RepA Protein Encoded by *Oat dwarf virus* Elicits a Temperature-Sensitive Hypersensitive Response–Type Cell Death That Involves Jasmonic Acid–Dependent Signaling

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The hypersensitive response (HR) is a component of disease resistance that is often induced by pathogen infection, but essentially no information is available for members of the destructive mastreviruses. We have investigated an HR-type response elicited in *Nicotiana* species by *Oat dwarf virus* (ODV) and have found that expression of the ODV RepA protein but not other ODV-encoded proteins elicits the HR-type cell death associated with a burst of H₂O₂. Deletion mutagenesis indicates that the first nine amino acids (aa) at the N terminus of RepA and the two regions located between aa residues 173 and 195 and between aa residues 241 and 260 near the C terminus are essential for HR-type cell-death elicitation. Confocal and electron microscopy showed that the RepA protein is localized in the nuclei of plant cells and might contain bipartite nuclear localization signals. The HR-like lesions mediated by RepA were inhibited by temperatures above 30°C and involvement of jasmonic acid (JA) in HR was identified by gain- and loss-of-function experiments. To our knowledge, this is the first report of an elicitor of HR-type cell death from mastreviruses.

Plants are continuously exposed to various potential pathogens. To escape pathogen attack, plants have evolved various defense mechanisms, including hypersensitive responses (HR). The HR is often characterized by a rapid, localized necrosis of tissue in the vicinity of pathogen entry points that often results in pathogen arrest and prevention of systemic invasion (Schmelzer et al. 1989). Usually, an HR is preceded by a burst of reactive oxygen species (ROS) and activation of various defense genes, followed by the onset of systemic acquired resistance (Dangl et al. 1996; Levine et al. 1994). Signaling molecules like salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) are known to play key roles in mediating HR-like plant defense responses (Martinez et al. 2000; Rao et al. 2000; Tuominen et al. 2004; Yoon et al. 2009).

Numerous reports indicate that particular pathogen proteins can elicit an HR-type response in plants, resulting in effector-triggered immunity or gene-for-gene resistance (Bonas and Van den Ackerveken 1997; Hammond-Kosack and Jones 1997; Jones and Dangl 2006; Keen 1990). To date, several viral gene products from RNA and DNA viruses have been shown to act as elicitors of HR-type cell death in hosts. The known viral inducers of plant defense are functionally diverse and mainly include capsid, movement, and replicase proteins (Bendahmane et al. 1995; Berzal-Herranz et al. 1995; Culver and Dawson 1989; Ghazala and Varrelmann 2007; Hu et al. 2012; Kim and Palukaitis 1997; Király et al. 1999; Knorr and Dawson 1988; Lim et al. 2014).

Geminiviruses are a group of plant DNA viruses characterized by either one or two circular single-stranded (ss)DNA genomic components (DNA-A and DNA-B) that range in size from 2.6 to 2.8 kb. Based on their genome organization and insect vectors, geminiviruses have been classified into seven genera (namely, *Begomovirus*, *Mastrevirus*, *Curtovirus*, *Topocuvirus*, *Becurtovirus*, *Turncurtovirus*, and *Eragrovirus*) (Adams et al. 2013). Previous evidence has shown that the nuclear shuttle (NSP), precoat (V2), and replication-associated (Rep) proteins associated with several begomoviruses can induce cell death in plants when expressed under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter or from a *Potato virus X* (PVX) vector (Garrido-Ramirez et al. 2000; Hussain et al. 2005; Jin et al. 2008; Mubin et al. 2010; Sharma and Ikegami 2010; Van Wezel et al. 2002).

Oat dwarf virus (ODV) belongs to the genus *Mastrevirus* (Muhire et al. 2013; Schubert et al. 2007). Mastreviruses are transmitted by leafhoppers, possess a monopartite genome, and most of them infect monocotyledonous plants. The genome of mastreviruses encodes four confirmed proteins: the movement protein (MP or V1) and the coat protein (CP or V2) on the viral-sense strand and the replication associated proteins (including RepA and Rep) on the complementary-sense strand (Gutierrez et al. 2004). Rep is expressed from spliced transcripts encompassing the open reading frames (ORF) C1 and C2, and RepA is expressed from the C1 ORF using an unspliced transcript (Wright et al. 1997). Although Rep and RepA share the same N terminus, each has a different C terminus. Reps are multifunctional proteins that participate in rolling circle replication and reprogramming of the plant cell cycle (Collin et al. 1996; Luque et al. 2002; Xie et al. 1995).

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In this study, we describe an incompatible interaction between ODV and *Nicotiana* species. We demonstrate that the RepA protein encoded by ODV acts as an elicitor of HR-type cell death. In addition, we find that the cell death elicited by the RepA protein is temperature sensitive and jasmonic acid-dependent.

RESULTS

ODV RepA elicits HR-type cell death in a transient expression assay.

It is a general phenomenon that microbial pathogens can successfully infect only a limited numbers of plant species. To assess the interaction of ODV with *Nicotiana* species, an infectious clone of ODV was introduced by *Agrobacterium* infiltration (agroinfiltration) of *Nicotiana benthamiana* leaves and symptom production was monitored. A strong necrosis restricted to infiltrated regions appeared within 3 to 5 days postinoculation (dpi). Subsequently, total nucleic acid was extracted from systemically infected leaves of *N. benthamiana* plants at 35 dpi and the presence of viral DNA was determined by polymerase chain reaction (PCR) analysis. The results revealed that viral DNA could be detected only in one of the more than 100 *N. benthamiana* plants inoculated (data not shown). Expression of *HSR203J* and *HIN1*, two well-characterized HR marker genes (Takahashi et al. 2004), were analyzed by real-time quantitative reverse transcription PCR (qRT-PCR) at 3 dpi. The results show that the *HSR203J* and *HIN1* genes are highly up-regulated in *N. benthamiana* leaves inoculated with ODV as compared with empty vector-inoculated leaves (Supplementary Fig. S1). This suggests that when ODV attempts to infect *N. benthamiana* plants, a disease-resistance response is triggered.

To determine which ODV-encoded gene product is responsible for induction of the necrosis phenotype, agroinfiltration was used to transiently express each ODV-encoded gene. For this purpose, the 35S-V1:GFP, 35S-V2:GFP, 35S-RepA:GFP, and 35S-Rep:GFP constructs under the control of the 35S promoter from CaMV were produced (Fig. 1A) and subsequently mobilized into *Agrobacterium tumefaciens*. Two to three days after agroinfiltration into *N. benthamiana* leaves, Western blot analysis using a green fluorescent protein (GFP)-specific antibody and confocal microscopy visualization demonstrated that all ODV-encoded genes fused to GFP were stably expressed (Fig. 1B; Supplementary Fig. S2). Only the construct expressing RepA (35S-RepA:GFP) elicited necrotic local lesions, resembling an HR-type cell death within 2 to 3 dpi, with infiltrated tissues becoming completely necrotic by 5 dpi (Fig. 1C). However, leaves inoculated with an untranslatable RepA mutant (35S-RepA^{UT}:GFP) containing an in-frame stop codon introduced immediately downstream of the RepA start codon failed to display an HR-type phenotype at any time after infiltration. The remaining ODV genes were also found to have no discernable effect after transient expression in *N. benthamiana* leaves (Fig. 1C). The above data demonstrate that the induction of local HR-like lesions is due primarily to expression of the

ODV RepA. To rule out the possibility that GFP fusion to Rep, V1, and V2 might inactivate the cell death-eliciting activity of them, 35S-V1, 35S-V2, 35S-RepA, and 35S-Rep constructs were also produced, and only the construct of 35S-RepA elicited an HR-type cell death in *N. benthamiana* leaves (Supplementary Fig. S3).

Rapid generation and accumulation of ROS is a characteristic feature and probably is a primary process required for cell death during HR. Thus, production of H₂O₂, a primary ROS, was assayed in infiltrated *N. benthamiana* leaves by in situ detection, using the 3,3-diaminobenzidine (DAB)-uptake method. Only 35S-RepA:GFP infiltrated leaf tissue became brown at 2 dpi. Disruption of RepA expression in 35S-RepA^{UT}:GFP reduced H₂O₂ accumulation to the level found in control tissue inoculated with empty vector (Fig. 1C), suggesting that RepA expression is sufficient to trigger H₂O₂ accumulation. To further determine whether RepA-induced necrosis is HR-type cell death, expression of *HSR203J* and *HIN1* were analyzed by qRT-PCR at 3 dpi. The results show that the *HSR203J* and *HIN1* genes are highly expressed in *N. benthamiana* leaves inoculated with 35S-RepA:GFP, whereas expression was only slightly above background levels in 35S-RepA^{UT}:GFP-inoculated leaves or leaves expressing any of the other ODV proteins (Fig. 1D).

ODV RepA elicits HR-type cell death in a PVX expression assay.

Previous evidence has shown that several elicitors of HR-type cell death prevent systemic infection and result in pathogen arrest when expressed from a PVX vector (Greenberg and Yao 2004; Scholthof et al. 1995). To further confirm the involvement of RepA in eliciting HR-type cell death, RepA and its mutant derivatives were expressed in *N. benthamiana* plants from PVX-based vectors. Typical symptoms produced in plants inoculated with PVX, PVX-RepA, and PVX-RepA^{UT} are shown in Figure 2A. Plants infected by PVX developed only mild symptoms on inoculated leaves, which exhibited a faint systemic veinal chlorosis at 6 dpi, followed by the appearance of mild chlorotic spots. Remarkably, PVX-RepA elicited severe necrotic lesions on all inoculated leaves as early as 3 dpi. At 6 dpi, veinal chlorosis was visible on the systemically infected leaves of all tested plants inoculated with PVX-RepA, with downward curling of newly emerging leaves and necrotic mottling appearing at 9 dpi. A severe necrosis and the collapse of developing tissue were also observed in emerging leaves infiltrated with PVX-RepA at 12 dpi. In contrast, plants inoculated with the untranslatable mutant (PVX-RepA^{UT}) initially developed veinal chlorosis and subsequent systemic mild chlorotic symptoms identical to those associated with PVX infection. Infected plants were assayed for the production of H₂O₂ by DAB staining at 9 dpi, when sporadic HR-like lesions were visible on the systemic infected leaves. H₂O₂ accumulated to high levels in systemically infected leaves after inoculation with PVX-RepA. In contrast, disruption of RepA expression in PVX-RepA^{UT} failed to induce an HR-type cell death in *N. benthamiana* plants, with H₂O₂ accumulation levels similar

Fig. 1. Transient expression of individual *Oat dwarf virus* genes from 35S-based binary vectors following agroinfiltration of *Nicotiana benthamiana* leaves. **A**, 35S-based expression cassettes. Stop codons introduced into the RepA are indicated by asterisks (*). **B**, Western blot analysis of green fluorescent protein (GFP) fusion protein accumulation in wild-type (WT) plants infused with transient expression constructs at 3 days postinoculation (dpi). Coomassie staining was used to detect Rubisco as a loading control. **C**, Photographs of leaves from *N. benthamiana* plants exhibiting symptoms (left) or production of H₂O₂ (right) 5 days after agroinfiltration with transient expression constructs. Leaves on the right were stained with 3,3-diaminobenzidine. **D**, Quantitative reverse transcription-polymerase chain reaction analysis of *HSR203J* and *HIN1* transcripts expressed in *N. benthamiana* leaves infused with transient expression constructs at 3 dpi. The *GADPH* gene was used as a reference gene. Columns represent the mean value, with standard error of the mean, from three independent experiments ($n = 9$). The relative mRNA levels of *HSR203J* in leaves agroinoculated with 35S-RepA:GFP was arbitrarily set as 1. The data were analyzed using one-way analysis of variance followed by Student's *t* test. Asterisks (**) indicate significant difference between treatments ($P \leq 0.01$).

as those found in PVX-inoculated tissues (Fig. 2B). Furthermore, Western blotting indicated that expression of RepA protein could be detected in leaves infected with PVX-RepA but not in those infected with PVX or PVX-RepA^{UT} (Fig. 2C). Thus, these results suggest that ODV RepA has a critical role in eliciting an HR-type cell death during systemic infection by PVX.

To rule out the possibility that the enhanced pathogenicity of a PVX chimera expressing RepA is due to elevation of the PVX titer, an enzyme-linked immunosorbent assay (ELISA) test was conducted in systemic leaves infected with PVX, PVX-RepA, or PVX-RepA^{UT} at 9 dpi. The results showed that only minimal differences occurred in the relative titer of PVX after infection with the three different constructs (Fig. 2D).

To further assess the effects of RepA expression on other *Nicotiana* species, *N. tabacum* plants were stably transformed with *A. tumefaciens* carrying 35S-RepA. All T₀ lines failed to induce necrosis on fully expanded leaves (Supplementary Fig. S4). Analysis by qRT-PCR and Northern blotting showed relatively high levels of the *RepA* transcript in these transgenic plants. However, Western blot analysis failed to detect expression of RepA in these plants, suggesting that the failure to induce necrosis on transgenic tobacco plants might result from low expression of RepA.

Identification of ODV RepA sequences essential for inducing HR-type cell death.

To map amino acid sequences in the RepA protein potentially involved in the induction of cell death, a series of RepA mutants containing N-terminal, central, or C-terminal truncations were fused to the N terminus of the enhanced GFP (eGFP) and were cloned under the control of the 35S promoter (Table 1; Fig. 3A). The 35S-RepA:GFP construct containing the full-length RepA coding sequence was used as a positive control. Western blot analysis was performed to check protein stability of the RepA mutant constructs after agroinfiltration into *N. benthamiana* leaves. The results showed that a single predominant protein with the predicted size of the RepA-GFP fusion protein was detected in *N. benthamiana* leaves inoculated with RepA mutant constructs, except 35S-RepA^{dm30-239}:GFP, which had reduced amounts of RepA-GFP fusion protein (Fig. 3B).

Induction of HR-type cell death in plants after agroinfiltration with these constructs is summarized in Table 1 and Figure 4. *N. benthamiana* and *N. tabacum* plants inoculated with the 35S-RepA^{dm1-9}:GFP or 35S-RepA^{dm1-29}:GFP, with either a 9- or 29-aa deletion at the N-terminal end of RepA, did not exhibit an HR-type cell death. In contrast, plants inoculated with 35S-RepA^{dm261-266}:GFP, with a 6-aa deletion at the C-terminal end of RepA, was still able to induce an HR-type cell death as effectively as the wild-type RepA. However, deletion of the C-terminal 26 aa of RepA (35S-RepA^{dm241-266}:GFP) completely abolished the HR-type cell death. These findings suggest sequences within the first 9 aa at the N terminus of RepA are essential for HR-type cell-death elicitation. In addition, sequences located between aa 241 and 260 at the C terminus of RepA, also have an important role in eliciting HR-type cell death. Previous reports indicate that the mastrevirus RepA protein contains a conserved amino acid sequence (LxCxE) that may interact with a cellular retinoblastoma-related protein (RB) homolog to induce a cellular S-like phase that is permissive for viral DNA replication (Xie et al. 1995). In order to determine whether this interaction contributes to the HR-like phenotype, we constructed a RepA mutant (35S-RepA^{dmRB}:GFP) in which the conserved LICHE motif was deleted. The mutant protein could still induce necrotic local lesions without affecting the onset and severity. These results suggest that RepA functions can be dissected and that the

cell-cycle functions can be separated from the induction of HR-type cell death.

To further dissect the functional region between aa 30 and 240, we constructed additional deletion mutants. These constructs included 35S-RepA^{dm30-239}:GFP with a deletion of aa 30 to 239; 35S-RepA^{dm129-239}:GFP with a deletion of aa 129 to 239; 35S-RepA^{dm150-239}:GFP with a deletion of aa 150 to 239; and 35S-RepA^{dm173-239}:GFP with a deletion of aa 173 to 239. All the mutations failed to elicit HR-type cell death in *N. benthamiana* and *N. tabacum* plants (Fig. 4A and B). However, two other RepA constructs with a deletion of aa residues 196 and 239 (35S-RepA^{dm196-239}:GFP) or aa 215 and 239 (35S-RepA^{dm215-239}:GFP) were capable of eliciting HR-type cell death as effectively as wild-type RepA (Fig. 4A and B). These results suggest that the region between aa 173 and 195 of RepA are essential for induction of HR-type cell death.

Subcellular localization of ODV RepA and its deletion mutants.

Martin et al. (2009) demonstrated that transgenic *N. benthamiana* lines expressing red fluorescent protein (RFP) markers are useful tools for experiments aimed at defining nuclear or endomembrane localization. To determine the intracellular location of ODV RepA protein in plant cells, the 35S-RepA:GFP construct was introduced into transgenic *N. benthamiana* plants expressing RFP-tagged nuclear histone protein H2B (RFP-H2B), using agroinfiltration. Confocal microscopy showed that H2B-RFP fluorescence was present in nuclei of *N. benthamiana* epidermal cells, and examination of GFP and RFP fluorescence by confocal microscopy at 48 h post-infiltration showed that the 35S-RepA:GFP fusion protein accumulated only in the cell nucleus. In contrast, the fluorescence patterns for 35S:GFP controls were evenly distributed throughout the cytoplasm and the nuclei of infiltrated cells (Fig. 5A). Nuclear localization of RepA was further examined in ODV-infected *N. benthamiana* plants. Tissue sections from leaves infected by ODV were probed with an antibody specific for RepA followed by a gold conjugate. Electron microscopy showed the gold particles were found primarily within the nuclei of infected cells (Fig. 5B). In contrast, immunogold labeling in the nucleus was not seen in sections from uninfected *N. benthamiana* plants (Fig. 5B). These results reinforce the finding that the RepA protein is localized to the nuclei of plant cells.

To determine whether modified RepA proteins were altered in their subcellular distribution, leaves of *N. benthamiana* line RFP-H2B were infiltrated with an *Agrobacterium* strain containing RepA deletion mutants fused with GFP. Examination of coexpressing GFP and RFP demonstrated that the subcellular distribution patterns of 35S-RepA^{dm241-266}:GFP, 35S-RepA^{dmC261-266}:GFP, 35S-RepA^{dmC215-239}:GFP, and 35S-RepA^{dmRB}:GFP were similar to the pattern observed in *N. benthamiana* leaf cells infiltrated with the wild-type RepA:GFP fusion protein (Fig. 6). However, the fusion proteins of RepA^{dm1-9}:GFP, RepA^{dm30-239}:GFP, RepA^{dm129-239}:GFP, RepA^{dm150-239}:GFP, RepA^{dm173-239}:GFP, and RepA^{dm196-239}:GFP were localized mainly in the cytoplasm, with a greatly reduced localization to the nucleus. The RepA^{dm1-29}:GFP fusion protein was completely restricted to the cytoplasm (Fig. 6). These results suggest that sequences located within aa 1 to 29 at the N terminus and aa 196 to 214 of RepA are required for nuclear localization and might contain a bipartite nuclear localization signal (NLS). Analysis of RepA protein with online prediction software (PSORT) failed to identify basic amino acids with a consensus NLS sequence, suggesting the presence of an atypical NLS.

Other ODV-encoded proteins are unable to prevent ODV RepA-induced HR-type cell death.

It is common for animal- or plant-infecting viruses to encode proteins that inhibit HR to permit virus survival. Several previously identified begomovirus-encoded viral proteins have been shown to have vital roles in abrogating HR-induced cell death in plants (Hussain et al. 2007; Mubin et al. 2010). However, our primary results indicate a characteristic HR-type phenotype is evident on *N. benthamiana* leaves inoculated with an ODV infectious clone, suggesting that none of the ODV-encoded proteins can suppress cell death initiated by RepA. To test this hypothesis, *N. benthamiana* leaves were coinoculated with *A. tumefaciens* harboring 35S-RepA and 35S-V1:GFP, 35S-V2:GFP, or 35S-Rep:GFP constructs. As shown in Figure 7A, coexpression of RepA with any of the ODV-encoded proteins (V1, V2, and Rep) exhibited cell death similar to that observed in leaves expressing 35S-RepA alone. A Western blot assay showed that the accumulation level of RepA was similar in all inoculated leaves, indicating that this was not due to instability of the expressed proteins (Fig. 7B). Subsequently, similar results were obtained on *N. tabacum*, *N. glutinosa*, and *N. tabacum* cv. Samsun leaves by coexpression experiments

(Fig. 7A; data not shown). Our results, therefore, suggest that ODV is unable to suppress HR.

High temperature inhibits HR-type cell death induced by ODV RepA.

Temperature sensitivity has been found to play a role in some defense responses against biotrophic and hemibiotrophic microbes (Zhu et al. 2010). Therefore, we analyzed the effects of temperature on HR-type cell death induced by RepA. *A. tumefaciens* containing 35S-RepA:GFP was infiltrated into fully expanded leaves of *N. benthamiana*, *N. glutinosa*, *N. tabacum*, and *N. tabacum* cv. Samsun leaves. These plants were then incubated in controlled growth chambers to maintain constant environmental conditions, except for temperatures of either 25 or 30°C. As shown in Figure 8A, RepA elicited necrotic local lesions on all tested host plants within 2 to 3 dpi, and the infiltrated tissues become completely necrotic after 5 to 6 dpi at 25°C. In contrast, an HR-type cell death could not be detected at 30°C up to 10 dpi on infiltrated *N. benthamiana*, *N. tabacum* cv. Samsun, *N. glutinosa*, and *N. tabacum* leaves. These results demonstrate that an elevated temperature can inhibit HR-type cell death elicited by RepA on *Nicotiana* species.

Fig. 2. Transient expression of *Oat dwarf virus* RepA from *Potato virus X* (PVX)-based vectors after agroinfiltration of *Nicotiana benthamiana* plants. **A**, Phenotypes produced in *N. benthamiana* plants agroinoculated with PVX, PVX-RepA or PVX-RepA^{UT} constructs. Photographs were taken at 9 days postinoculation (dpi). **B**, Induction of H₂O₂ production in leaves systemically infected with PVX, PVX-RepA, or PVX-RepA^{UT}. Leaves were stained with 3,3'-diaminobenzidine at 9 dpi. **C**, Western blot analysis of RepA accumulation in wild-type (WT) plants infused with transient expression constructs at 9 dpi. A Coomassie-stained gel was used to detect Rubisco as a loading control. PC indicates the positive control. **D**, Enzyme-linked immunosorbent assay analysis of the PVX titer in the systemic leaves shown in panel B. The error bars show standard deviations in one representative experiment ($n = 9$). WT indicates uninoculated wild-type *N. benthamiana* plants.

Previous reports have also shown that an elevated temperature could reduce the nuclear accumulation of two resistant (R)-like proteins, *Arabidopsis* SNC1 and tobacco N proteins, which likely contribute to repression of defense responses against

Pseudomonas syringae pv. tomato DC3000 or Tobacco mosaic virus (TMV) (Zhu et al. 2010). Therefore, we further tested the effects of temperature on subcellular localization of RepA. Our observations show that the RepA:GFP fusion protein is

Table 1. Induction of hypersensitive response-type cell death in *Nicotiana* species by infiltration of wild-type RepA or RepA mutant constructs^a

Constructs	<i>N. benthamiana</i>	<i>N. glutinosa</i>	<i>N. tabacum</i> cv. Samsun	<i>N. tabacum</i>
35S-RepA:GFP	15/15	15/15	15/15	15/15
35S-RepA ^{UT} :GFP	0/15	0/15	0/15	0/15
35S-RepA ^{dm1-9} :GFP	0/15	0/15	0/15	0/15
35S-RepA ^{dm1-29} :GFP	0/15	0/15	0/15	0/15
35S-RepA ^{dm261-266} :GFP	15/15	15/15	15/15	14/15
35S-RepA ^{dm241-266} :GFP	0/15	0/15	0/15	0/15
35S-RepA ^{dm30-239} :GFP	0/15	—	—	0/15
35S-RepA ^{dm129-239} :GFP	0/15	—	—	0/15
35S-RepA ^{dm150-239} :GFP	0/12	—	—	0/12
35S-RepA ^{dm173-239} :GFP	0/12	—	—	0/12
35S-RepA ^{dm196-239} :GFP	15/15	—	—	15/15
35S-RepA ^{dm215-239} :GFP	12/12	—	—	12/12
35S-RepA ^{dmRB} :GFP	15/15	15/15	15/15	15/15

^a Values represent the number of plants showing necrosis symptom/number of plants inoculated.

Fig. 3. Protein stability of the RepA mutant constructs after agroinfiltration into *Nicotiana benthamiana* leaves. **A**, The diagrams represent 35S-based expression cassettes fused to the N terminus of the green fluorescent protein (GFP). **B**, Western blot analysis of expression of RepA or its deletion mutants fused to GFP using a GFP-specific antibody at 3 days postinoculation. A Coomassie-stained gel was used to detect Rubisco as a loading control.

localized only in the nucleus at both 25 and 30°C (Fig. 8B), indicating that elevated temperature does not affect the sub-cellular localization of RepA.

An earlier study has shown that expression of reporter genes in plants from T-DNAs delivered into plant cells by *A. tumefaciens* is affected by high temperature (Del Toro et al. 2014). Therefore, an elevated temperature might have an effect on expression of RepA itself. We investigated the accumulation of RepA protein at 25 and 30°C. Western blot analysis showed there was a slight reduction of RepA:GFP fusion protein at 30°C compared with at 25°C (Fig. 8C). To rule out the possibility that HR-type cell death elicited by RepA could be dose-dependent instead of temperature-sensitive. We further conduct a transient expression experiment by agroinoculation into *N. benthamiana* leaves with a serial dilution of *A. tumefaciens* containing 35S-RepA:GFP at 25°C in parallel with expression of RepA at 30°C. As shown in Figure 8D, our observations showed that necrotic local lesions were induced in leaves

infiltrated by diluted *A. tumefaciens* (a final optical density at 600 nm [OD₆₀₀] of 0.2) within 3 to 5 dpi at 25°C. In contrast, at 30°C, an HR-like necrosis phenotype could not be detected in leaves infiltrated by undiluted *A. tumefaciens* (a final OD₆₀₀ of 2.0) up to 10 dpi. Western blot analysis indicated that relatively higher levels of RepA protein were observed in leaves infiltrated by undiluted *A. tumefaciens* (a final OD₆₀₀ of 2.0) at 30°C than those in leaves infiltrated by diluted *A. tumefaciens* (a final OD₆₀₀ of 0.2) at 25°C (Fig. 8E). These results ruled out the possibility that HR-type cell death induced by ODV RepA was dose-dependent.

To further elucidate if ODV-induced cell death is also affected by temperature, *A. tumefaciens* containing the ODV infectious clone was infiltrated into fully expanded leaves of *N. benthamiana* and *N. tabacum*, and inoculated plants were incubated at 25 or 30°C. As shown in Figure 8F, a strong necrosis was induced that was restricted to infiltrated regions in leaves within 3 to 5 dpi at 25°C. In contrast, an HR-like necrotic

Fig. 4. Symptom induction by wild-type RepA and RepA deletion mutants expressed from 35S-based binary vectors following agroinfiltration. **A**, Phenotypes produced in *Nicotiana benthamiana* and **B**, *N. tabacum* plants following agroinfiltration with constructs for transient expression of RepA or RepA deletion mutants. Photographs were taken at approximately 5 days postinoculation.

phenotype could not be detected at 30°C up to 10 dpi in infiltrated leaves. Western blot analysis showed a slight reduction in RepA expression at 30°C compared with at 25°C (Fig. 8G and H).

JA signaling is required for RepA-induced HR-type cell death.

In many cases, cell death triggered by environmental stresses is linked to hormonal regulation and SA is known to play an important role in defense signaling (Martinez et al. 2000; Rao et al. 2000; Tuominen et al. 2004; Yoon et al. 2009). To determine whether SA is required for RepA-mediated HR-type cell death, *A. tumefaciens* containing 35S-RepA:GFP was infiltrated into expanded leaves of wild-type or transgenic *N. benthamiana* plants constitutively expressing the salicylate hydroxylase gene (*NahG*) (Ying et al. 2010). Plants expressing *NahG* have reduced levels of SA. The time required for 50% of the inoculated leaves of *NahG*-transgenic plants to develop HR-type cell death was less than 4 dpi, which was similar to that of wild-type control plants ($P > 0.05$) (Supplementary Fig. S5). In addition, *N. benthamiana* plants were treated with SA to examine the involvement of SA in eliciting HR-type cell death. No significant difference in the time for 50% of the SA-treated leaves to develop HR-type cell death were observed, as compared with buffer-treated control plants ($P > 0.05$). Together, these results suggest RepA-induced HR-type cell death might be SA-independent.

JA has been reported to have a functional role as a signal molecule involved in HR (Yoon et al. 2009). Therefore, the *AOS* (allene oxide synthase) and *COII* (coronatine-insensitive 1) genes, key components of the JA-defense pathway (Wasternack and Hause 2013), were silenced in *N. benthamiana* plants using a virus-induced gene silencing (VIGS) vector (TRV:00) (Liu et al. 2002). One week after agroinfiltration, qRT-PCR analysis of *NbAOS* or *NbCOII* (orthologs of *AtAOS* or *AtCOII* cloned from *N. benthamiana*) transcripts confirmed that the levels of *NbAOS* mRNA were reduced by more than 90% (Fig. 9A) and the levels of *NbCOII* mRNA were reduced by more than 75% (Fig. 9B) in systemic silenced *N. benthamiana* plants as

compared with TRV:GUS-infected control plants. To assess the effect of silencing *NbAOS* or *NbCOII* on JA responses, the transcript level of the JA-responsive gene *PDF1.2* was monitored. qRT-PCR results showed that *NbPDF1.2* (ortholog of *AtPDF1.2* cloned from *N. benthamiana*) was down-regulated in both the *NbAOS*- and *NbCOII*-silenced plants (Fig. 9C). *A. tumefaciens* containing 35S-RepA:GFP was then infiltrated into upper, expanded leaves of the *NbAOS*- or *NbCOII*-silenced plants. In *NbAOS*-silenced plants, 50% of the inoculated leaves developed HR-type cell death at 6 dpi and all infiltrated leaves had HR-type cell death at 7.5 dpi (Fig. 9D). In *NbCOII*-silenced plants, 50% of the inoculated leaves developed HR-type cell death after 7dpi and all infiltrated leaves had HR-type cell death at 9.5 dpi (Fig. 9D). In contrast, 50% of the inoculated leaves from control plants developed HR-type cell death after only 5 dpi (Fig. 9D). Therefore, compared with control plants, a significant delay of RepA-induced cell death was observed in *NbAOS*- or *NbCOII*-silenced plants ($P < 0.0001$), suggesting that JA-mediated signaling is required for RepA-mediated plant cell-death function. To further examine the role of JA-regulated signaling in RepA-induced HR-type cell death, *N. benthamiana* plants were treated with methyl jasmonate (MeJA). As shown in Figure 9E, an acceleration of RepA-induced cell death was observed in MeJA-treated plants, as compared with buffer-treated control plants ($P < 0.0001$). We also observed a significant delay in cell death induced by ODV infection in *NbAOS*- or *NbCOII*-silenced *N. benthamiana* plants ($P < 0.0001$) (Fig. 9F) and an acceleration in cell death in plants treated with MeJA ($P < 0.0001$) (Fig. 9G). Western blot analysis showed no significant differences in accumulation levels of RepA:GFP proteins from *NbAOS*- and *NbCOII*-silenced or MeJA-treated plants inoculated with 35S-RepA:GFP as compared with control plants (Fig. 10A and B). Similarly, when *NbAOS*- and *NbCOII*-silenced or MeJA-treated plants were infected by ODV, the accumulation levels of RepA proteins were similar to those of control plants (Fig. 10C and D). This indicates that the transient expression of RepA was not altered in silenced or MeJA-treated plants.

Fig. 5. Subcellular localization of RepA protein. **A**, Expression of a RepA:GFP fusion protein in transgenic *Nicotiana benthamiana* plants expressing red fluorescent protein (RFP)-tagged histone H2B infiltrated with agrobacterium harboring the 35S-RepA construct. Photographs represent confocal images captured 48 h after agroinfiltration with a 40× objective using FITC (GFP) and Rhodamine (RFP-Histone) filter sets. Images in the lower panels represent merged images of the two filter sets. **B**, Subcellular localization of RepA protein in *N. benthamiana* leaf cells infected with *Oat dwarf virus* (ODV) detected by immunocytochemistry and electron microscopy using a RepA-specific antibody followed by an immunogold conjugate. The bottom image shows a higher magnification photograph of the red box indicated in the upper image, in which the gold particles (red arrow) are visible in the nuclei of infected cells.

Moreover, to rule out the possibility that the differences in HR-type cell-death induction observed by JA treatment and reduction in COI1 and AOS expression could be due to an effect on the *Agrobacterium* delivery system, we evaluated the growth rate of agrobacterium containing 35S-RepA:GFP in *NbAOS*- and *NbCOI1*-silenced as well as MeJA-treated plants. The results showed no significant difference in growth rate as compared with control plants ($P > 0.05$) (Supplementary Fig. S6). These results confirm the importance of JA-mediated signaling in RepA-mediated HR-type cell death.

ODV RepA is a weak suppressor of RNA silencing.

In some cases, a virus-encoded avirulence determinant has a specific role in suppression of RNA silencing (Chapman et al. 2004). To identify whether the ODV RepA protein acts as an

RNA silencing suppressor, *N. benthamiana* line 16c carrying a transgenic *GFP* was coinfiltrated with a mixture of *A. tumefaciens* harboring 35S-GFP and either 35S:RepA or a control construct. The coinfiltrated leaves were evaluated for GFP silencing under a hand-held UV light. By 3 dpi, GFP fluorescence was clearly diminished in leaves coinfiltrated with 35S-GFP plus the empty vector or with 35S-GFP plus 35S-RepA^{UT}. In contrast, relatively strong GFP fluorescence was observed in the leaves coinfiltrated with 35S-GFP plus 35S-RepA, although fluorescence was much weaker compared with leaves coinfiltrated with 35S-GFP plus 35S-P19 (Supplementary Fig. S7). Northern blot analyses of GFP transcripts confirmed that GFP mRNA levels were remarkably reduced in leaves coinfiltrated with 35S-GFP plus the empty vector or 35S-GFP plus 35S-RepA^{UT}. In contrast, the GFP mRNA

Fig. 6. Subcellular localization of RepA deletion mutants. Transgenic *Nicotiana benthamiana* plants expressing red fluorescent protein (RFP)-tagged histone H2B were inoculated with agrobacterium harboring different plasmids as indicated above the images. Confocal images were captured at 48 h after agroinfiltration. The green fluorescent protein and RFP signals and an overlay of both signals are shown.

accumulated to higher levels in leaves coinfiltrated with 35S-GFP plus 35S-RepA, although the mRNA level were much lower than in leaves coinfiltrated with 35S-GFP plus 35S-P19. Further analysis of siRNA accumulation indicated that 21- and 24-nucleotide GFP siRNAs, characteristic of RNA silencing, had accumulated to significantly higher levels at 3 dpi in leaves coinfiltrated with 35S-GFP plus the empty vector or 35S-GFP plus 35S-RepA^{UT}. In contrast, the GFP-specific siRNA levels were remarkably reduced in leaves coinfiltrated with 35S-GFP plus 35S-RepA or 35S-GFP plus 35S-P19. These results demonstrate that ODV RepA is capable of suppressing RNA silencing in *N. benthamiana* plants.

DISCUSSION

HR responses can be triggered by different pathogens, whose elicitors include extracellular proteins produced by fungi and bacteria and intracellular proteins produced by bacteria and viruses (Morel and Dangl 1997). The HR response is generally associated with specific recognition of a pathogen-encoded avirulence (avr) factor by a host-encoded *R* gene (incompatible interaction). The associated cell death is believed to contribute to containment of the pathogen. Previously, it has been shown that begomoviruses encode proteins that can act as HR-type cell-death elicitors (Mubin et al. 2010), which have been classified as virulence determinants. Here, we have demonstrated that transient expression of ODV RepA in tobacco plants from a pCHF3 binary vector or PVX-based vector elicits HR-like necrotic lesions and produces an H₂O₂ burst. To our knowledge, this is the first report of an HR-type cell death-inducing elicitor from a mastrevirus. It is certainly possible that the induction of cell death by RepA is not an HR-type cell death derived from gene-to-gene recognition and, therefore, the RepA-host interaction may not represent a classical avirulent response. Alternatively, the RepA-triggered necrosis may have relevance to an inherent function of RepA in deregulation or destabilization of cellular membranes. Such modifications of membranes could damage the cell and lead to necrosis

(Hashimoto et al. 2015). However, it is also possible that RepA may represent an *avr* gene encoded by ODV, which is recognized by an unknown *R* gene encoded by the host leading to an incompatible interaction.

Analysis of the phenotypes of RepA deletion mutants has revealed that the first nine aa at the N terminus of RepA play an important role in HR-type cell-death induction and that two regions located between aa 173 and 195 and between aa 241 and 260 near the C terminus are essential for the induction of HR-type cell death. Previous reports focused on begomoviruses have shown that transcription activation protein (TrAP) mutants associated with *Tomato yellow leaf curl China virus* (TYLCCNV) that are defective in nuclear import are unable to induce a necrotic response when expressed from PVX (Dong et al. 2003). Chowda-Reddy and associates (2009) also reported that the TrAP protein encoded by *East African cassava mosaic virus* is a nuclear localized protein and that NLS residues have a role in the TrAP-mediated disease phenotype. However, we also noted that the Rep mutant of *African cassava mosaic virus* (ACMV) that failed to trigger HR-type cell death was localized to the nucleus (Jin et al. 2008). Our localization studies clearly demonstrate that ODV RepA accumulates in nuclei of tobacco epidermal cells. Deletion analysis of RepA indicated that RepA mutants lacking a region located between aa 196 and 239 elicited necrosis during transient expression and these RepA mutants localized mainly in the cytoplasm as well as in the nucleus. A mutation lacking the C-terminal 26 aa did not induce necrosis, even though it still accumulated in the nucleus. Obviously, our results suggest that nuclear targeting of ODV RepA may be not essential for induction of the HR-type cell death, at least in the mastreviruses.

Hussain and associates (2005) reported that *Tomato leaf curl New Delhi virus* (ToLCNDV) NSP is an avirulence determinant that targets host HR defense responses in stably transformed *N. tabacum* plants. However, in our study, stable ODV RepA transformation resulted in asymptomatic symptoms in expanded leaves of *N. tabacum*. Not surprisingly, our results are consistent with earlier studies demonstrating that ACMV Rep

Fig. 7. Analysis of the roles of individual *Oat dwarf virus* (ODV) gene products in abolishing RepA-induced cell death. **A**, Representative leaves from *Nicotiana benthamiana* and *N. tabacum* plants coinoculated with agrobacterium cultures harboring the 35S-RepA construct alone or in conjunction with a culture harboring either 35S-V1:GFP, 35S-V2:GFP, or 35S-Rep:GFP. Photographs were taken at 6 days postinoculation (dpi). **B**, Western blot analysis of the expression of green fluorescent protein (GFP) fusion proteins and RepA protein using a GFP-specific antibody or ODV RepA-specific antibody in inoculated *N. benthamiana* leaves at 3 dpi. A Coomassie-stained gel was used to detect Rubisco as a loading control.

lost the ability to induce an HR-type cell death when expressed in transgenic plants (Hong and Stanley 1995). This raises an interesting question about how plants cope with this toxic protein during viral infection. It is known that begomovirus Rep represses its own expression by binding to a sequence in the intergenic region that interferes with the function of the Rep promoter (Eagle et al. 1994). We also found that the expression of ODV RepA protein could not be detected in transgenic *N. tabacum* plants, even though RepA transcripts were detectable. It is possible that posttranslational protein modifications of the ODV RepA gene occurred during transformation and that the only surviving transgenic lines express RepA variants that had lost the ability to induce an HR-type cell death.

Temperature sensitivity of disease resistance has been observed in both basal defense responses and in *R* gene-mediated defense response. For example, resistance to TMV conferred by the *N* gene has been shown to be abolished above 28°C (Király et al. 2008; Samuel 1931; Zhu et al. 2010). An HR against the fungal pathogen *Cladosporium fulvum* mediated by the Cf4 and Cf9 elicitors in tomato can be suppressed at 33°C (de Jong et al. 2002). Recently, the coat protein of *Alternanthera mosaic virus* was identified to be an elicitor of temperature-sensitive systemic necrosis in *N. benthamiana* (Lim et al. 2014). Also, Szajko and associates (2014) reported HR

against *Potato virus Y* in cultivated potatoes carrying Ny-1 and Ny-2 genes were only occasionally observed above 28°C. However, elevated temperatures have not been previously reported to suppress geminivirus-encoded protein-mediated resistance and our report is the first observation that RepA-mediated defense responses are inhibited at elevated temperatures. Another important question is to understand how temperature modulates ODV RepA-induced HR-type cell death. Previous reports showed that Cf-mediated defense responses at elevated temperatures are correlated with the level of perception of fungal avirulence factors (de Jong et al. 2002). Király and associates (2008) reported that suppression of TMV-induced hypersensitive-type necrotization in tobacco at high temperature is associated with downregulation of the ROS superoxide and activation of antioxidant genes and enzymes. Although our results showed that elevated temperatures have a slight effect on expression of RepA protein itself, we rule out the possibility that HR elicited by RepA is dose-dependent instead of temperature sensitive. However, it remains to be determined whether that temperature sensitivity is intrinsic to the RepA protein itself or whether it inactivates host gene products involved in recognition of the RepA gene product or components of the defense signal transduction pathways (Wang et al. 2009; Zhu et al. 2010).

Fig. 8. Effects of temperature on RepA-induced hypersensitive response-type cell death. **A**, Representative leaves from *Nicotiana benthamiana*, *N. tabacum* cv. *Samsun*, *N. glutinosa*, and *N. tabacum* plants incubated at 25 or 30°C 5 days after infiltration with agrobacterium harboring 35S-RepA:GFP. **B**, Subcellular localization of the RepA:GFP proteins in *N. benthamiana* plants infiltrated with agrobacterium harboring 35S-RepA:GFP and incubated at 25 or 30°C. The green fluorescent protein (GFP) signals and the overlay of signals from GFP with an unfiltered light source are shown. Images were taken by confocal microscopy at 48 h after infiltration with agrobacterium culture harboring 35S-RepA:GFP. **C**, Western blot analysis of the RepA:GFP proteins in *N. benthamiana* plants infiltrated with agrobacterium culture harboring 35S-RepA:GFP and incubated at 25 or 30°C. A Coomassie-stained gel was used to detect Rubisco as a loading control. **D**, Representative leaves from *N. benthamiana* plants incubated at 25 or 30°C 5 days after infiltration with serially diluted agrobacterium harboring 35S-RepA:GFP from optical density at 600 nm (OD_{600}) = 0.2 to OD_{600} = 2.0. **E**, Western blot analysis of the RepA:GFP proteins in *N. benthamiana* leaves infiltrated with serially diluted agrobacterium harboring 35S-RepA:GFP from OD_{600} = 0.2 to OD_{600} = 2.0 and incubated at 25 or 30°C for 3 days. A Coomassie-stained gel was used to detect Rubisco as a loading control. **F**, Representative leaves from *N. benthamiana* and *N. tabacum* plants were collected at 5 days after infiltration with agrobacterium harboring an *Oat dwarf virus* (ODV) infectious clone and incubated at 25°C or 30°C. **G**, Western blot analysis of the RepA proteins in *N. benthamiana* and **H**, *N. tabacum* plants infected with an ODV infectious clone and incubated at 25 or 30°C. A Coomassie-stained gel was used to detect Rubisco as a loading control.

JA has been repeatedly associated with induction of cell death (He et al. 2002; Rao et al. 2000; Yoon et al. 2009; Zhang and Xing 2008). In the present study, we obtained direct evidence for a role of JA signaling in regulating RepA-induced HR-type cell death. Our loss-of-function studies show that cell death induced by overexpression of RepA is delayed by silencing of *COI1*, which encodes an F-box protein that participates in JA perception and mediates almost all JA responses

(Xie et al. 1998). Liu and associates (2004) also have reported that silencing of *COI1* using VIGS leads to loss of HR resistance to TMV in *N. benthamiana* transgenic for the *N* gene. It was, therefore, surprising that Pacheco and associates (2012) reported silencing of *COI1* accelerated cell death induced by PVX recombinant virus expressing the *HC-Pro* gene from *Plum pox virus* in a susceptible host. These conflicting results may suggest that plant responses to compatible and incompatible

Fig. 9. Jasmonic acid (JA) signaling components required for cell death induced by *Oat dwarf virus* (ODV) RepA. **A**, Quantitative real-time polymerase chain reaction analysis of *NbAOS*, **B**, *NbCOI1*, or **C**, *NbPDF1.2* expression in *NbAOS*-silenced, *COI1*-silenced, and control *Nicotiana benthamiana* plants 1 week after agroinoculation with either TRV:GUS, TRV:NbAOS, or TRV:NbCOI1. The *GADPH* gene was used as a reference gene. The relative levels of *NbAOS* mRNA, *NbCOI1* mRNA, or *NbPDF1.2* mRNA in plants agroinoculated with TRV:GUS was arbitrarily set as 1. The error bars show standard deviations of ten individual plants. **D** and **F**, Effect of *NbAOS* and *NbCOI1* silencing or **E** and **G**, methyl jasmonate (Me-JA) treatment on RepA-induced hypersensitive response-type cell death. Data show the percentage of *N. benthamiana* leaves exhibiting cell death. When inoculated with either 35S-RepA:GFP (**D** and **E**) or ODV (**F** and **G**). The data are derived from at least 10 plants in each treatment. Plants infected with TRV:GUS or treated with absolute alcohol were used as controls. Error bars represent standard deviations of three independent experiments. The data were analyzed using two-way analysis of variance, followed by Student's *t* test, showing significant difference between treatments ($P < 0.0001$).

plant-pathogen interactions share a common step mediated by JA pathway products. Compared with JA, SA has been more intensively studied due to its crucial role as a signal molecule involved in HR (Delaney et al. 1994; Xu et al. 2012). It is worth noting that some published evidence also indicates that SA and JA can act either antagonistically or synergistically to control stress responses and that these effects depend on the plant species and the stimulus (Niki et al. 1998; Rao et al. 2000; Xu et al. 1994). However, our results demonstrate that either depletion of SA by the *NahG* transgene or SA treatment has no detectable effects on RepA-induced necrosis, suggesting that the HR-type cell death induced in tobacco by RepA is independent of SA accumulation. Our results are also in agreement with earlier studies of other plant-pathogen systems, in which the function of several *R* genes against fungi and bacteria in tomato are SA independent (Brading et al. 2000; Li et al. 2002).

RNA silencing is an important defense mechanism against viral infection based on homology-dependent RNA degradation (Csorba et al. 2015). To counteract host RNA silencing, viruses have evolved suppressor proteins (Xiong et al. 2009). It is worthwhile noting that many viral proteins identified as suppressors of RNA silencing, including the P19 protein encoded by *Tomato bushy stunt virus*, the 2b protein encoded by cucumoviruses, or the V2 protein encoded by *Tomato leaf curl Java*

virus have also been identified as HR elicitors in planta (Ryang et al. 2004, 2005; Scholthof et al. 1995; Sharma and Ikegami 2010). Our results provide additional evidence that RepA has the potential to interact with both RNA silencing and gene-for-gene disease resistance pathways in plants. Despite the fact that viruses encode proteins that mediate both plant defense and viral counter-defense, the mechanisms whereby these proteins interact with different defense pathways are largely unknown in plants. Our efforts are now underway to identify host factors interacting with RepA that potentially are involved in modulating both host resistance pathways.

MATERIALS AND METHODS

Expression constructs.

Four ODV ORF (V1, V2, RepA, and Rep) were amplified individually by PCR from a recombinant plasmid that contains a full-length infectious clone of ODV-Hak14 (provided by J. Schubert from the Federal Research Institute for Cultivated Plants, Quedlinburg, Germany), using specific primers pairs (V1-GFP-F/V1-GFP-R, V2-GFP-F/V2-GFP-R, RepA-GFP-F/RepA-GFP-R, Rep-OF/Rep-GFP-R, and RepA-GFP-F/Rep-OR) (Supplementary Table S1). An untranslatable RepA mutant was constructed by replacing the second codon (GCT) with TAG, by PCR using the primer pair RepA^{UT}-GFP-F/RepA-GFP-R.

Fig. 10. Effect of jasmonic acid (JA) signaling components on expression of the RepA protein. Western blot analysis of the expression of RepA:GFP fusion proteins from **A**, *NbAOS*- or *NbCOII*-silenced or **B**, methyl jasmonate (Me-JA) treated *Nicotiana benthamiana* plants infiltrated with agrobacterium harboring 35S-RepA:GFP. **C**, Western blot analysis of the expression of RepA proteins from *NbAOS*- or *COII*-silenced or **D**, Me-JA treated *N. benthamiana* plants infiltrated with agrobacterium harboring an *Oat dwarf virus* infectious clone. Plants infected with TRV:GUS or treated with absolute alcohol were used as controls. A Coomassie-stained gel was used to detect Rubisco as a loading control.

The PCR products were cloned into the pMD18T vector (Takara, Dalian, China) and were then sequenced to ensure that sequence errors had not been introduced. The resulting constructs were digested with *KpnI/BamHI* or *KpnI/SalI* and were inserted between the CaMV 35S promoter and the nopaline synthase terminator in the modified binary vector pCHF3 (Xiong et al. 2009), which contains an eGFP reporter gene amplified by primers GFP-F/GFP-R into the pCHF3 vector. To assess the pathogenicity of RepA in *Nicotiana* species, the RepA ORF and the untranslatable mutant were amplified by PCR with the primer pairs RepA-PVX-F/RepA-PVX-R and RepA^{UT}-PVX-F/RepA-PVX-R and were inserted into the pGR107 vector (Thomas et al. 2001) at the *Clal/SalI* sites to produce the PVX-RepA and PVX-RepA^{UT} constructs. For deletion analysis of the RepA protein, start and stop codons were added to the N- and C-terminal deletion mutants with specific primers (RepA-N1-GFP-F/RepA-GFP-R, RepA-N2-GFP-F/RepA-GFP-R, RepA-GFP-F/RepA-C1-GFP-R, and RepA-GFP-F/RepA-C2-GFP-R) to generate RepA^{dm1-9}, RepA^{dm1-239}, RepA^{dm241-266}, and RepA^{dm261-266} products, respectively. A deletion mutant (RepA^{dmRB}) in which the conserved LICHE motif was deleted was amplified by overlap PCR extension with primer pairs RepA-GFP-F/RepA-RB-GFP-R and RepA-RB-GFP-F/RepA-GFP-R. The RepA mutants with deletions in the central domain were constructed by overlap PCR extension with gene-specific primer pairs (RepA-GFP-F/RepA-M1-GFP-R and RepA-M1-GFP-F/RepA-GFP-R, RepA-GFP-F/RepA-M2-GFP-R and RepA-M2-GFP-F/RepA-GFP-R, RepA-GFP-F/RepA-M3-GFP-R and RepA-M3-GFP-F/RepA-GFP-R, RepA-GFP-F/RepA-M4-GFP-R and RepA-M4-GFP-F/RepA-GFP-R, RepA-GFP-F/RepA-M5-GFP-R and RepA-M5-GFP-F/RepA-GFP-R, and RepA-GFP-F/RepA-M6-GFP-R and RepA-M6-GFP-F/RepA-GFP-R) to produce RepA^{dm30-239}, RepA^{dm129-239}, RepA^{dm150-239}, RepA^{dm173-239}, RepA^{dm196-239}, and RepA^{dm215-239}, respectively. The resulting constructs were digested with *BamHI/KpnI* and were inserted into the binary vector pCHF3:GFP.

Agroinoculation of plants.

A. tumefaciens cultures harboring different constructs were incubated in yeast extract-peptone medium at 28°C overnight and were resuspended in induction medium (10 mM MES, pH 5.7, 10 mM MgCl₂, 200 mM acetosyringone) to a final OD₆₀₀ of 1.0. Bacterial cultures were infiltrated into the underside of leaves using a 1-ml syringe, and the infiltrated plants were grown in a greenhouse at 25 or 30°C under a 16-h-light and 8-h-dark cycle and were observed daily for symptom development.

Detection of H₂O₂ in plants.

H₂O₂ was visually detected in the leaves of plants by using DAB as substrate (Thordal-Christensen et al. 1997). Briefly, leaves were excised at the base of the petioles with a razor blade and were incubated for 8 h under light at 25°C in the presence of a 1 mg/ml solution of DAB (Sigma-Aldrich [St. Louis], pH 3.8). Leaves were then immersed in boiling 96% ethanol for 5 to 10 min, were cooled and preserved at room temperature in 70% ethanol, and were photographed.

Protein extraction and Western blots.

Leaves of *N. benthamiana* plants infiltrated by *A. tumefaciens* cultures harboring different constructs were ground in liquid nitrogen, and 100 mg of powder was mixed with 100 µl of concentrated (2×) sodium dodecyl sulfate (SDS) sample buffer. Total extracts were boiled for 5 min and, then, were centrifuged for 5 min at 13,000 × g before gel loading. Proteins were separated on 12.5% SDS-polyacrylamide gel electrophoresis, were transferred to nitrocellulose membranes, and were probed with a

rabbit anti-RepA polyclonal antibody, produced by expression of the RepA protein in *Escherichia coli*, or with a rabbit anti-GFP monoclonal antibody (Epitomics, Burlingame, CA, U.S.A.). The secondary antibody was a goat antirabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase (Sigma-Aldrich), which was used at 1:8,000 (vol/vol) dilution. Bands were visualized by incubating the membranes in NBT-BCIP solution (Promega, Madison, WI, U.S.A.).

ELISA.

Tissue samples from systemic leaves of *N. benthamiana* infected by PVX, PVX-RepA, or PVX-RepA^{UT} were ground and coated overnight in ELISA plates as described (Shang et al. 2011). Coated samples were incubated for 1 h with a 5,000-fold dilution of a mouse anti-PVX monoclonal antibody produced from purified virus. Goat antimouse IgG conjugated with alkaline phosphatase at a 1:8,000 dilution was subsequently applied into the wells and incubated for 1 h. After incubating in NBT-BCIP solution (Promega) for 0.5 h, ELISA absorbance values were measured using a Microplate Reader Model 680 (Bio-Rad, Hercules, CA, U.S.A.).

Stable plant transformation.

For stable transformation, the RepA coding sequence was amplified by PCR with the primer pair RepA-GFP-F/RepA-PVX-R, using a recombinant plasmid containing a full-length infectious clone of ODV-Hak14 as template. The amplified fragment was inserted into the pCHF3 vector to produce 35S-RepA. *N. tabacum* leaf explants were cocultured with agrobacterium, using the leaf disc transformation method as described (Ding et al. 2009). Regenerated transgenic plants grown to a height of 5 to 6 cm were transplanted into soil and were allowed to grow to maturity inside a greenhouse. Empty pCHF3 vector-transformed plants were used as wild-type controls.

Subcellular localization of RepA and its deletion mutants.

Leaves of 4-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* C58C1 harboring various constructs (Xiong et al. 2009). Approximately 48 h after infiltration, 1-cm² leaf samples were excised and green or red fluorescence was examined in epidermal cells, using a Leica TCS SP5 confocal microscope.

Immunocytochemistry and electron microscopy.

Tissue samples (1 × 4 mm) excised from leaves of wild-type or ODV-infected *N. benthamiana* plants were vacuum infiltrated and fixed for 2 h in a solution of 0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.0) at 4°C, as described (Xiong et al. 2009). Tissue was dehydrated using a gradient of methanol solutions and was rinsed in Lowicryl K4M (Fort Washington, PA, U.S.A.) (Simon et al. 1987). Tissues were embedded by polymerization at −20°C for 3 days and were incubated at room temperature for 12 h under an ultraviolet lamp. Ultrathin sections (70 nm) were cut from embedded tissue using an Ultracut E Ultramicrotome (Reichert-Jung, Vienna, Austria) and were mounted on formvar-coated grids. Thin sections were probed with an anti-RepA polyclonal antibody (50-fold dilution), followed by incubation with a Protein A gold conjugate (10 nm size) (Sigma) diluted in BL buffer as described (Kong et al. 2014). The sections were stained with uranyl acetate and lead citrate and were examined under an electron microscope (JEM-1200 EX; Jeol, Akishima, Japan).

VIGS and qRT-PCR analysis.

For VIGS, a partial fragment from *NbCOI1* or *NbAOS*, key JA signaling components, was amplified from tobacco cDNA,

using gene-specific primer pairs. The resulting PCR product was cloned into a TRV-derived vector (Liu et al. 2002) at the *Xba*I and *Bam*HI sites and was transformed into *A. tumefaciens* EHA105. *N. benthamiana* plants (4 weeks old) were coinoculated with equal amounts of agrobacterium cultures carrying the recombinant TRV-derived vectors. In the VIGS assay, the GADPH gene was used as an internal control. All plants were grown in a greenhouse at 25°C with supplementary lighting to give a 16-h photoperiod. One week after agroinfiltration (when the *NbCOII* or *NbAOS* gene was completely silenced), plants were subjected to HR assays. VIGS efficiency was evaluated by comparing transcript abundance in silenced plants with that in control plants, using qRT-PCR analysis (Huang et al. 2009). All gene expression analyses were repeated three times.

Transient expression of RepA on MeJA-treated plants.

For each plant, 500 µM MeJA dissolved in 0.1% alcohol was applied to the underside of leaves on *N. benthamiana* plants 1 day before plants were inoculated with the 35S-RepA construct (Rao et al. 2000). Control plants were treated with absolute alcohol alone.

Suppression of local RNA silencing.

Assays of RNA silencing suppression of various genes were conducted by agroinfiltration as described previously (Xiong et al. 2009). Briefly, 4-week-old transgenic *N. benthamiana* plants expressing GFP (line 16c) were infiltrated with equal volumes of *A. tumefaciens* (strain GV3101) cultures (individually diluted to OD₆₀₀ = 1 before mixing) of each harboring the relevant binary plasmid and were then cultured in growth chambers at 25°C. Local RNA silencing in agroinfiltrated plants was determined by examination of GFP expression in infiltrated leaves under a hand-held 100-W, long-wave UV lamp (UV Products, Upland, CA, U.S.A.), photographed using a Nikon 5000 digital camera (Nikon, Tokyo) with a Y48 yellow filter. In each treatment, eight to 10 plants were infiltrated and the experiments were repeated at least three times.

RNA extraction and analysis.

Total RNA was extracted from *N. benthamiana* leaf tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). High-molecular mass RNA (20 µg) was separated in a 1% denaturing agarose gel and was transferred to Hybond-N⁺ membranes (Amersham Pharmacia, Piscataway, NJ, U.S.A.). Northern blot analysis was conducted as described previously (Cui et al. 2004). RNA bound to the membranes was hybridized with [³²P]-dCTP-labeled probes synthesized using the Primer-A-Gene labeling kit (Promega).

Enrichment and detection of low-molecular mass RNAs were performed as described (Dalmay et al. 2000). Low-molecular-mass RNAs (20 µg) were separated in a 15% polyacrylamide gel containing 7 M urea and were transferred to Hybond-N⁺ membranes (Amersham Pharmacia). The membranes were hybridized with four synthetic 40-nucleotide GFP segments end-labeled with [³²P]-γ-ATP as probes for siRNA hybridization performed as described previously (Xiong et al. 2009).

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