell death. This C-terminal region also was required for homodimerization and phosphorylation of recombinant StMORC1 and SlMORC1, and its transient expression induced spontaneous cell death in *N. benthamiana*. Thus, this C-terminal region likely plays important roles in both determining and modulating the biological activity of MORC1 proteins.

Plants are constantly exposed to many kinds of pathogens and pests. As a result, they have evolved multiple layers of defenses, and they rely on a complex network of systemic signals emanating from the infection sites to induce defenses in distal portions of the plant (Dempsey and Klessig 2012; Jones and Dangl 2006). The first layer of active defense against pathogen infection is based on the recognition of pathogen-associated molecular pattern (PAMPs; also known as microbe-associated molecular patterns). PAMPs are slowly evolving signature-pattern molecules derived from pathogens such as flg22 and elf18 from bacteria, chitin and β-glucans from fungi, and cellulose-binding domains, elicitins (such as infestin1 [INF1]), and Pep-13 from oomycetes (Ingle et al. 2006). Recently, the necrosis and ethylene-inducing peptide 1-like protein from oomycete pathogens was shown to be a PAMP (Oome and Van den Ackerveken 2014). Recognition of PAMPs by their corresponding transmembrane pattern recognition receptors (PRRs) triggers PAMP-triggered immunity (PTI) in the inoculated leaf (Jones and Dangl 2006; Zipfel 2008).

Although PTI is sufficient to halt further colonization by many microbes, successful pathogens deploy effector proteins that suppress PTI. As a countermeasure, plants have evolved a second set of receptors, termed resistance (R) proteins, which specifically recognize their cognate effector protein or detect effector-mediated alteration of a host target. As a result of this direct or indirect effector recognition, a second layer of immunity, called effector-triggered immunity (ETI; also called *R* gene-mediated resistance), is activated. ETI is an accelerated and amplified PTI response, resulting in disease resistance and usually a hypersensitive cell death response (HR) at the infection site (Jones and Dangl 2006); this response can help restrict pathogen growth and spread (Dodds and Rathjen 2010; Jones and Dangl 2006). In addition to activating defenses at the initial infection site, both PTI and ETI can trigger systemic acquired resistance (SAR), which is manifested as a long-lasting, broad-spectrum resistance to subsequent pathogen infection in the distal, uninfected tissues (Dempsey and Klessig 2012; Grant and Lamb 2006).

In comparison with PTI and ETI, by far the most common form of disease resistance is nonhost resistance, in which an entire plant species displays immunity against all variants of a pathogen species. Despite its great importance, the molecular basis for this type of resistance is not well understood (Ellis 2006; Schweizer 2007). However, recent studies suggest that the activation of defenses during nonhost resistance can involve recognition of PAMPs by PRR receptors, as well as recognition of pathogen effectors by their corresponding R protein receptors (Schulze-Lefert and Panstruga 2011; Schwessinger and Zipfel 2008). Thus, it is possible that both PTI and ETI contribute to nonhost resistance.

Resistance to *Turnip crinkle virus* (TCV) in *Arabidopsis* is governed by the resistance protein HRT (HR to TCV) and its cognate avirulence factor, the viral coat protein. A genetic screen to identify components involved in the TCV resistance signaling pathway previously identified *Arabidopsis* microrchidia (MORC)1, formerly named CRT1 ("compromised for recognition of TCV 1") (Kang et al. 2008). AtMORC1, like a subset of other proteins belonging to the GHKL superfamily of ATPases, contains a GHKL ATPase domain and an S5 fold domain that, together, are the hallmark of MORC (Iyer et al. 2008). MORC proteins were first identified in mice as nuclear proteins required for male spermatogenesis (Inoue et al. 1999). A variety of prokaryotic and eukaryotic proteins containing the GHKL ATPase motif have

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since been shown to play roles in rearranging DNA structure (gyrase or topoisomerases), heat shock response (Hsp90), signal transduction (histidine kinase), or DNA mismatch repair (MutL) (Iyer et al. 2008). Recently, *Arabidopsis* MORC proteins were implicated in gene silencing or chromatin remodeling in response to epigenetic signals (Brabbs et al. 2013; Lorković 2012; Moissiard et al. 2012).

In addition to these functions, MORC proteins in plants have been implicated in immunity. Analyses of the *Arabidopsis* double-knockout mutant *morc1 morc2* (previously designated *crt1-2 crh1-1*), which lacks *MORC1* and its closest homolog *MORC2* (formerly named *CRH1*), indicated that AtMORC1 and AtMORC2 are required for multiple layers of immunity, including PTI, ETI, SAR, and nonhost resistance (Kang et al. 2010, 2012). By contrast with *Arabidopsis* MORC orthologs, which positively affect immunity, the MORC orthologs in barley were recently shown to negatively affect basal resistance and ETI against both biotrophic and necrotrophic pathogens (Langen et al. 2014). Currently, it is unclear whether the opposing effects of these MORC orthologs are due to differences between their protein sequences (they share less than 50% amino acid identity) or are caused by differences in the cellular environments in which they function. This latter alternative is particularly relevant considering that *Arabidopsis*, a dicot, and barley, a monocot, are highly divergent plant species (Langen et al. 2014).

The oomycete pathogen *Phytophthora infestans* is the causal agent of the late blight disease in its solanaceous host species potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*); it was responsible for the Great Irish Potato Famine of the 1840s. Even today, late blight is one of the world's most devastating crop diseases, with worldwide losses exceeding several billion dollars annually (Haverkort et al. 2008). This destructive pathogen, if left unchecked, can destroy entire tomato and potato crops within days. This pathogen's success is due not only to its extreme virulence but also to its remarkable capacity to rapidly overcome *R*-gene-mediated resistance, which has led several authors to label it the "*R* gene destroyer" (Haas et al. 2009; Vleeshouwers et al. 2011).

Previous studies revealed that MORC GHKL ATPases are required for *Arabidopsis* to activate nonhost resistance to *P. infestans* (Kang et al. 2012). To assess whether MORC1 also functions during basal resistance to *P. infestans* in solanaceous host species, transgenic potato, tomato, and *Nicotiana benthamiana* expressing altered levels of their respective MORC1 orthologs were generated and their resistance to this pathogen was tested. These analyses revealed that the MORC1 proteins in these closely related species have divergent, species-specific functions that positively or negatively influence immunity. Domain-swapping studies further indicated that MORC1 function is determined by specific amino acid residues in the C-terminal region. The combined findings that the C-terminal region (i) can be phosphorylated in vitro, (ii) is required for dimerization of recombinant MORC1 proteins, and (iii) induces spontaneous cell death when transiently expressed in *N. benthamiana* suggest that this region plays a key role in modulating MORC1 activity.

RESULTS

Identification of MORC homologs from solanaceous genomes.

In *Arabidopsis*, the MORC family consists of seven members. To identify solanaceous MORC homologs, the tomato (*S. lycopersicum*) genome sequence, available on the Sol Genomics Network (SGN), was scanned with *Arabidopsis* MORC cDNA sequences. The resulting tomato homologs were then used for BLAST analysis against potato (*S. tuberosum*) and *N. benthamiana* genomic sequences. Six MORC genes were identified in each solanaceous species tested. Based on their amino acid sequence, they shared the greatest level of identity with AtMORC1, AtMORC4, AtMORC6, and AtMORC7 (Kang et al. 2008), and they are distributed through the three phylogenic clades of the *Arabidopsis* MORC family (Fig. 1) (Langen et al. 2014). *N. benthamiana*, which is an allotetraploid resulting from the hybridization of two unknown progenitors (Bombarely et al. 2012), appears to have two different alleles for most of its homologs, which presumably are derived from the two progenitor lines. Interestingly, only one MORC family member (MORC1) belongs to clade I in these solanaceous species, whereas *Arabidopsis* has three members (AtMORC1, AtMORC2, and AtMORC3) and barley has two (HvMORC1 and HvMORC2) (Langen et al. 2014).

Given the importance of MORC1 in modulating immunity in *Arabidopsis* and barley, this study focused on assessing the functions of solanaceous MORC1 proteins. Sequence analysis indicated that StMORC1 shares 96% amino acid identity and 98.5% similarity with SlMORC1, with only 12 conservative and 15 nonconservative amino acid differences between these proteins; it shares 87 and 91% amino acid identity and similarity, respectively, with NbMORC1; SlMORC1 and NbMORC1 share 85 and 91% amino acid identity and similarity, respectively (Supplementary Fig. S1).

Similar to the previously described MORC1 proteins in *Arabidopsis* and barley (Kang et al. 2008; Langen et al. 2014), the solanaceous MORC1 proteins contain the GHKL ATPase and S5 fold domains that are the hallmark of MORC proteins (Watson et al. 1998; Iyer et al. 2008). An unstructured region or linker (L1) connects these two domains. The CRT1 subfamily members of MORC proteins also contain a coiled-coil (CC) domain, which forms a putative basic leucine zipper (bZIP) domain, as described by Langen and associates (2014). In agreement with this, bioinformatics analysis predicted that the C-terminal region of the solanaceous MORC1 proteins contains a CC domain, which forms a putative bZIP domain; this zipper could mediate protein–protein interactions, including dimer formation (data not shown). The CC domain is connected to the S5 fold domain via a second linker (L2).

The MORC1 proteins from the Solanaceae family exhibit ATPase activity, which is inhibited by the GHKL ATPase-specific inhibitor radicicol.

The GHKL ATPase domains of *Arabidopsis* and barley MORC1, like those of other GHKL ATPases, including Hsp90, MutL, PMS2, and DNA gyrase B, contain three key conserved motifs (Chène 2002). Motif B1 (ExxxNxxD) and motif B3 (GxxGxG/A) interact with the phosphate group of ATP, whereas motif B2 (DxGxG) cradles the adenine moiety. These three motifs also are present in the solanaceous MORC1 proteins. Analysis of recombinant C-terminal His_6-tagged full-length (FL) $StMORC1_{1-644}$ and $SlMORC1_{1-644}$ expressed in *Escherichia coli* revealed that they, like their *Arabidopsis* and barley counterparts, exhibit ATPase activity in vitro (Fig. 2A). This activity was inhibited by the antibiotic radicicol (Fig. 2A), which was previously shown to specifically inhibit the ATPase activity of other GHKL ATPase family members, including Hsp90, by occupying the ATP-binding site (Chène 2002).

AtMORC1 and its homolog AtMORC6 exist primarily as heterodimers in vivo (Moissiard et al. 2014). Sequence analyses of StMORC1 and SlMORC1 predicted that their C-terminal region forms a dimeric but not trimeric CC, suggesting dimerization of MORC1 (data not shown). Thus, the ability of recombinant StMORC1 and SlMORC1 to form homodimers in solution was tested using gel filtration chromatography followed by immunoblotting. The FL proteins predominantly eluted in fractions corresponding to approximately 150 KDa, consistent with the size of dimers (Fig. 2B). To determine whether dimerization requires

the predicted C-terminal CC region, recombinant StMORC1$_{59-501}$ and SlMORC1$_{59-501}$, which lack the predicted CC domain and part of L2, were subjected to gel filtration chromatography. The truncated versions eluted as monomers (50 KDa) (Fig. 2B). Thus, the CC domain appears to be required for homodimerization of recombinant StMORC1 and SlMORC1 in vitro.

Whether the C-terminal region is important for ATPase activity also was assessed. StMORC1$_{59-501}$ and SlMORC1$_{59-501}$ exhibited ATPase activity at levels similar to those of the FL versions (Fig. 2C). These results suggest that the CC domain-dependent homodimerization of StMORC1 and SlMORC1 is not required for ATPase activity in vitro.

StMORC1 of potato positively affects basal resistance.

Because AtMORC1 and its closest homolog, AtMORC2, are required for nonhost resistance to *P. infestans* in *Arabidopsis* (Kang et al. 2012), we tested whether StMORC1 contributes to basal resistance against this pathogen in potato. RNA interference (RNAi) was used to generate transgenic *S. tuberosum* 'Désirée' lines in which the endogenous *StMORC1* gene was completely silenced (Supplementary Fig. S2A). Resistance to *P. infestans* was then tested using a detached-leaflet assay. *StMORC1*-silenced potato exhibited increased susceptibility to several virulent strains of *P. infestans*, as manifested by the development of substantially larger lesions than control plants transformed with an empty vector (EV) construct. Indeed, the collapsed tissue covered nearly the entire leaf by 6 days post-inoculation (dpi), precluding an accurate determination of the number of sporangia (Fig. 3A; Supplementary Fig. S2B). In addition, when whole plants were inoculated in the greenhouse with the US22 strain of *P. infestans*, the causal strain of the 2009 late blight epidemic in the northeastern United States (Fry et al. 2013), a 40% increase in diseased area was observed in the *StMORC1*-silenced lines as compared with the EV control plants (Supplementary Fig. S2C). RNAi-mediated silencing of *StMORC1* in the major commercial potato variety, 'Russet Burbank IDA', also compromised resistance to several virulent strains of *P. infestans* (data not shown), indicating that silencing *StMORC1* increases susceptibility to *P. infestans* in more than one potato cultivar.

In contrast, transgenic Désirée potato lines overexpressing (OE) an N-terminal 6Myc-tagged StMORC1 protein driven by an estradiol-inducible promoter (Supplementary Fig. S3A and B) showed reduced susceptibility to *P. infestans* in detached-leaflet assays (Fig. 3B). Both lesion size and the number of sporangia were reduced in these plants as compared with the EV controls. These OE lines also exhibited reduced disease symptom development following whole-plant inoculation in the greenhouse (data not shown). Together, these results argue that StMORC1, like its *Arabidopsis* ortholog, positively affects immunity.

The tomato SlMORC1 negatively affects basal resistance.

To assess whether *SlMORC1* of tomato also participates in basal resistance against *P. infestans*, we generated several independent transgenic *S. lycopersicum* 'M82' lines in which the endogenous *SlMORC1* gene was completely silenced (Supplementary Fig. S4A). Unexpectedly, these plants exhibited enhanced resistance to the virulent US22 strain in detached-leaflet assays, as manifested by the development of smaller lesions and fewer sporangia as compared with the EV controls (Fig. 3C). These *SlMORC1*-silenced tomato lines also exhibited enhanced resistance to US23, the virulent strain of *P. infestans* that currently predominates in the United States (data not shown). Analysis of the segregating T_2 progeny from the RNAi10 T_1 line revealed that all silenced progeny for *SlMORC1* displayed enhanced levels of *P. infestans* resistance (Supplementary Fig. S4B and C).

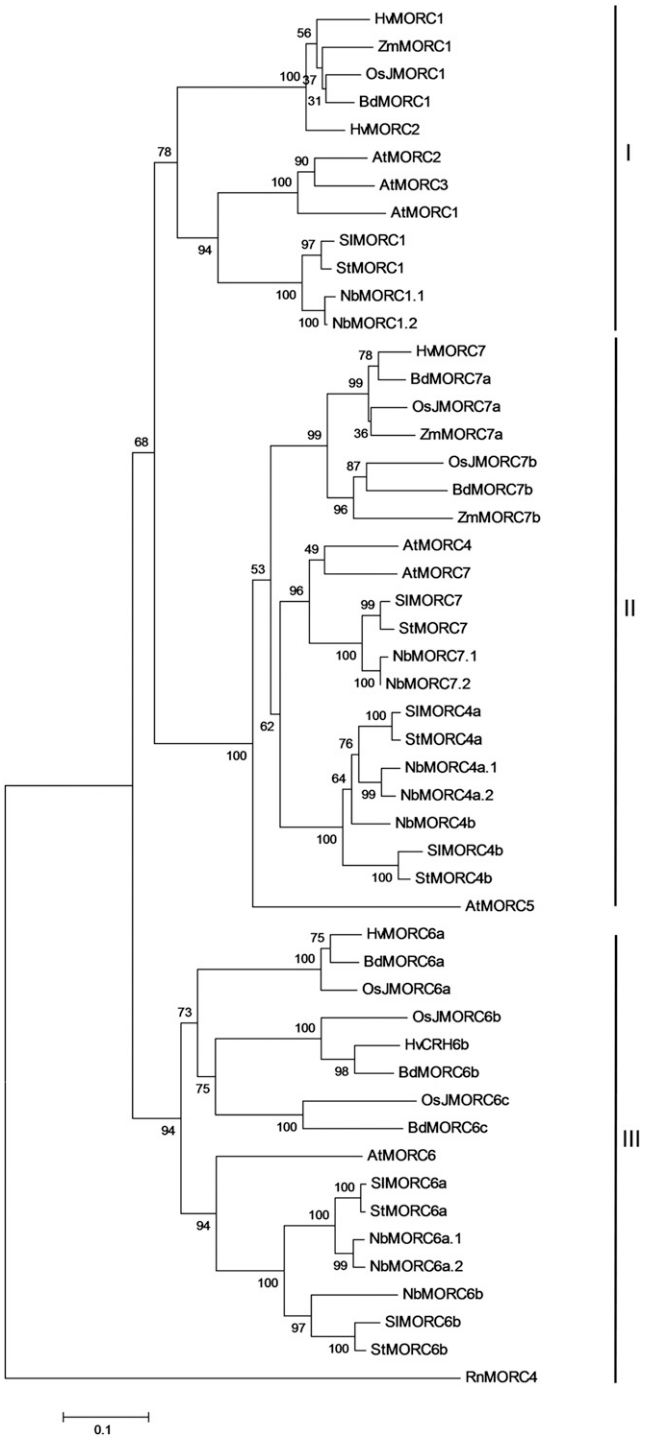

Fig. 1. Phylogenetic analysis of the MORC gene family in the Solanaceae family. The analysis involved 50 amino acid sequences corresponding to MORC proteins from the following species: *Hordeum vulgare* (Hv), *Zea mays* (Zm), *Brachypodium distachyon* (Bd), *Oryza sativa* (Os), *Arabidopsis thaliana* (At), *Nicotiana benthamiana* (Nb), *Solanum tuberosum* (St), and *S. lycopersicum* (Sl). The closest homolog from *Rattus norvegicus*, RnMORC4, was used as an outgroup (Langen et al. 2014). The evolutionary history was inferred using the neighbor-joining method using MEGA5. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Previously identified clades of the MORC family in plants are also indicated (I, II, and III).

In contrast, tomato M82 transgenic OE lines, in which the estradiol-inducible promoter controlled expression of an N-terminal 6Myc-tagged SlMORC1 (Supplementary Fig. S5A), showed increased susceptibility to several virulent strains of *P. infestans*. In detached-leaflet assays, these plants developed larger lesions and supported a greater number of sporangia than the EV controls (Fig. 3D; Supplementary Fig. S5B). Analysis of the T_2 segregating progeny from the OE25 line showed that the T_2 progeny overexpressing SlMORC1 protein displayed increased susceptibility to *P. infestans* (data not shown).

To test whether altering *SlMORC1* expression affects basal resistance to another common tomato pathogen, resistance to *Pseudomonas syringae* pv. *tomato* DC3000, the causal agent of bacterial speck disease, was monitored. Analysis of the *SlMORC1*-silenced T_2 progeny from the RNAi2 T_1 transgenic line revealed substantially less bacterial growth at 3 dpi than was detected on the EV4 control (Supplementary Fig. S4D). Thus, suppressing SlMORC1 expression appears to lead to broad-spectrum resistance in tomato.

NbMORC1 negatively affects basal resistance in *N. benthamiana.*

The solanaceous species *N. benthamiana* is a model plant widely used to study plant–microbe interactions because it is amenable to functional studies, including virus-induced gene silencing (VIGS) and transient gene expression (Bombarely et al. 2012). To determine the role of *NbMORC1* in basal resistance to *Phytophthora infestans*, we silenced this gene in *N. benthamiana* using VIGS and then challenged the silenced leaves of four plants with *P. infestans*. Reverse-transcription polymerase chain reaction (RT-PCR) analysis revealed that *NbMORC1* expression in the *Tobacco rattle virus* (TRV):*NbMORC1* plants (M1 to M8) was strongly reduced as compared with that in the TRV:EV controls (EV1 to EV8) (Fig. 4A). Analysis of sporangia density revealed that the TRV:*NbMORC1*-silenced plants (M1 to M4) were more resistant to *P. infestans* than the TRV:EV controls (EV1 to EV4) (Fig. 4B and C).

P. infestans produces a 10-kDa extracellular protein, the elicitin INF1, which is considered to be an oomycete PAMP (Chaparro-Garcia et al. 2011; Gilroy et al. 2011; Heese et al. 2007). In *N. benthamiana*, INF1 triggers defense responses, including cell death and reactive oxygen species accumulation, and its recognition activates PTI, which is suppressed by several *P. infestans* effectors (Bos et al. 2006; Kamoun et al. 1998; Oh et al. 2009). Consistent with the enhanced level of *P. infestans* resistance previously observed, *NbMORC1*-silenced *N. benthamiana* also displayed an accelerated INF1-induced cell death response. Extensive cell death was detected in TRV:*NbMORC1* silenced leaves 3 days after transient expression of a constitutively expressed INF1 construct, whereas even low levels of necrosis were not observed in the TRV:EV control lines until 4 days after INF1 expression (Fig. 4D). However, by 6 days post INF1 expression, the levels of necrosis in the TRV:*NbMORC1* and TRV:EV lines were comparable.

MORC1 negatively affects ETI to *P. infestans* in some species of Solanaceae.

Because MORC1 and its closest homologs affect ETI in *Arabidopsis* and barley (Kang et al. 2008, 2010; Langen et al. 2014), we tested whether StMORC1 and SlMORC1 contribute to ETI in members of the Solanaceae family by generating transgenic J101K6A6K41 ('K41') potato (Bhaskar et al. 2008), in which *StMORC1* was partially silenced (Supplementary Fig. S6A). K41 contains the *Rpi-blb1* (*RB*) resistance gene, which encodes a CC nucleotide-binding site leucine-rich repeat (NBS-LRR) protein that recognizes the *P. infestans* RXLR effector

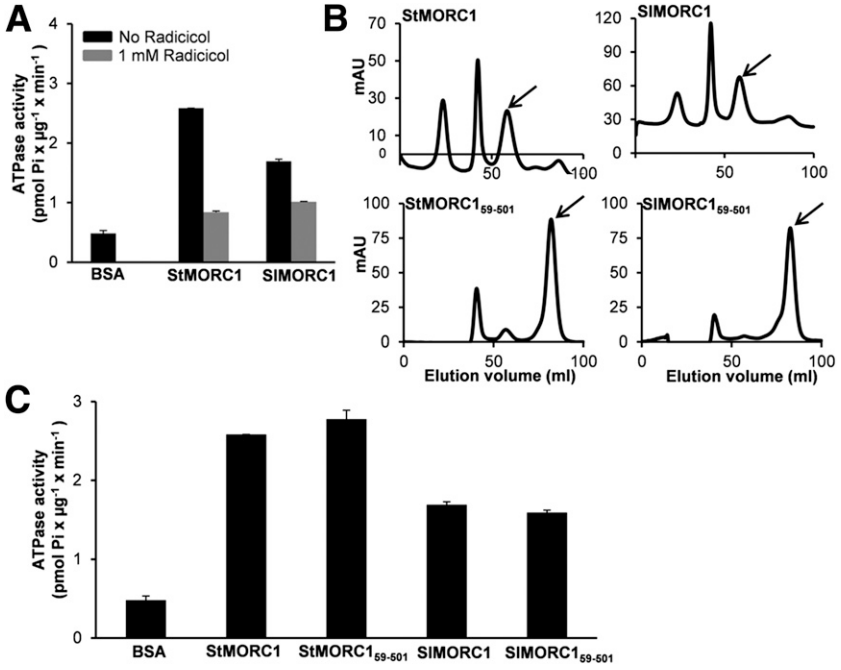

Fig. 2. Potato and tomato MORC1 proteins display radicicol-inhibitable ATPase activity that is independent of their oligomerization state. Recombinant proteins were generated as C-terminal 6xHis-tagged fusion proteins using the pET24d expression vector. **A,** ATPase activity of purified StMORC1 and SlMORC1 in the absence or presence of 1 mM radicicol. Data represent the mean of two independent measurements, with the error bars representing the standard deviation (SD). These results are representative of three different experiments with similar results. Bovine serum albumen (BSA) was used as a negative control. **B,** Analysis of the oligomerization state of purified full-length (FL) and truncated 6xHis-tagged potato and tomato MORC1 proteins (St- and $SlMORC1_{59\text{-}501}$) by gel-filtration chromatography. Arrows indicate peaks of corresponding proteins. **C,** ATPase activity of purified FL and truncated St- and $SlMORC1_{59\text{-}501}$ proteins. Data represent the mean of two independent measurements, with the error bars representing the SD. The results are representative of three different experiments with similar results. BSA was used as a negative control.

Avrblb1. Using a detached-leaflet assay, transgenic *StMORC1*-silenced K41 plants and EV controls were inoculated with the US22, US11, or US23 isolates of *P. infestans.* No visible differences were observed between the silenced and control plants after *P. infestans* inoculation, regardless of which isolate was used (Supplementary Fig. S6B; data not shown). Thus, StMORC1 does not appear to affect *RB*-mediated resistance to *P. infestans*. However, because silencing was not complete, we cannot rule out the possibility that the residual levels of StMORC1 are sufficient for ETI. Similar to the results in potato, VIGS-mediated silencing of *NbMORC1* in *N. benthamiana*, which contains the *RB* gene, did not affect recognition of transiently expressed Avrblb1. Comparable levels of HR cell death were observed in both *NbMORC1*-silenced and EV control leaves (Supplementary Fig. S6C and D).

To test the effect of *SlMORC1* on ETI in tomato, this gene was silenced in the tomato breeding line 'NC 2 CELBR', which carries the *P. infestans* resistance genes *Ph2* and *Ph3* in the homozygous state (Gardner and Panthee 2010). Although *Ph2* has yet to be isolated, *Ph3* encodes a CC-NBS-LRR type R protein (Zhang et al. 2014). Analysis of plants from two transgenic NC 2 CELBR lines in which *SlMORC1* was completely silenced (Fig. 5A) revealed that they developed even smaller HR lesions than the EV control plants after inoculation with *P. infestans* strain US22 (Fig. 5B and C). Microscopic analysis further revealed that *P. infestans* penetration was completely suppressed in the NCRNAi5 line at all time points assayed; a similar lack of penetration was generally observed in the NC2RNAi1 line, although a small amount of penetration was detected in some plants at 24 h postinoculation (Fig. 5D). By contrast, the EV control plants supported substantially greater levels of penetration at every time point analyzed. Together, these results argue

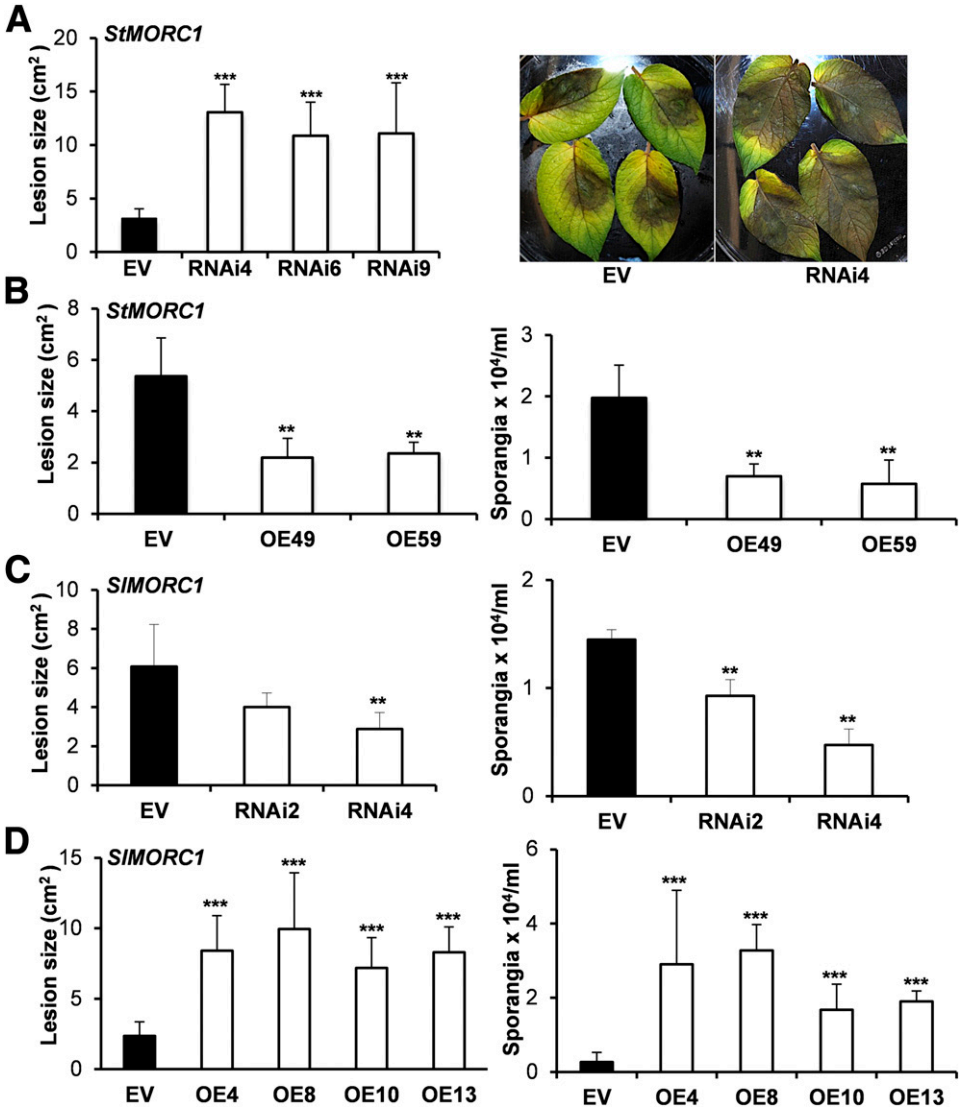

Fig. 3. Altered expression of StMORC1 and SlMORC1 influences basal resistance to *Phytophthora infestans* in a species-specific manner. Basal resistance was monitored in Désirée potato or M82 tomato expressing altered levels of *MORC1* (white bars) and in the corresponding empty vector (EV) transgenic control plants (black bars) following inoculation with the US22 strain of *P. infestans*. Disease symptoms were measured based on the size of the blighted area (cm^2) at 5 days postinoculation (dpi) and the number of sporangia per milliliter at 6 dpi. Experiments were done twice with similar results. **A,** Silencing *StMORC1* increases susceptibility to *P. infestans*. Error bars for the EV plants are shown as the standard deviation (SD) corresponding to five independent EV plants; the error bars for the *StMORC1*-silenced plants (RNAi) are shown as the SD of two plants per line. Pictures of the blighted area were taken at 7 dpi with *P. infestans*. **B,** Overexpression (OE) of StMORC1 enhances resistance to *P. infestans*. Error bars for EV plants are shown as the SD corresponding to five independent EV plants; the error bars for the OE plants are shown as the SD of two plants per line. **C,** Silencing *SlMORC1* in tomato enhances resistance to *P. infestans*. Error bars for the EV plants are shown as the SD corresponding to five independent EV plants; the error bars for the silenced plants are shown as the SD of two plants. **D,** OE of *SlMORC1* increases susceptibility to *P. infestans*. Error bars for the EV plants are shown as the SD corresponding to five independent EV plants; the error bars for the OE plants are shown as SD of two plants per line. Asterisks ** and *** indicate $P \leq 0.005$ and 0.0005, respectively; two-tailed *t* test.

that *SlMORC1* negatively affects *Ph2*- and *Ph3*-mediated ETI to *P. infestans* in tomato.

Transient expression of solanaceous MORC1 proteins in *N. benthamiana* confers species-specific effects on INF1-induced cell death.

The discovery that highly conserved MORC1 proteins from closely related solanaceous species play divergent roles in basal immunity, and possibly ETI, provided a unique opportunity to assess whether MORC1 function is governed by the protein itself or the cellular environment in which it functions. To distinguish between these possibilities, constructs encoding estradiol-inducible StMORC1, SlMORC1, or NbMORC1 proteins were transiently expressed in *N. benthamiana* (Fig. 6A). Following induction of MORC1 expression, a second construct supplying constitutively expressed INF1 was infiltrated and INF1-induced cell death was monitored. At 3 days after infiltration (dai) of the second construct, the INF1-expressing tissue in StMORC1-expressing *N. benthamiana* was completely necrotic, whereas that in the EV control exhibited only approximately 20% complete necrosis and 80% partial necrosis (Fig. 6B and C). It should be noted that, whereas initiation of cell death is accelerated in StMORC1-expressing *N. benthamiana* as compared with the EV control, by 4 days after INF1 expression, both lines exhibited comparable levels of cell death (data not shown). In comparison, *N. benthamiana* expressing SlMORC1 or NbMORC1 exhibited reduced levels of INF1-induced cell death as compared with the EV control plants. At both 3 and 4 days after INF infiltration, approximately 75% of the leaf tissue in the SlMORC1- and NbMORC1-expressing plants remained necrosis free, whereas the INF1-expressing tissue in EV control plants displayed a combination of partial and complete necrosis (Fig. 6B and C; data not shown). These results, like those from our silencing and OE studies, indicate that StMORC1 positively affects the activation of defense responses, whereas SlMORC1 and NbMORC1 negatively affect them. Moreover, because the species-specific differences in MORC1 function were observed when these proteins were all expressed in *N. benthamiana*, they appear to be due to differences in the proteins themselves, rather than the cellular environment in which they function.

The C-terminal region of MORC1 determines its species-specific effect on INF1-induced cell death.

To identify the domain or region of MORC1 responsible for determining its species-specific effect, domain-swapping experiments between StMORC1 and SlMORC1 were performed and their effect on INF1-induced cell death was assessed using the *N. benthamiana* transient assay described

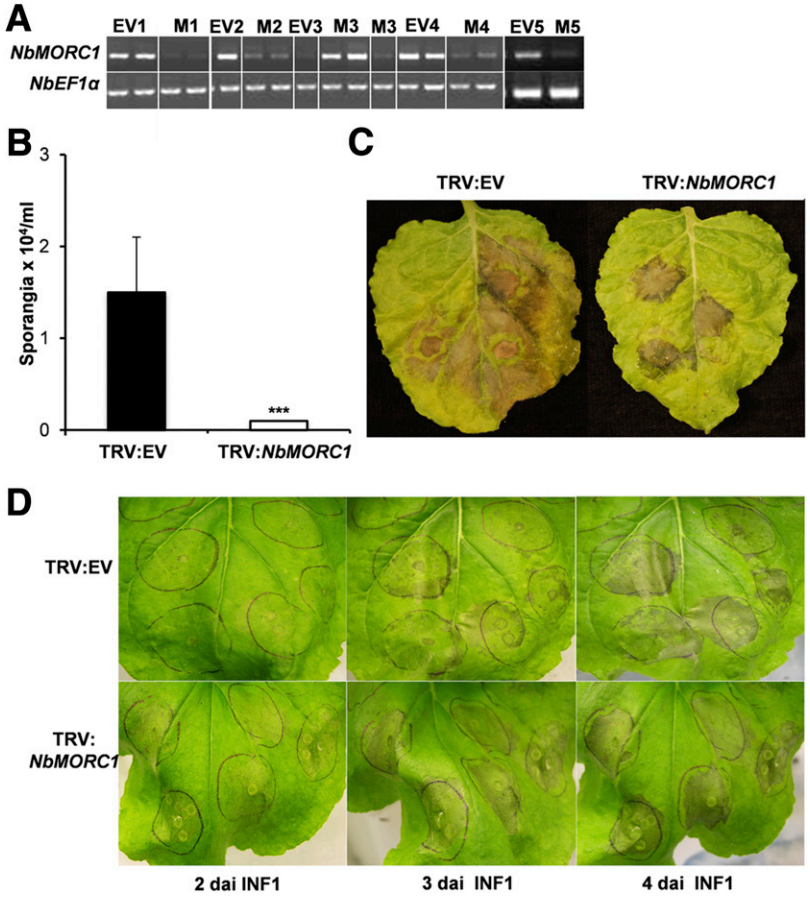

Fig. 4. Virus-induced gene silencing (VIGS) silencing of NbMORC1 in *Nicotiana benthamiana* enhances resistance to *Phytophthora infestans* and accelerates INF1-induced cell death. The *NbMORC1* gene was silenced by *Tobacco rattle virus* (TRV)-based VIGS; TRV:empty vector (EV) was used as a negative control. Plants were infiltrated with TRV:*NbMORC1* (M1-M8) and TRV:EV (EV1-EV8). **A,** Level of *NbMORC1* expression was assessed by semiquantitative polymerase chain reaction 4 weeks after infiltration. *NbEF1*α was used as an internal control. **B,** Leaves from each silenced plant (M1 to M4) or the EV controls (EV1 to EV4) were inoculated with the US22 strain of *P. infestans* 4 weeks after TRV infiltration. Sporangia numbers were counted at 14 days postinoculation (dpi). Data are average ± standard deviation (SD) ($n = 7$, where n denotes the number of independent samples); *** indicates $P \leq 0.0001$, two-tailed t test. **C,** Photographs of the blighted area were taken at 14 dpi with *P. infestans*. **D,** Four weeks after TRV infiltration, the INF1 cell death elicitor was transiently expressed (optical density = 0.075) in the TRV:EV (EV5 to EV8) control plants and in the TRV:*NbMORC1* (M5 to M8) silenced plants. Pictures of the INF1-infiltrated area were taken at 2, 3, and 4 days after infiltration (dai) to monitor the development of the cell death.

above. Seven chimeras between StMORC1 and SlMORC1 and two C-terminal deletion proteins were generated (Fig. 7A). Immunoblot analysis indicated that all of these proteins, which contained a 3xHA tag at their N terminus, accumulated to comparable levels when transiently expressed in *N. benthamiana* leaves (Fig. 7B). In the presence of transiently expressed INF1, the species-specific effect these chimeras exerted on cell death was mapped to the C-terminal region, which includes the L2 and

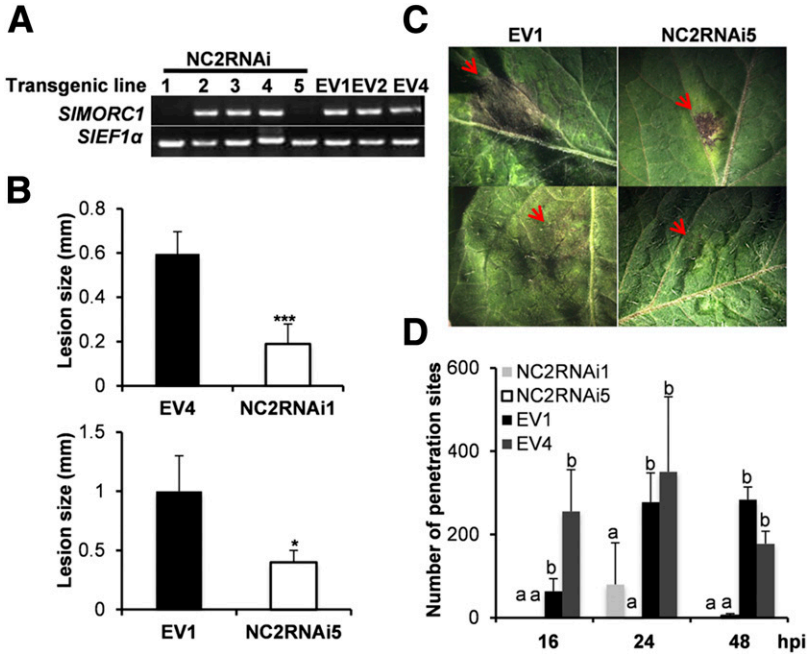

Fig. 5. Silencing *SlMORC1* further enhances *Ph2-/Ph3*-mediated resistance in tomato. **A,** *SlMORC1* expression in NC2 CELBR transgenic tomato expressing a silencing construct or an EV control construct was assessed using semiquantitative polymerase chain reaction. *SlEF1α* was used as an internal control. Note only NC2RNAi1 and NC2RNAi5 lines contained the silencing constructs (data not shown) and therefore had their endogenous *MORC1* gene silenced **A, B,** Transgenic *SlMORC1*-silenced lines NC2RNAi1 and NC2RNAi5, together with the corresponding EV1 and EV4 control plants were inoculated with the US22 isolate of *Phytophthora infestans* (4,000 sporangia/ml) using a detached-leaflet assay. Lesion size was measured 6 days postinoculation (dpi). Data are the average ± standard deviation (SD) ($n = 13$). ***$P \leq 0.0005$, two-tailed t test. **C,** Photographs of inoculated areas undergoing HR taken at 6 dpi. Arrows indicated the inoculated area. **D,** *P. infestans* penetration was determined by microscopic analysis of leaf discs punched from the inoculated area at 16, 24, and 48 h postinoculation (hpi). Leaf discs were stained with trypan blue to visualize *P. infestans* penetration. Experiments were done twice with similar results. Data are the average ± standard deviation (SD) ($n = 3$). Turkey's HSD test was performed to test statistical significant differences between EV plants and silenced plants for all the time points analyzed ay $P < 0.05$.

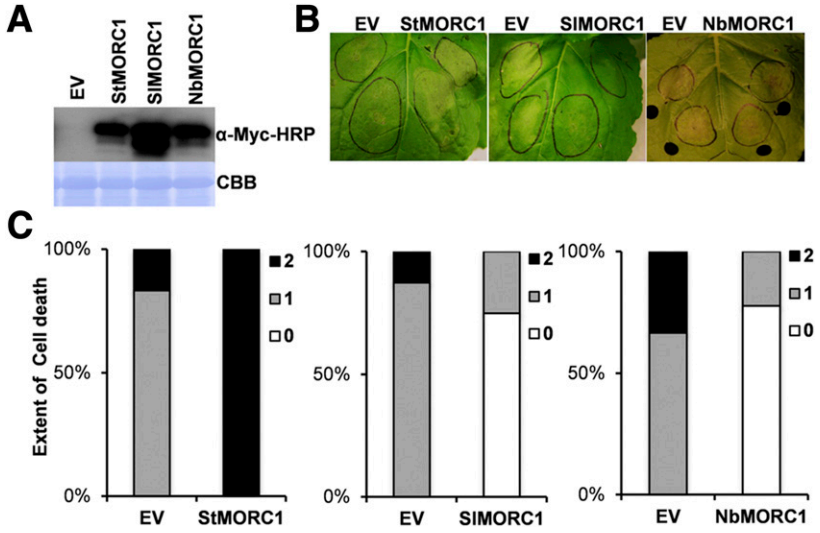

Fig. 6. Transient expression of StMORC1, SlMORC1, or NbMORC1 in *Nicotiana benthamiana* alters the development of INF1-induced cell death. N-terminal myc-tagged StMORC1, SlMORC1, and NbMORC1 driven by the β-estradiol-inducible promoter were transiently expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated transformation. An empty vector (EV) construct also was infiltrated as a negative control. β-Estradiol was sprayed 24 h after *Agrobacterium* infiltration for induction of protein production. INF1 under the expression of a constitutive promoter was transiently expressed 24 h post β-estradiol induction. **A,** Leaf samples were harvested 24 h after β-estradiol treatment and proteins were detected using immunoblot analysis to determine the accumulation of StMORC1, SlMORC1, and NbMORC1 using an anti-Myc-HRP antibody. Coomassie brilliant blue (CBB) staining was used a loading control. **B,** Photographs of *N. benthamiana* leaves were taken 3 days after infiltration (dai) with INF1. **C,** Extent of INF1-induced cell death was quantified as 0 = none, 1 = partial, and 2 = complete from the data in B at 3 dai and was represented graphically. In each experiment, half of a leaf of three plants was infiltrated with the EV construct and the other half with the corresponding construct. Data correspond to a total of eight INF1-infiltrated areas for each construct. This experiment was repeated three times with similar results.

CC domain. Swapping the L2 and CC region of StMORC1 with that of SlMORC1 to create Chi3 switched the activity of this protein from accelerating INF1-induced cell death to suppressing it (Fig. 7C and D). Likewise, the reciprocal swap to generate Chi6 changed the activity of the SlMORC1 protein from suppressing INF1-mediated cell death to accelerating it. The species-specific activity could not be switched by interchanging either L2 or CC independently (Chi2, Chi7, Chi4, or Chi5), indicating that the specific determinants governing MORC1 activity reside in both regions. Interestingly, StMORC1 and SlMORC1 constructs lacking the CC domain lost the ability to positively or negatively influence INF1-induced cell death. *N. benthamiana* expressing these deletion constructs displayed levels of cell death comparable with those of the EV controls (Fig. 7C and D), indicating the importance of this domain for the biological activity of these proteins. Together, these results provide additional evidence that the species-specific differences in MORC1 activity during defense induction are due to differences in the proteins themselves.

Four amino acids in the C-terminal region determine MORC1's species-specific activity during INF1-induced cell death.

Of the 15 nonconservative amino acid differences between StMORC1 and SlMORC1, 5 reside in L1 (which connects the ATPase and the S5 fold domains), 8 are in L2, and 2 are in the CC domain. Because the L2-CC region appears to be responsible for the species-specific phenotype, site-directed mutagenesis was used to convert most of the nonconserved amino acids in this region from potato to tomato, and vice versa. The resulting constructs were screened using the INF1-induced cell death assay (Supplementary Table S2). To facilitate investigation

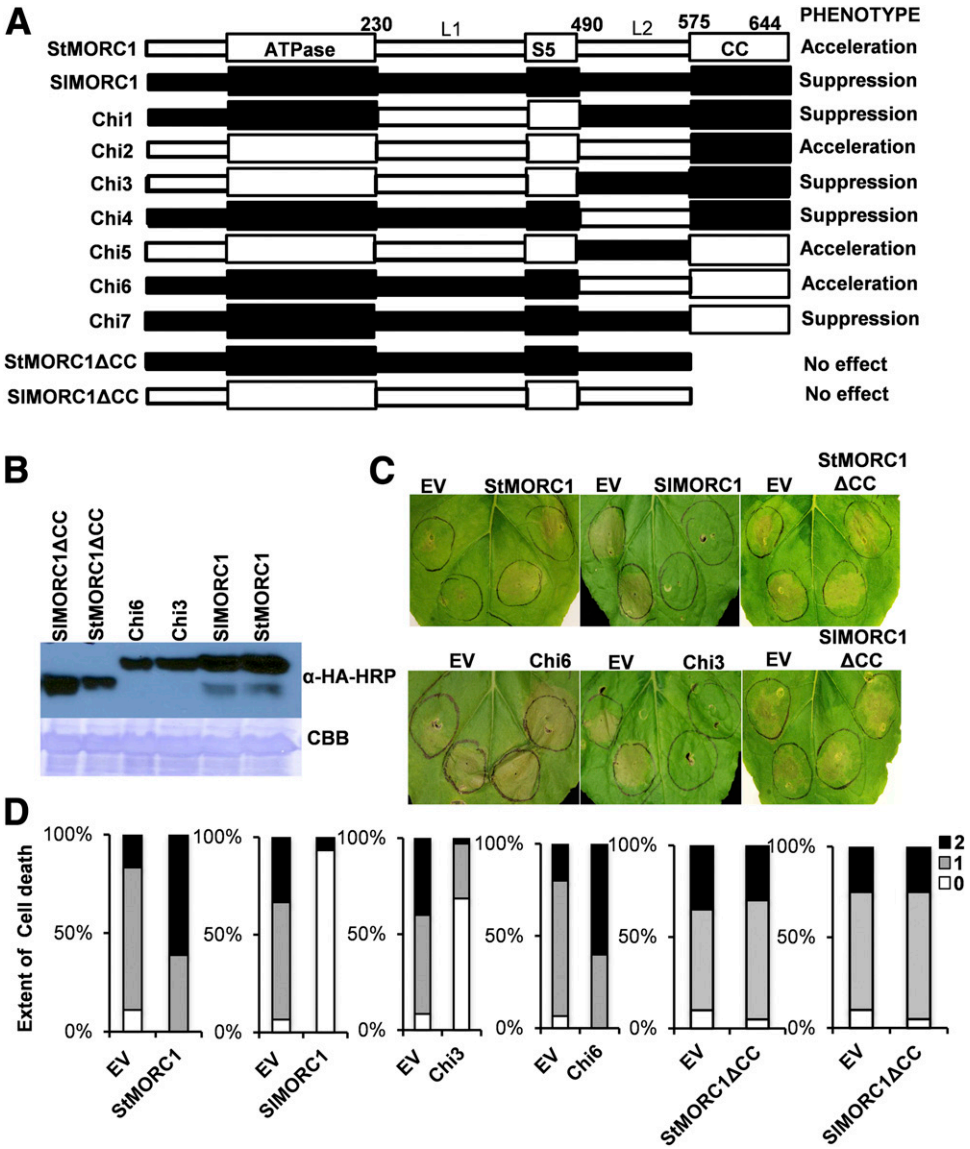

Fig. 7. C-terminal region governs MORC1's species-specific effect on INF1-induced cell death in *Nicotiana benthamiana*. **A,** Schematic diagram of the domain structure of StMORC1 (white), SlMORC1 (black), the seven chimeras generated by swapping regions between StMORC1 and SlMORC1, and their two CC truncated versions. The ATPase, S5 fold, and CC domains, as well as two linker regions, L1 and L2, are indicated. The relative amino acid position of the regions swapped is indicated. The effect of the transiently expressed proteins on INF1-induced cell death is also indicated. **B,** Proteins were detected by immunoblot analysis using an anti-HA-HRP antibody. Coomassie brilliant blue (CBB) staining serves as loading control. **C,** Photographs of INF1-induced cell death in *N. benthamiana* transiently expressing the various proteins and the empty vector (EV) control. Pictures showing INF1-induced cell death were taken at 4 dai, except for StMORC1, which was taken at 3 days postinoculation. **D,** Extent of INF1-induced cell death was quantified as 0 = none, 1 = partial, and 2 = complete at 3 dai and is represented graphically. Similar experiments were repeated three times. Data correspond to more than three plants per construct, with n = 18 for StMORC1 and SlMORC1, n = 30 for Chi3 and Chi6, and n = 20 for the CC truncated versions.

of the amino acid residues in the L2 region, mutational analysis was performed on the previously constructed Chi2 chimera, in which StMORC1 sequences were fused to the SlMORC1 CC domain (Fig. 7). Six of the eight nonconserved residues in the L2 region of Chi2 were replaced with the corresponding amino acid from SlMORC1 (Fig. 8A). Similarly, the role in species specificity of amino acid residues in the CC region was assessed by performing mutational analysis on the Chi5 chimera, which contains StMORC1 sequences, except for the SlMORC1 L2 region (Fig. 7A). The two nonconserved residues in the CC domain were independently replaced with the corresponding amino acids from SlMORC1 (Fig. 8A). All proteins were fused to a 3xHA tag at their N-terminus and immunoblot analysis was used to monitor their transient expression levels in *N. benthamiana* (Fig. 8B). Analysis of INF1-induced cell death indicated that swapping five amino acids in the L2 region (L516S, G543R, I562T, G563E, and E567K) of Chi2 or one amino acid in the CC domain (C605R) of Chi5 was sufficient to convert StMORC1's activity from accelerating cell death to suppressing it (Fig. 8C and D).

For the reciprocal study, mutational analysis was performed on Chi7, which contains SlMORC1 sequences, except for the CC domain of StMORC1, and Chi4, which consists of SlMORC1 sequences, except for the L2 region of StMORC1 (Fig. 7A). The same six nonconserved amino acids were switched to the corresponding StMORC1 residues, as described above (Supplementary Fig. S7A). Following immunoblot analysis, which indicated that these 3xHA-tagged chimeras were expressed at comparable levels in *N. benthamiana* (Supplementary Fig. S7B), their effect on INF1-induced cell death was assessed. Results from this set of chimeras revealed that changing S516L, L534F, R543G, or K567E in the L2 region of Chi7, or R605C in the CC domain of Chi4, was sufficient to convert SlMORC1's activity from suppressing to accelerating INF1-mediated cell death (Supplementary Fig. S7C and D).

Together, these studies revealed four amino acids whose reciprocal alteration in the chimeras changed MORC1 activity: three reside in the L2 region (S/L516, R/G543, and K/E567) and one in the CC domain (R/C605). To assess the minimum number of residues required to confer species specificity, FL nonchimeric StMORC1 or SlMORC1 in which different combinations of these four residues were changed were generated and tested in the cell death assay (Fig. 9). Changing residue 605 in the CC domain and only one or two of the three nonconserved amino acids in L2 (e.g., $StMORC1_{RR}$) was not sufficient to alter the cell death phenotype; instead, all four residues (516, 543, 567, and

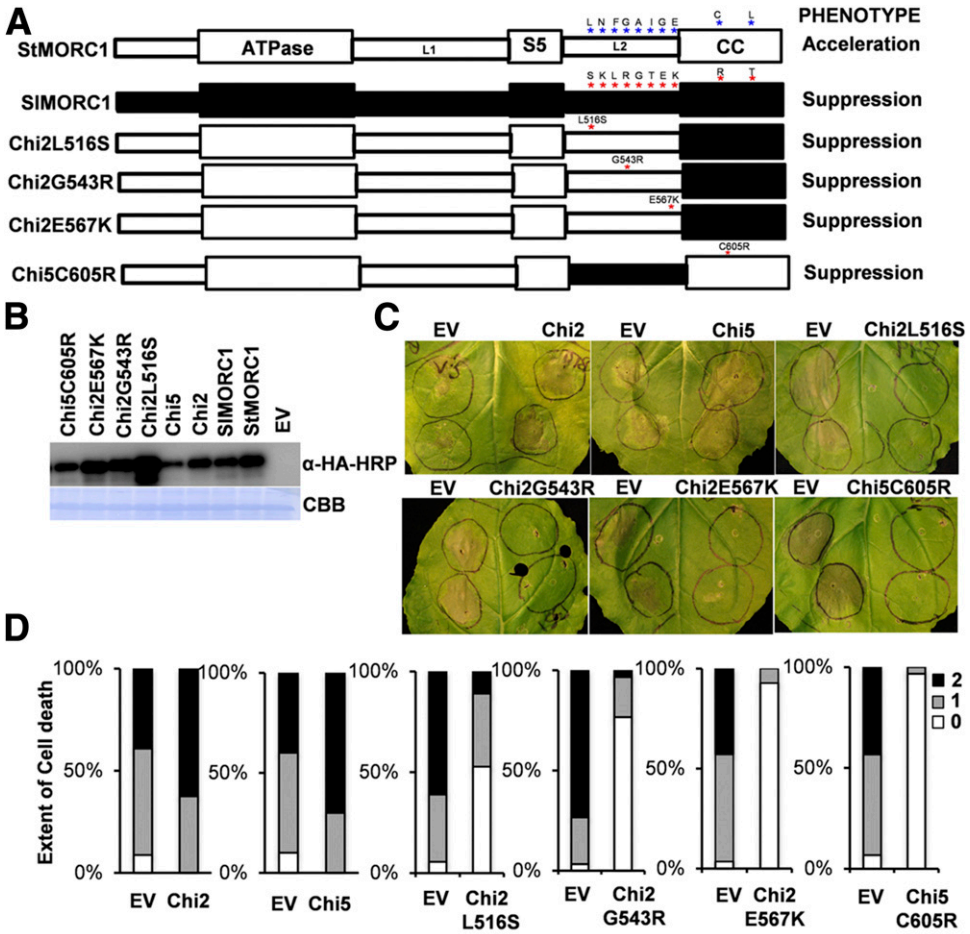

Fig. 8. Species-specific effects of StMORC1 and SlMORC1 on INF1-induced cell death are due to amino acid differences in the L2 and CC regions. **A,** Schematic diagram showing the 10 nonconserved amino acids in the L2-CC region of StMORC1 (blue asterisks) and SlMORC1 (red asterisks), as well as the four constructs generated by swapping specific amino acid residues in Chi2 and Chi5 with the corresponding amino acids from SlMORC1. The resulting INF1-induced cell death phenotype conferred by these chimeras is indicated. **B,** Immunoblot analysis showing similar expression of all N-terminal 3xHA-tagged fusion proteins. Proteins were detected using an anti-HA-HRP antibody. Coomassie brilliant blue (CBB) staining serves as loading control. **C,** Pictures of the INF1-induced cell death area were taken at 4 days after infiltration (dai). **D,** Extent of cell death was monitored in *Nicotiana benthamiana* plants transiently expressing the fusion proteins or an empty vector (EV) control. INF1-induced cell death was quantified as 0 = none, 1 = partial, and 2 = complete at 3 dai and is represented graphically. Similar experiments were repeated at least twice with similar results. Data correspond to more than three plants per construct, with $n = 24$ for Chi2 and Chi5 and $n = >28$ and <36 for the other constructs.

605) needed to be changed (Fig. 9C and D). Interestingly, three of these four amino acids are conserved between SlMORC1 and NbMORC1 (S516, K567, and R605C); this is consistent with the similar effect these two proteins have on plant immunity and INF1-induced cell death.

The C-terminal region of MORC1 is phosphorylated and displays effector or signaling activity.

Sequence analysis of StMORC1 and SlMORC1 predicted that several serine and threonine residues in the C-terminal region could be phosphorylated. To test whether these proteins are phosphorylated in vitro, recombinant N-terminal 6xHis-tagged FL StMORC1 and SlMORC1 proteins, as well as truncated versions of both proteins lacking (i) both N- and C-terminal sequences (St- and $SlMORC1_{59\text{-}501}$), (ii) an N-terminal version of SlMORC1 ($SlMORC1_{1\text{-}235}$), or (iii) the L2-CC region of the SlMORC1 ($SlMORC1_{485\text{-}644}$) (Fig. 10A) were expressed in *E. coli* and then incubated with *N. benthamiana* protein extracts in a phosphorylation assay. Both FL MORC1 proteins and the $SlMORC1_{485\text{-}644}$ constructs were phosphorylated in vitro, whereas the St- and $SlMORC1_{59\text{-}501}$ constructs and $SlMORC1_{1\text{-}235}$ were not (Fig. 10B). Moreover, FL St- and SlMORC1 were not phosphorylated in absence of *N. benthamiana* protein extracts, suggesting that these proteins do not posses autophosphorylating activity in vitro.

Unexpectedly, transient expression of $SlMORC1_{485\text{-}644}$ or $StMORC1_{485\text{-}644}$ in *N. benthamiana* induced spontaneous cell death. Because expression of the FL St- and SlMORC1, St- and $SlMORC1_{1\text{-}575}$, and St- and $SlMORC1_{1\text{-}490}$ did not induce spontaneous cell death (Fig. 10C; data not shown), the activity of the C-terminal region appears to be modulated by an interaction with either another part of the MORC1 protein (intramolecular) or by host protein factors (intermolecular) that require part of the N-terminal three-quarters of the MORC1 protein for interaction.

DISCUSSION

MORC proteins in *Arabidopsis* and barley have been shown to regulate multiple layers of plant immunity. Here, we demonstrate that StMORC1, SlMORC1, and NbMORC1 also are involved in modulating disease resistance in potato, tomato, and *N. benthamiana*, respectively. StMORC1, like MORC1 from *Arabidopsis* (AtMORC1), was found to exert a positive effect on plant immunity, whereas SlMORC1 and NbMORC1, like barley (HvMORC1), influenced immunity in a negative manner (Kang et al. 2008, 2010, 2012; Langen et al. 2014).

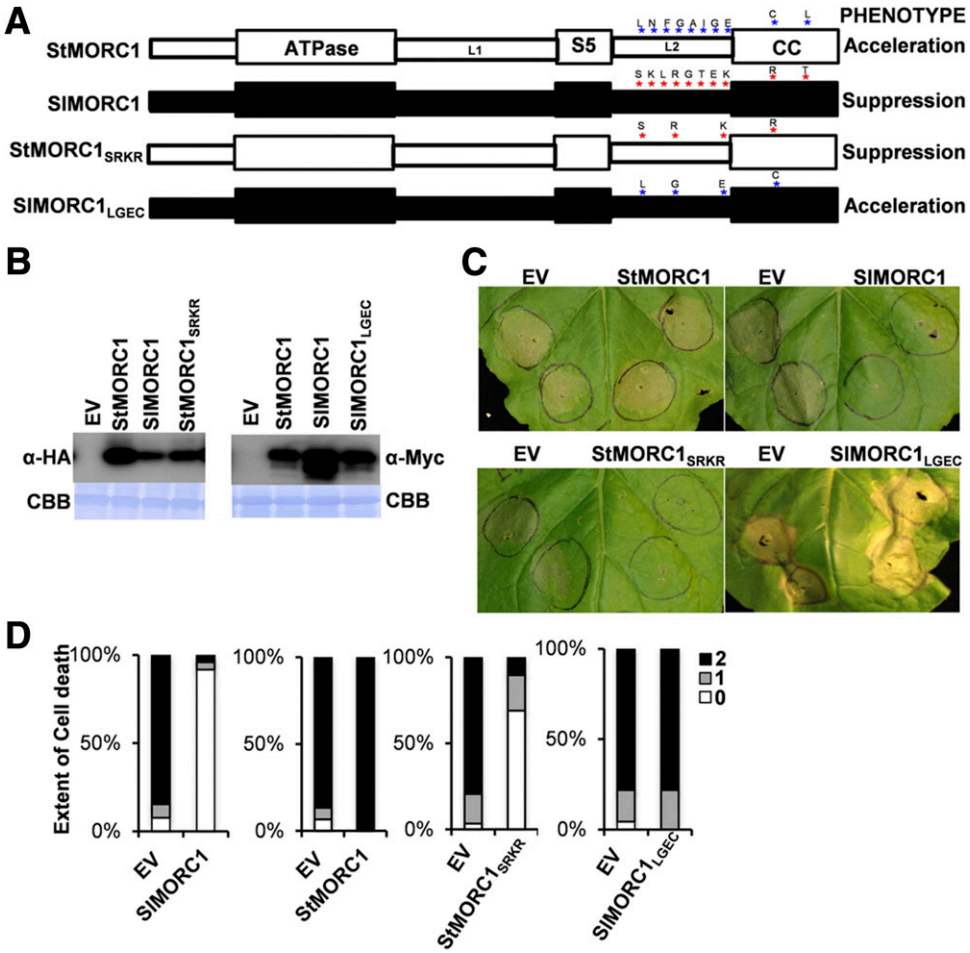

Fig. 9. Four amino acid residues in the C-terminal region are sufficient to determine the species-specific activity of MORC1 proteins. **A,** Graphic representation of StMORC1 and SlMORC1 proteins showing the 10 nonconserved amino acids (aa) in the L2-CC region, together with the constructs created by swapping 3 aa in the L2 and 1 aa in the CC region of StMORC1 with those from SlMORC1 ($StMORC1_{SRKR}$), and vice versa ($SlMORC1_{LGEC}$). The corresponding effect on INF1-induced cell death is indicated. **B,** Accumulation of the N-terminal 3xHA and N-terminal 6×Myc fusion proteins used in this study. Proteins were detected by immunoblot analysis using an anti-HA-HRP or anti-Myc-HRP antibody. Coomassie brilliant blue (CBB) staining serves as loading control. **C,** Photographs showing INF1-induced cell death were taken at 4 days after infiltration (dai). **D,** Extent of the INF1-induced cell death was quantified at 4 dai and represented graphically as 0 = none, 1 = partial, and 2 = complete. Similar experiments were repeated at least twice with similar results. Data correspond to more than three plants per constructs, with n = >23 and <30 for each construct.

Langen and coworkers previously suggested that the opposite effects that AtMORC1 and HvMORC1 exert on plant immunity are likely due to differences in the proteins themselves, because expression of HvMORC1 did not restore ETI or basal resistance in the *morc1 morc2 Arabidopsis* mutant. However, because *Arabidopsis* and barley are highly divergent plant species, the possibility that differences in cellular environment influence MORC1 activity (e.g., lack of an appropriate interacting partner) could not be ruled out. In this study, using highly conserved MORC1 homologs (>90% amino acid similarity) from different solanaceous species, we extended our previous findings by providing strong direct evidence for the species-specific effects of MORC1 on plant immunity and on PAMP-induced cell death. Moreover, we rigorously demonstrated that the MORC1 species-specific effects are governed by the proteins themselves and not by their cellular environment. Upon transient expression in the same plant species (*N. benthamiana*), *StMORC1, SlMORC1,* and *NbMORC1* retained their species-specific effect in the INF1-induced cell death assay. The identification of four amino acids in the C-terminal region (three in L2 and one in the CC domain), whose reciprocal swapping was sufficient to alter species specificity, provides further support for this conclusion.

To investigate the mechanism through which silencing of SlMORC1 leads to enhanced *Ph2*- and *Ph3*-mediated ETI to *P. infestans* in tomato, microscopic analysis of the HR lesions was performed. A substantial reduction in *P. infestans* penetration was observed in the leaves of *SlMORC1*-silenced NC 2 CELBR lines; this, in turn, would limit the formation of biotrophic structures called haustoria. Haustoria are the sites where oomycete and fungal effector proteins, including avirulence proteins, are secreted (Catanzariti et al. 2007; Petre and Kamoun 2014; Whisson et al. 2007). Thus, reduced haustoria

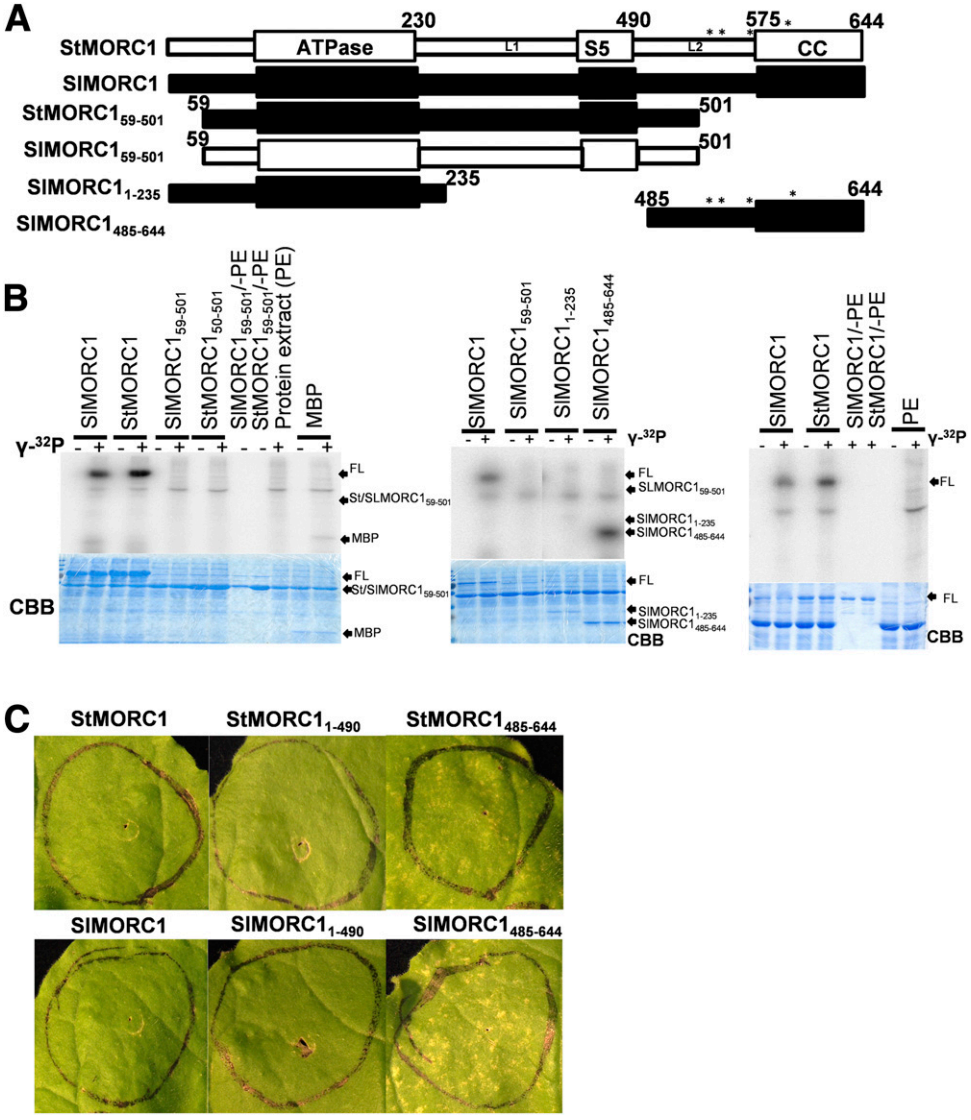

Fig. 10. L2-CC region of StMORC1 and SlMORC1 is phosphorylated in vitro and displays cell death signaling activity. **A,** Schematic of full-length (FL) StMORC1 and SlMORC1 and various deletion mutants. The relative amino acid position of the regions deleted is indicated for each construct. The 4 amino acid residues in the C-terminal region that are sufficient to determine the species-specific activity of MORC1 proteins are indicated as asterisks. **B,** All recombinant proteins were combined with *Nicotiana benthamiana* protein extracts in an in vitro phosphorylation assay in the presence (+) or absence (−) of radioactive ATP (γ-^{32}P). Protein extract (PE) alone was used as a negative control and myelin binding protein (MBP) was used as a positive control. SlMORC1$_{59\text{-}501}$/-PE was included as a loading control for this protein using Coomassie brilliant blue (CBB) staining. FL St- and SlMORC1 proteins were incubated with γ-^{32}P in the absence of PE to detect autophosphorylation activity. Black arrows indicate the expected size of each of the proteins tested. **C,** Photographs of *N. benthamiana* leaves transiently expressing FL StMORC1 or SlMORC1, St- and SlMORC1$_{1\text{-}490}$, and St- and SlMORC1$_{485\text{-}644}$. Three days after β-estradiol induction, cell death was detected in leaves infiltrated with either L2-CC construct (St- and SlMORC1$_{485\text{-}644}$) but not the others.

formation in these plants would decrease the area undergoing an HR triggered by recognition of the yet-to-be-isolated AvrPh2 and AvrPh3 effectors, which would manifest as a dramatic reduction in HR lesion size. This enhanced resistance phenotype suggests that SlMORC1 affects ETI at an early stage in the pathogenesis process, possibly at the level of pathogen perception or penetration. By contrast, ETI mediated by the *RB* resistance gene in potato and *N. benthamiana* may not require MORC1. In potato, resistance to *P. infestans* infection was comparable in *StMORC1*-silenced and EV control plants carrying this resistance gene; likewise, VIGS-mediated silencing of *NbMORC1* in *N. benthamiana* did not affect recognition of transiently expressed *Avrblb1*. However, because *StMORC1* expression was not completely suppressed in the transgenic potato lines, we cannot rule out the possibility that ETI was affected by residual StMORC1 activity.

Similar to *Arabidopsis* and barley, MORC proteins in solanaceous plants are encoded by a gene family. In this study, we identified six MORC genes from tomato, potato, or *N. benthamiana* that encode homologs to *Arabidopsis* MORC1, MORC4, MORC6, and MORC7. These MORC genes are distributed among the three clades previously reported (Langen et al. 2014). The RNAi constructs used in this study were designed to specifically silence MORC1 by targeting the same region in the potato and tomato genes. To rule out the possibility that additional MORC family members also were silenced, RT-PCR analyses with specific primers for *StMORC6a*, the closest homolog of *StMORC1* and a member of clade III, and *StMORC7*, a member of clade II, were performed. No cosilencing was detected in potato (data not shown). Thus, the altered levels of immunity observed in *MORC1*-silenced potato lines appear to be due to the specific loss of MORC1, rather than multiple MORC family members.

The observation that transient expression of the L2-CC region of StMORC1 or SlMORC1 in *N. benthamiana* induces spontaneous cell death suggests that this region is critical for MORC1's biological activity. Consistent with this possibility, transiently expressed *StMORC1* or *SlMORC1* constructs lacking the CC domain (residues 1 to 575) were unable to positively or negatively influence INF1-induced cell death. Because spontaneous cell death was not induced by transient expression of FL *StMORC1* or *SlMORC1*, the cell-death inducing activity of the C-terminal region may be regulated by another part of the MORC1 protein that, perhaps, shields it from interaction with other factors. The combined observations that (i) the C-terminal L2-CC region, spanning residues 502 to 644, is required for homodimerization of recombinant StMORC1 and SlMORC1 proteins, and (ii) the L2-CC region is phosphorylated by *N. benthamiana* extracts in vitro raise the possibility that phosphorylation of MORC1's C terminus regulates its ability to form protein–protein interactions and, thus, influences its activity. Indeed, the biological activity and protein–protein interactions of other GHKL ATPases, such as Hsp90 and topoisomerase II, are regulated by phosphorylation of their C-terminal region (Cardenas and Gasser 1993; Muller et al. 2013; Retzlaff et al. 2009). Based on the results presented in this study, combined with the finding that the C-terminal region of *Arabidopsis* AtMORC1 interacts with more than a dozen different proteins (H.-G. Kang and D. F. Klessig, unpublished data), we propose that the opposing species-specific effects that StMORC1 and SlMORC1 exert on disease resistance and in the INF1-induced cell death assay are due to specific protein–protein interactions with positive and negative modulators of immunity, and that these interactions are, at least in part, specified by four critical amino acid residues in the L2-CC region.

GHKL ATPase domains are present in a variety of prokaryotic and eukaryotic proteins that have functions related to DNA structure rearrangement (gyrase or topoisomerase) and DNA mismatch repair (MutL) (Iyer et al. 2008). More recently, two independent studies in *Arabidopsis* have implicated MORC1, MORC2, and MORC6 in chromatin-based transcriptional gene silencing (Brabbs et al. 2013; Lorković et al. 2012; Moissiard et al. 2012, 2014). We previously showed that *Arabidopsis* and barley MORC1 exhibit DNA- or RNA-binding activity and endonuclease activity, and that the subpopulation of nuclear-localized MORC1 increases after defense activation (Kang et al. 2012; Langen et al. 2014). Together, these findings argue that MORC1 in plants has an important function in the nucleus. Although the relationships between MORC1, -2, or -6's role in silencing genes and transposons and their function in immunity are presently unclear, several possibilities can be envisioned. Nuclear translocated MORC1 could directly or indirectly modulate gene- or transposon-silencing pathways, which have been shown to play key roles in transcriptional reprograming during plant immune responses against pathogens and pests (Weiberg et al. 2014). Alternatively, elevated levels of nuclear MORC1's enzymatic activities could lead to chromatin remodeling or DNA modifications, which could affect transcriptional reprograming or genome stability and, thereby, result in altered immunity.

MATERIALS AND METHODS

Plant materials.

Unless otherwise stated, plants were grown in a growth chamber under cycles of 16 h of light and 8 h of darkness at 22°C with 70% relative humidity. Seed of *N. benthamiana* and T_2 progeny of transgenic tomato plants were germinated in trays and plants were transferred to large pots 2 weeks post germination, then grown under cycles of 16 h of light and 8 h of darkness, 65% humidity, and a temperature of 24°C during daylight and 22°C at night. Transgenic tomato T_1 (*S. lycopersicum*) M82 and NC 2 CELBR, and potato (*S. tuberosum*) Désirée, Russet Burbank IDA, and K41 plants were propagated in vitro on root-inducing media (Van Eck et al. 2007) and transferred after 21 days to pots and grown in a growth chamber. Transgenic potato and tomato plants were grown in the growth chamber for 3 weeks, then transferred to a greenhouse for 1 or 2 weeks before pathogen inoculation.

Sequence analyses.

To identify MORC homologs in tomato, MORC cDNA sequences corresponding to all MORC family members from *Arabidopsis thaliana* were used as queries for a tBLASTx search with the *S. lycopersicum* genome (ITAG2.4) available in the SGN database. MORC cDNA sequences from tomato were used as query for tBLASTx analysis against the *S. tuberosum* scaffold database (PGSC DM v3) available at the SGN. Similar analyses were done using the *N. benthamiana* scaffold database (PGSC DM v0.4.4). For potato and *N. benthamiana* MORC gene and protein predictions, FGENESH (Solovyev et al. 2006) was used. The evolutionary history of the solananceous MORC family was inferred using the neighbor-joining method (Saitou and Nei 1987) and was conducted in MEGA5 (Tamura et al. 2011). The bootstrap consensus tree was inferred from 1,000 replicates. The evolutionary distances were computed using the Poisson correction method. The analysis involved 50 amino acid sequences corresponding to MORC proteins from different plant species (Supplementary Table S1). Multiple sequence analyses were done using MultAlin (Corpet 1988).

Cloning and plasmid constructs.

All oligonucleotides used for cloning, plasmid construction, and site-directed mutagenesis are listed in Supplementary Table S2. $SlMORC1_{1-235}$ and $SlMORC1_{485-644}$ fragments were amplified from the pET28a-SlMORC1 vector using primer pairs 1/2 and 3/4, respectively. The resulting PCR products were digested with *Nde*I and *Sac*I and cloned in the expression vector pET28a (EMD Millipore, MA, U.S.A.) for protein expression. To generate the

β-estradiol-inducible OE constructs, cDNAs encoding StMORC1 and SlMORC1 were amplified using primer pairs 5/6 for cloning into both the pER8:myc and the pER8:HA vector. cDNA encoding NbMORC1 was amplified using primer pairs 7/8 for cloning into the pER8:myc vector. The resulting PCR products were digested with *Avr*II and *Spe*I and cloned in their corresponding pER8 vectors. For gene-silencing constructs, a 608-bp sequence from *StMORC1* and *SlMORC1* was amplified in the sense orientation using primer pairs 9/10 and 9/11, respectively. Antisense StMORC1 and SlMORC1 sequences were amplified using primer pairs 12/13 and 12/14, respectively. PCR sense fragments were digested with *Xho*I and *Eco*RI and the antisense with *Xba*I and *Bam*HI and cloned into the pHANNIBAL vector (Wesley et al. 2001). The *St- and SlMORC1* sense and antisense cassette from pHANNIBAL was transferred to the binary vector pART27 (Gleave 1992) using the *Not*I site for *Agrobacterium*-mediated transformation. For domain swapping between StMORC1 and SlMORC1, primers were designed by choosing a conserved region to amplify fragments from both StMORC1 and SlMORC1 and with at least 10 overlapping region within the primers for fusion PCR. The desirable fragments were amplified using set of primers indicated in Supplementary Table S3. PCR fragments were gel purified and used for fusion PCR to generate desirable chimera sequences using primer pair 5/6. The PCR product corresponding to each chimera was digested with *Avr*II and *Spe*I and cloned in the pER8:HA vector. St- and $SlMORC1_{1-575}$ (St- and SlMORC1ΔCC) fragments were amplified using primer pair 5/15. The resulting PCR products were digested with *Avr*II and *Spe*I and cloned in their corresponding pER8:HA vector. Site-directed mutagenesis of the chimeras, *StMORC1*, and *SlMORC1* in the pER8:HA vector was performed as described by Zheng and associates (2004) using primers listed in Supplementary Table S3.

Plant transformations.

For potato transformation, pER8:*StMORC1,* pER8:EV, pART27:*StMORC1*, and pART27:EV were transformed into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen, CA, U.S.A.) by electroporation. *Agrobacterium* spp. containing the constructs were used to transform potato internode segments following the protocol described by Van Eck and associates (2007) and Manosalva and associates (2010). For tomato transformation, pER8:*SlMORC1*, pER8:EV, pART27:*SlMORC1*, and pART27:EV were transformed into *A. tumefaciens* strain LBA4404 (Invitrogen) by electroporation. These constructs were transformed in tomato as described by Pattison and Catalá (2012). Protein induction of StMORC1 and SlMORC1 in the corresponding transgenic plants was done 1 day before pathogen inoculation by spraying plants with a solution containing 30 μM β-estradiol and 0.01% Tween-20.

RNA extractions and RT-PCR analysis.

Total RNA was isolated using the Qiagene RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNAse treatment was done using the DNA-free Kit (Ambion, CA, U.S.A.) following the manufacturer's instructions. First-strand cDNA was synthetized from 1 μg of RNA using SuperScript II (Invitrogen) and amplified using gene-specific primer pairs for *StMORC1* (16/17), *SlMORC1* (20/21), and *NbMORC1* (24/25). Control reactions to normalize RT-PCR amplifications were run with primer pairs for the constitutively expressed *translation elongation factor* 1α gene from potato (18/19), tomato (22/23), and *N. benthamiana* (26/27).

Pathogen isolates and plant inoculations.

Two isolates of *P. infestans* A1 mating type (US050007, US-11 genotype and US-23 genotype) and two isolates of the A2 mating type (US100021, US-8 genotype and US100041, US-22 genotype) were maintained on tomato leaves and used as a suspension of 4,000 sporangia/ml for potato and tomato inoculations. For inoculations of *N. benthamiana*, a suspension of 20,000 sporangia/ml was used. Detached-leaflet assay inoculations were performed as described by Manosalva and associates (2010). For whole-plant inoculations, 5-week-old potato plants were spray inoculated with a *P. infestans* suspension of 4,000 sporangia/ml until drenched and maintained under a cycle of 16 h of light and 8 h of darkness at a temperature of 18°C. At 2 h after inoculation, atomizing humidifiers were turned on to ensure that the humidity was greater than 80%. Percentage of diseased area was calculated at 6 dpi based on the rating system key of W. E. Fry. *Pseudomonas syringae* pv. *tomato* DC3000 was grown on King's B solid medium containing rifampicin at 100 μg/ml for 2 days at 28°C. The bacteria were scraped from plates and suspended in sterile 10 mM $MgCl_2$, and the optical density at 600 nm (OD_{600}) was adjusted to 0.2 (approximately 5×10^8 CFU/ml). Serial dilutions were done in 10 mM $MgCl_2$ to generate a bacterial suspension of 5×10^5 CFU/ml. Tomato plants were vacuum infiltrated with the bacterial suspension containing 0.02% Silwet L-77. Plants were dipped upside down in 4 liters of bacterial suspension and a vacuum was applied for 1 or 2 min followed by a slow release to infiltrate the leaves uniformly. Plants were then incubated in a growth chamber with 16 h of illumination and 60% humidity at 22°C. Bacterial count was done 3 dpi.

Trypan blue staining.

Leaf tissue was boiled for 5 min in a 1:1 mixture of ethanol and staining solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue, dissolved in 10 ml of distilled water) for staining. The leaf tissue was destained in chloral hydrate at 2.5 g/ml in distilled water. Microscopic analysis was done using a Leica DM5500 Epifluorescence Microscope.

VIGS in *N. benthamiana*.

For VIGS, the TRV2:*NbMORC1* (*NbCRT1*) and TRV2:EV constructs described by Kang and associates (2010), as well as a TRV2:*EC1* negative control construct described by Chakravarthy and associates (2010) were used. VIGS in *N. benthamiana* was performed as described by Velásquez and associates (2009). Four weeks after VIGS, *N. benthamiana* were used for pathogen inoculations and *Agrobacterium*-mediated transient expression.

***Agrobacterium*-mediated transient expression in *N. benthamiana*.**

A. tumefaciens strain GV2260 was transformed with pER8 plasmids containing either the FL MORC1 cDNA or the MORC fragments expressed under control of a β-estradiol-inducible promoter using electroporation. Positive transformants were grown on Luria Bertani (LB) medium plates containing appropriate antibiotics for 48 h at 29°C. Bacteria were scrapped from plates using a sterile spatula and resuspended in sterile infiltration media (10 mM $MgCl_2$, 10 mM MES [pH 5.5], and 200 μM acetosyringone) and the OD_{600} was determined. The suspension was diluted in infiltration media to the desired OD_{600}, then incubated at room temperature for 2 h. Leaves of 5-week-old *N. benthamiana* plants were infiltrated with agrobacteria carrying EV or FL *SlMORC1* at OD_{600} = 0.7, with *StMORC1* at OD_{600} = 0.5, and with all of the other constructs at OD_{600} = 0.4. These OD_{600}s were selected based on immunoblot analysis at 2 and 3 days post estradiol induction to ensure similar levels of protein expression among all constructs. Protein induction was done 1 dai by spraying plants with a solution containing 30 μM β-estradiol and 0.01% Tween-20. Immunoblot analysis was performed to monitor the accumulation of StMORC1, SlMORC1, the chimeras,

and the C-terminal truncations. Total proteins were extracted from leaf samples at 24 h after β-estradiol treatment and proteins were detected using an anti-HA-HRP antibody. For INF1-induced cell death, small areas within the EV and MORC1-infiltrated areas were reinfiltrated with *A. tumefaciens* strain GV3101 carrying INF1 driven by the constitutive 35S promoter at an OD_{600} = 0.075 or 0.1 in infiltration media 24 h after β-estradiol induction. Cell death was scored at 3 and 4 dai with GV3101. Following VIGS in transgenic *N. benthamiana* plants containing the *RB* gene, pTRBO:*Avrblb1* construct was delivered by agroinfiltration 4 weeks after infiltration with the TRV constructs. Agrobacteria containing these constructs were resuspended in infiltration media to OD_{600} = 0.4. *RB*-mediated cell death was recorded and photographed at 5 dpi.

Protein purification.

Protein coding sequences corresponding to FL StMORC1, FL SlMORC1, $StMORC1_{59-501}$, $SlMORC1_{59-501}$, $SlMORC1_{1-235}$, and $SlMORC1_{485-644}$ were cloned into pET28a (EMD Millipore) to generate recombinant proteins with an N-terminal His_6 tag. The error-free clones were confirmed by sequencing and then transformed into the Rosetta2 (DE3) (EMD Millipore) *E. coli* strain for protein expression. The bacteria were grown at 37°C in 2 liters of LB containing kanamycin at 50 μg/ml and chloramphenicol at 34 μg/ml for Rosetta 2 (DE3) cells to an OD_{600} of 0.6, before addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.1 to 1 mM to induce gene expression. Induced cultures were incubated overnight at 20°C. The cells were then harvested by centrifugation and the pellet was resuspended in lysis buffer (50 mM Tris [pH 8.5], 500 mM NaCl, 10% glycerol, 20 mM Imidazole, 0.5% triton X-100, and 1 mM phenylmethyl sulphonyl fluoride). Resuspended cells were disrupted by sonication. The clarified supernatant was incubated with Ni-NTA His resin (EMD Millipore) for 1 h and then washed with lysis buffer containing increasing concentrations (20, 30, and 40 mM) of imidazole. The proteins remaining bound to the Ni-NTA resin were eluted in lysis buffer supplemented with 250 mM imidazole. The eluted proteins were subjected to gel filtration chromatography on a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare, PA, U.S.A.) equilibrated in a buffer containing 50 mM Tris (pH 8.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), and 10% glycerol. Fractions containing the purified proteins were collected, pooled, and further purified using anion exchange chromatography on a SOURCE 15Q 4.6/100 PE column (GE Healthcare) equilibrated in a buffer containing 25mM Tris (pH 8.0), 1 mM DTT, and 10% glycerol and eluted using increasing concentration of NaCl in the buffer.

ATPase assay.

The ATPase assay was performed using a colorimetric kit (Innova Bioscience, U.K.) in 50 mM Tris/HCl (pH 7.5), 2.5 mM $MgCl_2$, and 10 mM KCl; and 10 mM NaCl containing 0.5 mM ATP in the absence or presence of 1 mM radicicol with StMORC1 (11.1 μg), SlMORC1 (9.6 μg), StMORC159-501 (4.5 μg), and SlMORC159-501 (6.0 μg). Reaction mixtures were incubated for 90 min at 37°C. ATPase activity was calculated on the basis of a standard curve obtained with serial dilutions of free inorganic phosphate and adjusted for protein concentrations. The specific Pi released was adjusted for protein concentration and was measured as picomoles of Pi per 1 μg of protein per minute.

In vitro phosphorylation assay.

Approximately 100 mg of frozen *N. benthamiana* tissue was ground with steal beads with 250 μl of ice-cold of GTEN extraction buffer (25 mM Tris HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, and 10% glycerol) containing 10 mM DTT, 2% (wt/vol) polyvinyl-polypyrrolidone, and 1× plant general protease inhibitor (Sigma-Aldrich, St. Louis). Cell debris was cleared by centrifugation at 13,000 × *g* for 5 min. The supernatant was transferred to a 1.5-ml microcentrifuge tube and centrifuged again for 2 min. Cleared *N. benthamiana* extract (5 μl) was used for the kinase assay by combining it with 2.5 μg of Ni-NTA purified recombinant proteins expressed as N-terminal His_6 fusions and kinase buffer (25 mM Tris HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 20 μM ATP) in presence or absence of 0.2 μCi ^{32}P-γ ATP) in a total volume of 20 μl. The reaction was incubated at room temperature for 10 min and then stopped by adding 10 μl of sodium dodecyl sulfate (SDS) loading buffer (4× Laemmli buffer). After separating 10 μl of the reaction on SDS polyacrylamide gel electrophoresis, the gel was subjected to three 10-min washes with Milli-Q water and stained overnight at room temperature with biosafe Bio-Rad protein stain (Bio-Rad, CA, U.S.A.). The gel was then washed for 30 min with Milli-Q water and dried on a gel dryer for 90 min at 60°C before autoradiography at room temperature for 3 days. The phoshorimager screen was scanned with a STORM scanner.

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