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Tomato leaf curl Java virus V2 protein is a determinant of virulence, hypersensitive response and suppression of posttranscriptional gene silencing

Pradeep Sharma [a,b,*], Masato Ikegami [a]

[a] *Department of Life Science, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan*
[b] *Division of Crop Improvement, Directorate of Wheat Research, Karnal 132 001, India*

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ABSTRACT

We previously identified the *Tomato leaf curl Java virus*-A (ToLCJV-A[ID]) from Southeast Asia as a new member of the emerging group of monopartite begomoviruses that require a betasatellite component for symptom induction. In this study, the role of V2 in viral pathogenesis and posttranscriptional gene silencing (PTGS) was studied. Our results showed V2 of ToLCJV-A[ID] elicits a reaction resembling the hypersensitive response (HR) associated with the induction of necrosis and a systemic burst of H_2O_2 production when expressed from a potato virus X vector in *Nicotiana* species and tomato. Transient expression of ToLCJV-A[ID] V2 after agroinfiltration of *Nicotiana benthamiana* and tomato also triggered HR-like cell death, demonstrating that ToLCJV-A[ID] V2 is a target of host defense responses. Deletion of 58 amino acids (aa) from the N-terminus did not affect the HR, suggesting that this region has no role in the HR, while deletion of 58 aa from the C-terminus of V2 abolished both the HR response and V2 silencing suppressor activity, suggesting that these sequences are required for the HR-like response and suppression of PTGS. This finding demonstrated that ToLCJV-A[ID] V2 is a pathogenicity determinant that elicits an HR-like response.

Introduction

Plant viruses belonging to the family *Geminiviridae* have circular single-stranded DNA (ssDNA) genomes and are encapsidated within geminate particles. Infection results in considerable negative impact on economically important crops. Tomato leaf curl disease (ToLCD) is among the most important limiting factors that affect tomato production in tropical and sub-tropical regions of the world including in Southeast Asia. Based on genome organization, host range and insect vector, geminiviruses are divided into four genera, *Begomovirus*, *Mastrevirus*, *Curtovirus* and *Topocuvirus* (Stanley et al., 2005). Most begomoviruses have genomes consisting of one component (monopartite) although some have two components (bipartite), designated DNA A and DNA B in the Old World (OW). *Tomato leaf curl Java virus*-A (ToLCJV-A[ID]) has a genome consisting of a single component and associates with a betasatellite (Fig. 1). The ToLCJV-A [ID] genome consists of a single ~2.7-kb circular ssDNA that contains six open reading frames (ORFs), encoded on both strands of the double-stranded replicative form DNA. ORFs V1 and V2 are in the sense direction; the V2 ORF encodes the pre-coat protein. The arrangement of the DNA component of the monopartite begomoviruses is with ORFs C1, C2 and C3 partially overlapping and a small ORF, C4, located within C1 ORF, in a different reading frame.

Symptom modulating betasatellites associated with geminiviruses have come to our attention only recently but have proven to be widespread, associated with many diseases throughout the OW, and economically significant, particularly in developing countries. Following the identification of a novel ssDNA satellite (betasatellite) associated with ageratum yellow vein disease (Saunders et al., 2004), the vast majority of begomoviruses previously assumed to be monopartite have shown to be satellite requiring. ToLCD is caused by many viruses, many of them are associated with betasatellite. The ToLCJV-A [ID] is associated with Tomato leaf curl Java betasatellite [Indonesia: Indonesia1:2003] (ToLCJB-[ID:ID1:03]) and encodes a βC1 ORF on the complementary sense strand of DNA encoding a ~13-kDa protein. This betasatellite DNA depends on ToLCJV-A[ID] for its replication and encapsidation (Kon et al., 2006). Further, Kon et al. (2007) showed that the betasatellite associated with the ToLCJV-A [ID] encodes a pathogenicity determinant and suppressor of post-transcriptional gene silencing (PTGS). C1 and C3 are involved in viral replication (Dry et al., 2000; Hanley-Bowdoin et al., 2000), C2 is an RNA silencing suppressor (Dong et al., 2003; Kon et al., 2007; Wartig et al., 1997) that can also function as a host range factor in the related *Tomato yellow leaf curl China virus*-Boashan1[China:Yunnam10: Tobacco:2000] (TYLCCNV-Bao1 [CN: Yn10:Tob: 00]) and C4 induces disease symptoms (Jupin et al., 1994; Rigden et al., 1994; Kreke et al., 1998) and is localized to the host cell periphery where it interacts with plasmodesmata (Rojas et al., 2001). Recently, we have shown that V1 (coat protein) of ToLCJV-A[ID] is involved in nucleocytoplasmic shuttling within cells of the host as well as laboratory

* Corresponding author. Division of Crop Improvement, Directorate of Wheat Research, Karnal 132 001, India. Fax: +81 22 717 8659.
E-mail address: neprads@gmail.com (P. Sharma).

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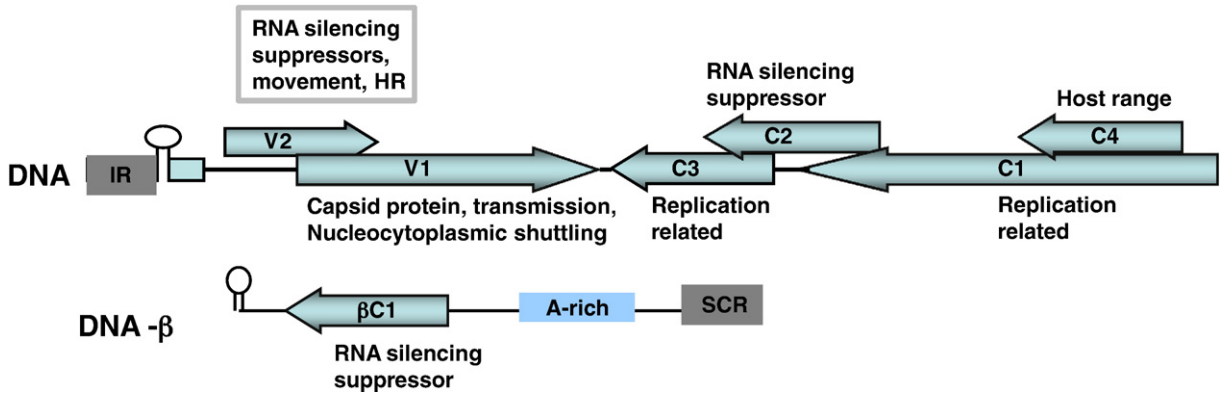

Fig. 1. Genome organization of the monopartite ToLCJV-A[ID] and its associated betasatellite component. For clarity, the circular components are presented as a linear molecule. The open reading frames are shown as boxes and their potential roles in the viral infection cycle are indicated. The white open box shows the function of V2 studied in this investigation. V represents virion sense, C is complementary sense, IR is intergenic region, SCR is satellite conserved region and A-rich is adenine-rich region.

yeast strains used to study the protein (Sharma and Ikegami, 2009). The V2 ORF occurs only in OW begomoviruses but absent in New World begomoviruses and mutational analysis has shown that it plays a role in viral spread and infectivity (Padidam et al., 1996; Wartig et al., 1997; Rojas et al., 2001; Bull et al., 2007; Rothenstein et al., 2007; Chowda-Reddy et al., 2008). There is no consistent picture of its function suggesting that the V2 of different viruses may differ in their function.

Gene-for-gene disease resistance is an important plant defense mechanism against pathogens. It is induced when the host plant carrying a resistance gene is challenged by a pathogen carrying a corresponding avirulence gene (Keen, 1990). The production of an oxidative burst (including O^{-2} and H_2O_2) is one of the earliest responses to incompatible interactions between pathogens and plants and is caused by the activation of a membrane-bound NADPH oxidase (Keen, 1990). Some of the geminiviral encoded gene products (such as nuclear shuttle protein) are avirulence factors that induce a hypersensitive response (HR) in hosts. Examples of geminivirus pathogens and hosts are *Bean dwarf mosaic virus*-[Colombia:1987] (BDMV-[CO:87]) in *Phaseolus vulgaris* (Garrido-Ramirez et al., 2000; Seo et al., 2004) and *Tomato leaf curl New Delhi virus*-India[India:New Delhi:AVT1] (ToLCNDV-IN[IN:ND:AVT1]) in *Solanum esculentum* (Hussain et al., 2005). The programmed cell death (PCD) resulting from an HR is believed to be associated with resistance to pathogens, generally induced at infection sites or within defined areas surrounding the site of infection that effectively limits pathogen spread (Morel and Dangl, 1997; Garrido-Ramirez et al., 2000; Jurkowski et al., 2004; Hussain et al., 2005). Such a response is clearly advantageous to the plant as it limits the effects of pathogenic infection, although pathogens have developed countermeasures to overcome such defense mechanisms. Systemic leaves of nepovirus- and caulimovirus-infected plants exhibit strong virus resistance, similar to that observed with PTGS, thus providing evidence for a second type of natural defense mechanism against viruses in plants (Covey et al., 1997; Ratcliff et al., 1997).

RNA silencing is a versatile and complex gene regulation and defense mechanism in a broad range of eukaryotic organisms. It is activated in cells by double-stranded (ds) RNAs, followed by cleavage of the inducing RNAs into short (21- to 24-nucleotide) fragments. These, in turn, mediate multiple different regulatory and defense functions in the cells (Brodersen and Voinnet, 2006). In PTGS, they target the RNA-induced silencing complex (RISC) to degrade homologous RNA transcripts or to arrest their translation. The importance of these suppressors is reflected by the fact that many of them previously have been identified as pathogenicity factors or as cell-to-cell or long-distance movement proteins, essential for infectivity in indicated hosts (Voinnet, 2001; Sharma and Ikegami, 2008). Silencing suppressor proteins encoded by unrelated RNA and DNA viruses bear no similarity to each other in either coding sequence or protein structure, suggesting separate origins and variable functional mechanisms for each suppressors type; no such activity has been described for the V2 protein of ToLCJV-A[ID]. However, V2 of *Tomato yellow leaf curl virus*-Israel [Rehovot:1986] (TYLCV-IL[IL:Reo:86] functions as an RNA silencing suppressor that inhibits the innate immune response of the host plant (Zrachya et al., 2007). V2 was proposed to interact with SGS3 and thus inhibit its function, based on the results of yeast two-hybrid and fluorescence microscopy experiments in plant cells (Glick et al., 2008). In addition, its role in relation to SGS3 in binding small RNAs has been elucidated recently (Fukuda and Doudna, 2009).

In this study, we report that the V2 protein encoded by ToLCJV-A[ID] acts as an important virulence determinant by suppressing PTGS. Furthermore, our results also suggest the role of V2 in elicitation of HR-like cell death.

Results

V2 is a symptom determinant

To investigate the pathogenicity of V2, we used the PVX vector for expression (Baulcombe et al., 1996). The full-length V2 gene was cloned in the PVX vector (Fig. 2) and inoculated into *Nicotiana benthamiana*. Inoculated plants were maintained in a growth chamber with 16 h light (at 24 °C) and 8 h of darkness (20 °C) and symptoms were recorded periodically. Three to four days after inoculation, mild chlorotic patterns appeared on the inoculated leaves followed by downward curling of newly emerging leaves. However, systemic symptoms developed in upper leaves at 6–7 days postinoculation (dpi) and necrotic lesions became prominent on the inoculated leaves after 1 week (Fig. 3Aii). This was followed by apical necrosis that ultimately led to the death of the plant. The results obtained with *Nicotiana glutinosa* plants were comparable but less dramatic, because PVX constructs that expressed V2 usually failed to cause the death of the plant. Nevertheless, necrosis was always associated with the presence of V2, especially in lower leaves (data not shown). In contrast, *N. benthamiana* plants inoculated with a construct containing a mutated V2 (PVX-mV2) developed milder chlorotic lesions on the inoculated leaves 3–5 dpi followed by mosaic symptoms on systemically infected leaves (Fig. 3Aiii) that were indistinguishable from those seen in plants infected with the parental PVX vector at 6–7 dpi. Taken together, these results demonstrate that V2 of ToLCJV-A[ID] is a symptom determinant and is sufficient to elicit severe necrotic disease in *Nicotiana* species that support a systemic infection.

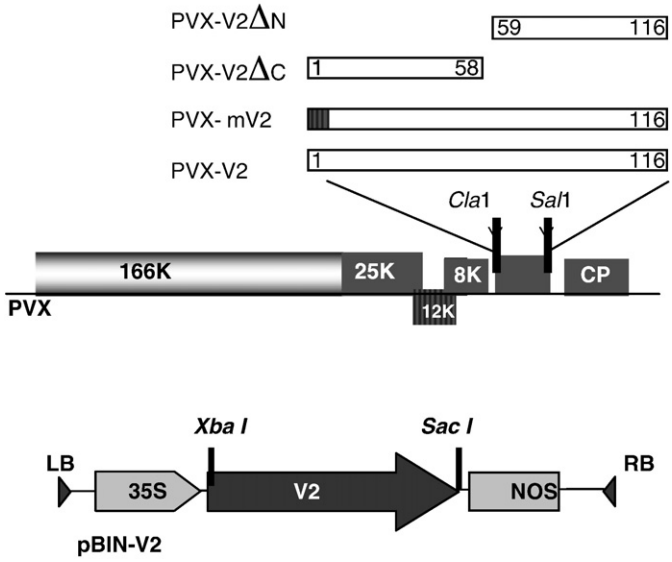

Fig. 2. *Potato virus X* based expression cassettes of V2 of ToLCJV-A[ID]. The deletion mutants of V2 of ToLCJV-A[ID] are indicated by amino acid coordinates. For VIGS in plants, the PVX-based GFP expression vector (PVX-GFP) was used. The first four major ORFs in the PVX sequence are indicated by their size (K = kDa) of their putative products (166K, 25K, 12K and 8K) and the coat protein gene by CP. Expression of inserts in the PVX is controlled by a duplicated CP promoter, indicated by dark lines. PVX-mV2 has a nonsense mutation in the N-terminus region, and PVX-V2ΔN having 58 aa deletion at the N-terminus, while PVX-V2ΔC contains a deletion of 58 aa at the C-terminus of ToLCJV-A[ID]. For transient expression, binary vector pBI121 was used. LB is the T-DNA left border, RB is the T-DNA right border, 35S represents the *Cauliflower mosaic virus* 35S promoter, NOS is the nopaline synthase terminator. The diagrams are not to scale.

V2 elicits an HR

Previously, we showed infectivity of ToLCJV-A[ID] clones in *N. benthamiana* and tomato (Kon et al., 2006). The symptoms induced were mild leaf curling; when ToLCJV-A[ID] was co-inoculated with its associated betasatellite (ToLCJB-[ID:ID1:03]), downward leaf curling was more severe and growth was stunted. However, the virus does not induce hypersensitive cell death or systemic necrosis.

In order to assess the effects of V2 expression on *N. benthamiana*, *N. glutinosa* and tomato, plants were co-infiltrated with Agrobacterium cultures harboring a construct of ToLCJV-A[ID] V2 under the control of the *Cauliflower mosaic virus* 35S promoter (35S:V2). Infiltrated leaves developed HR-like lesions at the site of inoculation, which covered the infiltration patch in *N. benthamiana* but was very limited in tomato and *N. glutinosa* (Fig. 3B). Plants inoculated with empty vector (pBIN61) did not show any HR-like symptoms. For the remaining genes of C1, C2, C3, C4 and V1, transient expression in tomato leaves was found to cause no discernible phenotype (data not shown). For *N. benthamiana* plants stably transformed with V2 under the control of the 35S promoter (pBI121), all the transformed T_0 lines (seven lines) showed severe systemic necrosis; infection induced a massive cell death response that killed the transgenic plants (data not shown). Hence, it was not possible to recover seeds.

Deletion analysis of V2

Next, we determined the region of ToLCJV-A[ID] V2 potentially involved in HR. Two deletion mutants, V2ΔC and V2ΔN, were generated and cloned into the PVX vector, yielding PVX-V2ΔC and PVX-V2ΔN. The results of the deletion mutant constructs are summarized in Table 1. Plants inoculated with construct PVX-V2ΔN having a 58 amino acid (aa) deletion at the N-terminus remained capable of inducing systemic symptoms and the HR, although much less efficiently (Fig. 3C). In contrast, a construct with a deletion of V2 at the C-terminus (PVX-V2ΔC) did not exhibit HR-like symptoms. Taken together, these results suggest that sequences within the C-terminus of V2 play a role in eliciting the HR.

To determine whether these attenuated symptoms could be due to low replication and systemic spread, a northern blot test was conducted in inoculated as well as systemically infected leaves of plants infected with each construct. The results showed that these mutants replicate in inoculated leaves (Fig. 3D, IL). Both PVX-V2 and PVX-V2ΔN accumulated in systemically infected leaves (Fig. 3D, SL, lanes 1 and 3). However, near absence of PVX-V2ΔC in systemically infected leaves suggests that this mutant is likely unstable and deficient in viral movement or replication (Fig. 3D, SL, lane 2).

Accumulation of H_2O_2 in plants

Plant cell death at infection sites and pathogen localization in necrotic tissues are typical features of the HR. Therefore, we investigated plants infiltrated with either empty vector pBIN16 as a negative control or inoculated with a PVX-based vector expressing V2 for the production of H_2O_2 by *in situ* detection using the 3,3′-diaminobenzidine (DAB) uptake method. In the presence of H_2O_2, DAB polymerizes to produce a deep brown product that can be visualized after ethanol decolorization of the tissue. Mock- and virus-inoculated plants were assayed at 5–7 dpi, when the virus-infected plants had either necrotic or HR-like lesions visible on the inoculated leaves, although systemic symptoms had not yet developed. Fig. 4 shows that H_2O_2 accumulated to a high level in the infiltrated leaves of tomato (Fig. 4ii) and *N. benthamiana* (Fig. 4iv) and the systemically infected PVX-inoculated leaves of *N. benthamiana* (Fig. 4vi) and *N. glutinosa* (Fig. 4viii). Furthermore, we also detected H_2O_2 in all epidermal cells undergoing HR. Here, it is noteworthy that the plants inoculated with PVX-V2ΔN developed a vascular HR (data not shown), while PVX-V2ΔC did not display induction of any such vascular HR-like response.

V2 is a suppressor of PTGS

Recently, V2 and its homolog AV2 have been shown to be suppressors of PTGS (Chowda-Reddy et al., 2008; Zrachya et al., 2007; Glick et al., 2008). Our data also indicate that the ToLCJV-A[ID] V2 protein is a pathogenicity determinant, and further suggest a function in PTGS. Therefore, we investigated the ability of ToLCJV-A[ID] V2 to suppress PTGS using PVX-based constructs. The V2 protein and GFP were expressed separately from a PVX vector in *N. benthamiana* 16c as described previously (Kon et al., 2007). Examination of mock-inoculated plants at 18 dpi showed that green fluorescence was maintained under long-wave UV light. Inoculation with the PVX vector produced mild chlorosis on systemically infected leaves but had no effect on GFP expression in 16c plants. In contrast, stable transgenic plants expressing GFP that were inoculated with PVX-GFP had greatly decreased green fluorescence under UV light at 18 dpi. However, *N. benthamiana* 16c plants inoculated with PVX-V2/PVX-GFP showed leaf curling and severe stunting with green fluorescence under UV illumination (Fig. 5A). The levels of GFP mRNA and GFP-specific siRNA in infected plants were assayed by northern blotting. GFP mRNA was readily detected in mock-inoculated GFP-transgenic plants and in GFP-transgenic plants inoculated with either PVX vector or PVX-V2/PVX-GFP. GFP-transgenic plants infected with PVX-GFP had significantly reduced GFP mRNA levels (Fig. 5B, lane 2). In addition to GFP mRNA levels, we also assessed the levels of GFP-specific siRNA. To test whether the reduced levels of mRNA were indeed the result of PTGS, the relative levels of GFP-specific siRNA were assessed. The accumulation of GFP-specific siRNAs was higher in plants inoculated with PVX-GFP (Fig. 5B, lane 2). However, this accumulation was drastically reduced in GFP-transgenic plants inoculated with PVX-V2/PVX-GFP (Fig. 5B, lane 4). Taken together, these results

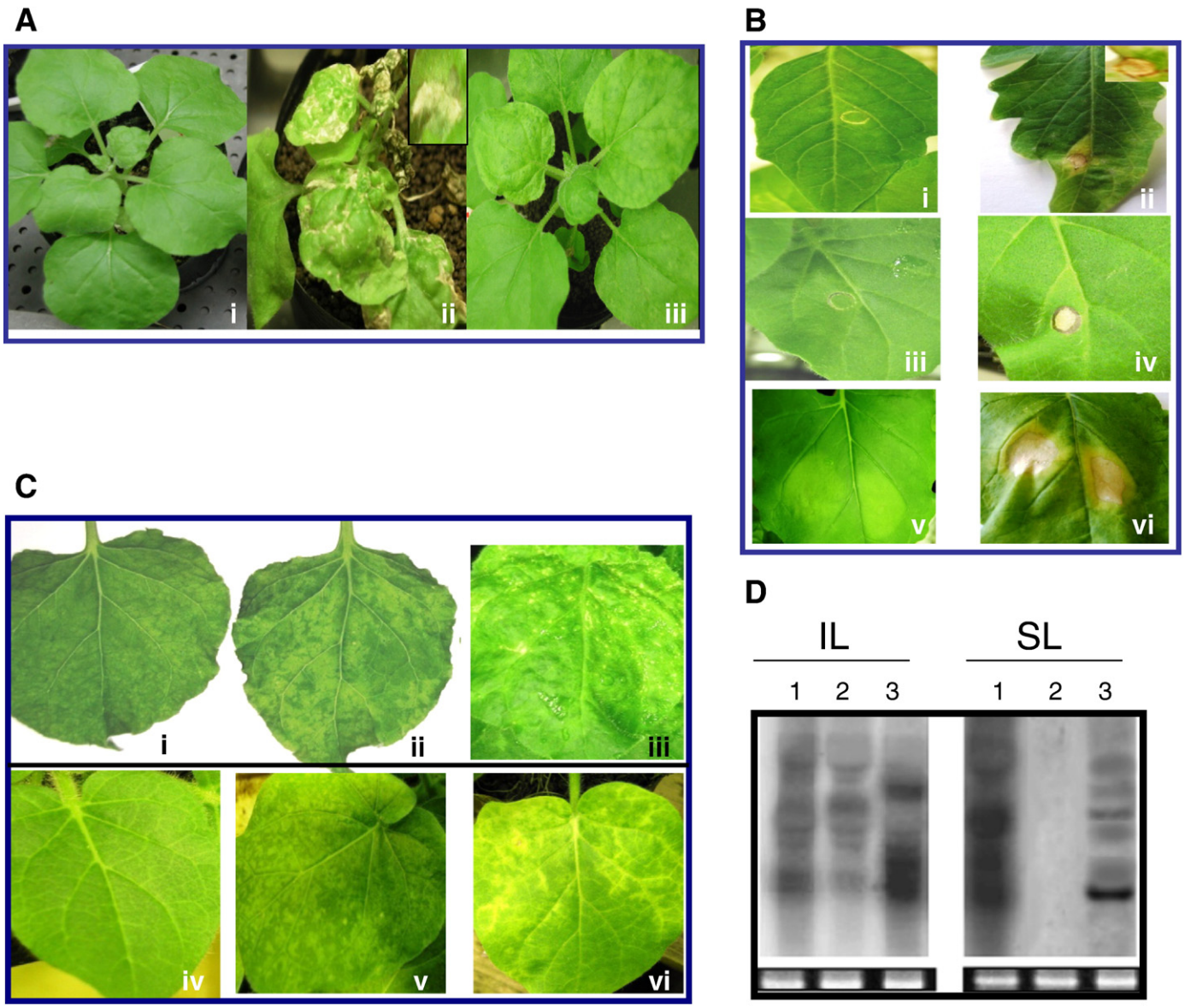

Fig. 3. Symptoms exhibited by plants following inoculation or co-infiltrated with PVX constructs and northern blot analysis. (A) Symptoms elicited on *N. benthamiana* plants infected with PVX-V2. Plants inoculated with mock virus (i), PVX-V2 (ii), and or its derivative PVX-mV2 was photographed 7 dpi. (B) Induction of hypersensitive response (HR). Leaves of tomato, *N. glutinosa* and *N. benthamiana* plants were co-infiltrated with *A. tumefaciens* C58C1 carrying pBIN35S (left lane, i, iii and v) and pBIN35S-V2 (right lane, ii, iv and vi). Photographs were taken at 7 dpi. (C) *N. benthamiana* (upper lane) and *N. glutinosa* leaves (bottom lane) infected with PVX derivatives. Plants were inoculated with PVX alone (panels i and iv), or with PVX-V2ΔC (panels ii and v) and PVX-V2ΔN (panels iii and vi). (D) Northern blot hybridization of total RNA extracted from inoculated and newly emerged leaves of plants inoculated with PVX-V2 (lane 1), PVX-V2ΔC (lane 2) and PVX-V2ΔN (lane 3). High levels of RNA transcripts were observed in inoculated (i) and newly emerging (ii) leaves infected with PVX-V2 and PVX-V2ΔN compared with the low RNA levels observed in PVX-V2ΔC, IL is inoculated leaves and SL is systemically infected leaves. Ethidium bromide staining of the ribosomal RNA is shown as an indication of amount of RNA loaded.

Table 1
Symptoms induced by V2 expressed from the PVX vector following inoculation.

Gene constructs	*N. benthamiana* (systemic necrosis)	*N. tabacum* (local necrosis)	*N. glutinosa* (systemic necrosis)	*S. esculentum* (systemic necrosis)
PVX	0/5	0/5	0/5	0/5
PVX-V2	5/5	3/5	4/5	5/5
PVX-mV2	0/3	0/3	0/3	0/3
PVX-V2ΔN	4/6	2/5	4/6	3/6
PVX-V2ΔC	0/4	0/4	0/4	0/4

Data shown are number of plants infected with the indicated virus/total number of inoculated plants.

indicate that the ToLCJV-A[ID] V2 protein is an efficient and strong suppressor of RNA silencing.

Further studies were carried out in order to determine whether truncated N- and C-termini are able to suppress PTGS. These V2ΔN or V2ΔC mutants and GFP were expressed separately from a PVX vector in an *N. benthamiana* line 16c (Fig. 5A). Consistent with the fluorescence results, northern blot analysis revealed that the levels of GFP mRNA accumulation were very low in GFP-transgenic plants infected with PVX-V2ΔN/PVX-GFP, similar to the level of GFP mRNA accumulation in GFP-transgenic plants infected with PVX-GFP (Fig. 5B). The GFP-specific siRNA was readily detected in GFP-transgenic plants infected with PVX-V2ΔC/PVX-GFP, although the level of GFP-specific siRNA accumulation was slightly lower than that of GFP-specific siRNA accumulation in GFP-transgenic plants infected with PVX-GFP. In contrast, GFP-transgenic plants inocula-

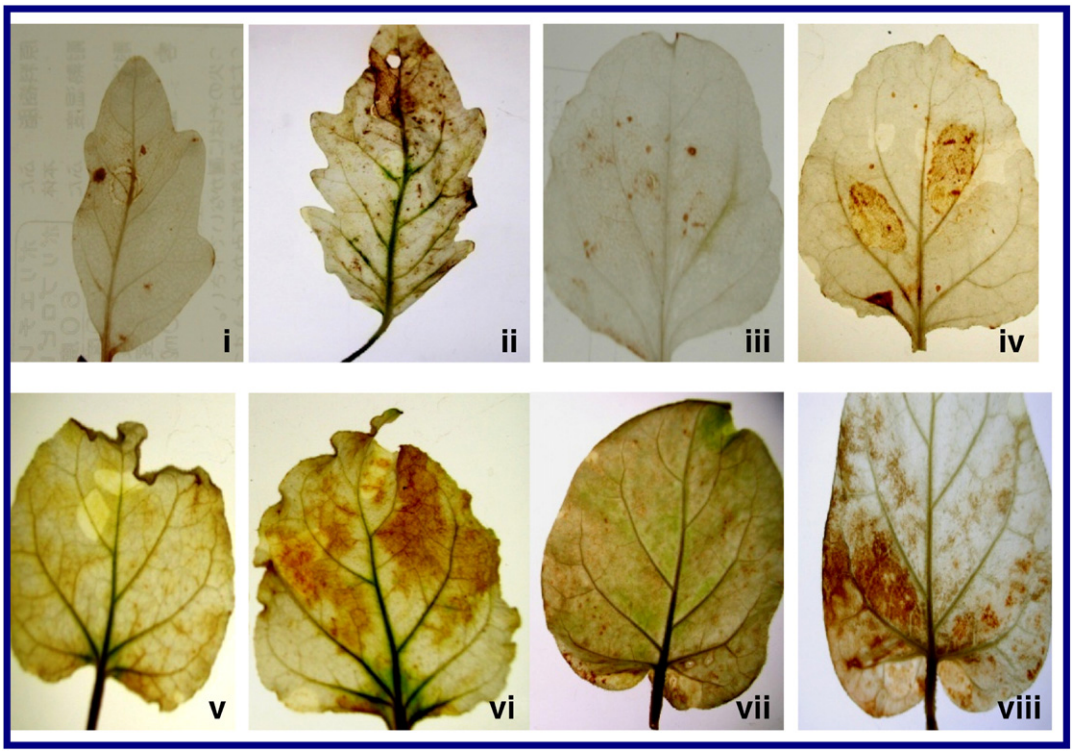

Fig. 4. Induction of H_2O_2 production in agroinfiltrated (top panels, i–iv) and systemically infected (bottom panels v–viii) tomato, *N. benthamiana*, and *N. glutinosa* leaves. Mock-inoculated (panels v and vii) and agroinfiltrated (panels i and iii) plants, plants agroinfiltrated with pBINV2 (panels ii and iv), and plants infected with PVX-V2 (panels vi and viii) were photographed at 7 dpi.

ted with the mutant PVX-V2ΔN/PVX-GFP exhibited downward leaf curling and maintained green fluorescence under long-wave UV light when photographed at 18 dpi (Fig. 5A). However, the upper leaves of the inoculated plants as indicated by decreased florescence under UV light. The steady-state levels of GFP mRNA and GFP-specific siRNA in infected plants were assayed by northern blotting (Fig. 5B), indicating that inoculation with PVX-V2ΔN correlates with a slight increase in GFP mRNA and reduction in siRNA accumulation compared to plants inoculated with PVX-GFP alone. These results confirm that the C-terminal domain is likely required for V2 to suppress RNA silencing, which is consistent with the results obtained with *Tomato yellow leaf curl virus*-Israel[Rehovot:1986] (TYLCV-IL[IL:Reo:86] V2 (Zrachya et al., 2007).

Discussion

The work described here represents first study of the phenotype induced by the V2 gene when expressed in the absence of other viral proteins, and it has provided new insight into the ToLCJV-A[ID]-host interaction. In comparison, a normal ToLCJV-A[ID] infection, in which all of the viral proteins are available, causes symptoms in *S. esculentum* and *N. benthamiana* that include downward leaf curling and mottling, and when co-inoculated with its associated betasatellite, produces severe crinkling, leaf curling and stunting (Kon et al., 2006, 2007). Here, using a PVX-based vector via an *Agrobacterium* infiltration method, we identified a novel activity of V2 in eliciting an HR-like cell death phenotype in tobacco and tomato, indicative of a role in the induction of a plant defense response. Plants have evolved a vast array of defense mechanisms to prevent or limit infection by viruses, fungi and bacteria. In some situations, the barrier appears to be formed prior to pathogen attack, whereas in others, it requires induction by the pathogen or the presence of pathogen-generated elicitors. One such inducible mechanism that is associated with specific pathogen recognition and resistance to infection by a range of plant pathogens is the HR (Dangl et al., 1996; Morel and Dangl, 1997).

Plants inoculated with PVX-mV2 showed milder chlorotic and mosaic symptoms, possibly as a result of the larger size of the replicating viral RNA. In contrast, V2 modulating phenotype is clearly distinct from the chlorotic lesions usually associated with PVX infection. V2 expression induced necrotic lesions on inoculated leaves, in which the affected tissues collapsed, became desiccated and turned brown, resembling an HR response. Cell death and a reduction in cell wall permeability presumably caused plant pigments to become trapped within the lesions, allowing the phenotype to be readily identified in decolorized tissues. Moreover, V2 expression caused a burst of H_2O_2 production, a feature typical of an HR but not normally associated with PVX infection of tobacco and tomato. Interestingly, the AV2-modulating phenotype in the PVX system does not occur with EACMCV-CM[CM:98] infection, which is likely to be due to a different level of V2 expression in different plants (Chowda-Reddy et al., 2008). The expression of proteins of RNA viruses and begomoviruses using the PVX-based system results in identification of pathogenicity determinants, as implied by the induction of a novel phenotype that is not always associated with native virus infection (Voinnet et al., 1999; van Wezel et al., 2002). Nevertheless, the induction of the V2-modulating HR-like response via *Agrobacterium* infiltration of tobacco and tomato plants negates such a phenotype being a general result of events occurring during PVX infection. The present results showed that V2 is a symptom determinant similar to corresponding proteins in other monopartite (Wartig et al., 1997; Rojas et al., 2001) and bipartite begomoviruses (Chowda-Reddy et al., 2008). The necrosis in transgenic *N. benthamiana* plants is systemic, as it starts from the base of newly emerged leaves and spreads through the entire leaf lamina and to other leaves (T. Hatanaka, T. Ogawa, T. Ito and M. Ikegami, personal communication). Thus, V2 of ToLCJV-A[ID] is an avirulence factor that interacts with the product of a resistance gene recruited by the host defense system, triggering a defense response involving an HR in tobacco species and tomato. This result is consistent with findings for other begomoviruses, for example, NSP of BDMV-[CO:87]

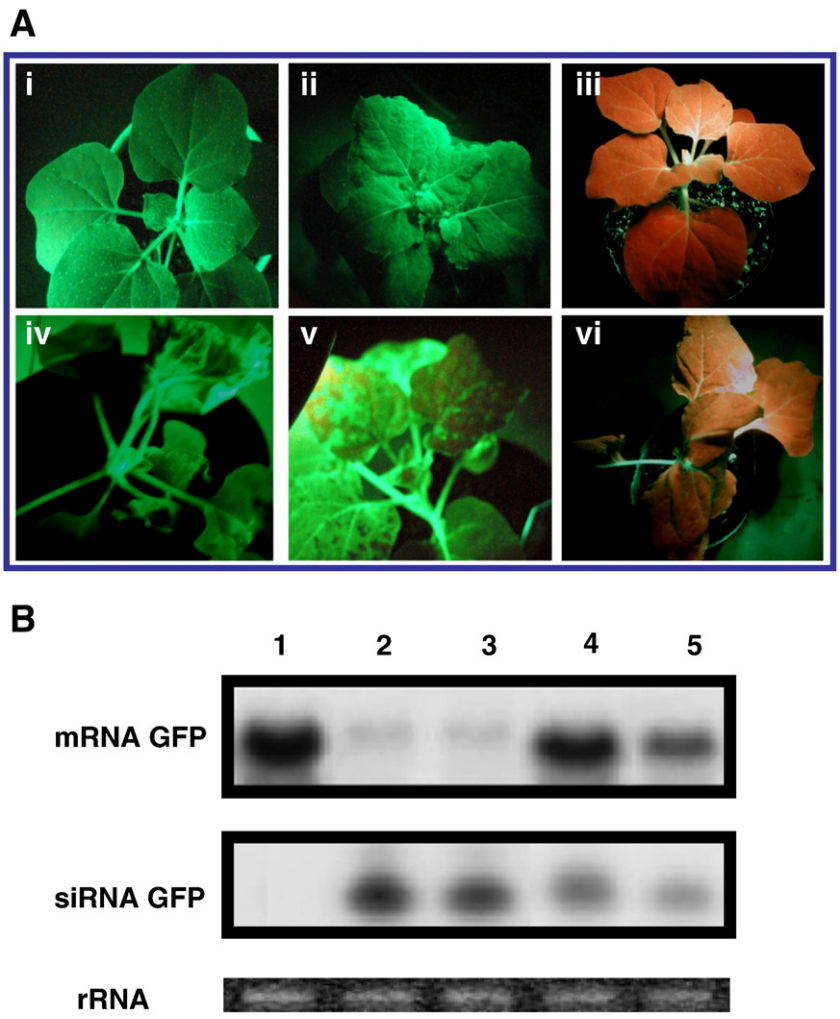

Fig. 5. Suppression of posttranscriptional gene silencing (PTGS) by V2 and its derivatives using a PVX assay. (A) Photographs taken under UV light of *N. benthamiana* 16c plants through a yellow filter at 18 days postinoculation. Plants were inoculated with (i) mock, (ii) PVX alone (PVX), (iii) PVX harboring GFP (PVX-GFP), or (iv) PVX in combination with PVX-V2 (PVX-GFP/V2), (v) PVX-V2ΔN (PVX-GFP/V2ΔN) or (vi) PVX-V2ΔC (PVX-GFP/V2ΔC). (B) Northern blot analysis of GFP mRNA and siRNA extracted from 16c plants expressing GFP and inoculated with PVX alone (1), PVX-GFP (2), PVX-V2ΔC (3), PVX-V2 (4), or PVX-V2ΔN (5). Ethidium bromide staining of rRNA was used as a loading control for mRNA.

(Garrido-Ramirez et al., 2000) and C2 of TYLCCNV-Bao1 [CN: Yn 10: Tob:00] (van Wezel et al., 2001), the C1 product of ACMV-[CM:98] (van Wezel et al., 2002) and NSP of ToLCNDV-IN[IN:ND:AVT1] (Hussain et al., 2005). Similarly, recently Zhou et al. (2007) demonstrated that the N-terminus of the begomovirus NSP determines a virulence or avirulence factor in *P. vulgaris*. Studies conducted by van Wezel et al. (2002) have shown that the individual expression of AC4 and C4 proteins did not cause an HR-like phenotype and systemic necrosis, they had no major influence on the Rep-mediated phenotype in inoculated leaves, and they did not induce burst of H_2O_2 production, indicating that neither protein acts as a determinant or enhancer of the phenotype. However, both proteins altered the systemic phenotype associated with Rep expression from cholorosis and sporadic mild necrosis, to severe necrosis resulting in plant death. In this study, our results demonstrate that expression of the V2 protein rather than the overlapping CP protein is responsible for the induction of the necrotic phenotype. Indeed, CP did not induce necrosis when expressed from this PVX construct. Recently, we have elucidated the role of CP in nucleocytoplasmic shuttling within cells of host.

To further investigate the ability of ToLCJV-A[ID] V2 to induce the HR, N- and C- terminal deletion constructs were expressed in the PVX vector. The N-terminal deletion attenuated but did not abolish the ability of V2 to elicit necrosis in tobacco and tomato. In contrast, C-terminal deletion failed to induce the HR, indicating that a domain within 58 aa of the C-terminus is likely important in V2 recognition and induction of the HR response.

PTGS is an antiviral defense mechanism of plants and is manifested by homology-dependent RNA degradation (Voinnet, 2001). To counter this defense, viruses have evolved proteins that are capable of suppressing PTGS (Voinnet et al., 1999). A number of such proteins have been studied (Roth et al., 2004; Sharma and Ikegami, 2008). Of these viral proteins, V2 is the second reported RNA silencing suppressor, after CMV 2b (Zhang et al., 2006) that is likely to target a protein component of the host RNA silencing machinery directly. Unlike 2b, which interacts with the AGO1 endonuclease component of the RISC (Zhang et al., 2006), V2 binds SGS3 (Glick et al., 2008) and blocks host cell RNA silencing by targeting a unique step of the RNA interference pathway (Fukunaga and Doudna, 2009). The discovery that dsRNA substrates containing 5′ overhangs have an important function in antiviral defense in plants suggests the potential for new approaches to combating viral infection. Seo et al. (2004) has demonstrated that the HR is not correlated with BDMV. The HR may be a secondary defense response that develops subsequent to the initial defense response. Plants employ a second form of defense against viruses that works on a gene-for-gene basis, the classical disease resistance model. In this case, a virus-encoded elicitor, the avirulence determinant, is the target of a host-encoded resistance

gene, which triggers an HR. PCD elicitor activity, the presence of determinants for the systemic spread of the virus, and suppression of PTGS are functions reported for the 2b protein of cucumoviruses (Brigneti et al., 1998; Li et al., 1999; Ryang et al., 2005; Soards et al., 2002). Our results show that V2 suppresses PTGS when both inducer (GFP) and suppressor (V2) are expressed from a PVX vector. This RNA silencing suppression ability of ToLCJV-A[ID] V2 is therefore in agreement with recent reports indicating that TYLCV-IL[IL:Reo:86] (V2) and EACMCV-CM[CM:98] (AV2) suppress RNA silencing (Zrachya et al., 2007; Chowda-Reddy et al., 2008; Glick et al., 2008). These results and our previous report (Kon et al., 2007) show that ToLCJV-A [ID], with its associated betasatellite, encodes at least three suppressors of gene silencing—V2, C2 and βC1—thus explaining the virulence associated with tomato leaf curl disease in Java, Indonesia (Kon et al., 2006). Our new data indicate that V2 has the potential to interact with both the gene silencing and gene-for-gene disease resistance mechanisms in plants and that its C-terminal region of 58 aa is probably essential for activation of virus resistance. Our deletion mutant results show that this C-terminal region is critical for the PTGS suppressor activity of V2, suggesting that the same or overlapping domains of V2 are essential in the interaction with ToLCJV-A[ID] virus resistance pathways. These observations suggest that V2 is a multifunctional protein. To our knowledge, no geminiviral elicitors have been reported to modulate the induction of the HR as well as PTGS.

Interestingly in this work, we observed that agroinfiltration of *N. benthamiana* with ToLCJV-A[ID] DNA A alone or with satellite β02 triggered a local necrotic response, while agroinfiltration with satellite β02 alone induced no such response (Fig. 6). The lack of HR suggests that betasatellite may encode protein to overcome the HR elicited by V2, and addressing this possibility will form the basis of further studies.

Materials and methods

Clone reconstruction

The constructs used in this study (Fig. 2) were derived from a recombinant plasmid that contains a full-length infectious clone of ToLCJV-A[ID] (pBToX1.4) (Kon et al., 2006). PCR was carried out as described by Sharma et al. (2005). The primer sequences used for amplification of V2 protein and deletion mutants of V2 protein are given in Supplementary Table S1. For deletion analysis of V2 protein, start and stop codons were added to the N-terminal and C-terminal deletion mutants, respectively. A nonsense mutation was created in V2 to make mutated V2. To assess the pathogenicity of V2 in *Nicotiana* species and tomato, the V2 ORF was expressed from the PVX vector at *Cla*I-*Sal*I sites (Baulcombe et al., 1996) and under the control of the *Cauliflower mosaic virus* 35S promoter in vector pBIN61, which has *Xba*I-*Sac*I sites. Additionally, the GFP gene (738 nt) was amplified from total DNA of *N. benthamiana* line 16c and cloned into the PVX vector to obtain PVX-GFP. The GFP constructs were mechanically inoculated into *N. benthamiana* 16c as described by Kon et al. (2007). All PCR products were amplified using high-fidelity PrimeSTAR DNA polymerase (Takara, Japan); the products were verified by sequencing using ABI Prism BigDye (Perkin Elmer, Wellesley) to make sure that no sequence errors had been introduced. To express ORF V2, it was PCR-amplified from pBToX1.4 using primers XbaV2F and SacV2R (Supplementary Table S1). The PCR product was first cloned into pUC118 to obtain pUCV2. The V2 ORF was released using *Xba*I-*Sac*I digestion and was cloned into the binary vector pBIN61 (kindly provided by Dr. Vicki Vance, France).

Agroinfiltration of tissues

The expression constructs were electroporated into *Agrobacterium tumefaciens* strain C58C1. *Agrobacterium* with the respective transformation plasmids was cultured on solid YEP medium containing 50 mg/l kanamycin and 25 mg/l streptomycin for 2–3 days at 28.5 °C. A single colony of the *Agrobacterium* strain was inoculated and cultured in YEP broth medium supplemented with 50 mg/l kanamycin and 25 mg/l streptomycin. The bacteria were cultured at 28.5 °C for 24 h with continuous shaking at 135 rpm. Then the bacterial cultures were diluted 1:100 in fresh LB medium, grown to an optical density at 600 nm of 0.5, resuspended in infiltration solution containing 100 mM MES, pH 5.6, and 150 μM acetosyringone. Bacterial cultures were mixed well and incubated for 2 h at room temperature before co-infiltrating them with mixed bacterial cultures onto the underside of leaves using a 5 ml syringe. The inoculated plants were maintained in a growth chamber at 25 °C with continuous lighting to give a 16-h photoperiod.

H_2O_2 detection in planta

H_2O_2 was detected visually in leaves using the 3,3′-diaminobenzidine (DAB)–HCl (Sigma, MO) uptake method (Orozoco-Cardenas and Ryan, 1999; Thordal-Christensen et al., 1997). Briefly, leaves were excised at the base of the stems with a 1 mg/ml solution of DAB in Tris buffer, pH 3.8. After 8-h incubation in the light at 25 °C, the leaves were immersed in 96% ethanol and boiled for 5–10 min. This treatment decolorized the leaves except for the deep brown polymerization product produced by the reaction of DAB with H_2O_2. After cooling, the leaves were preserved at room temperature in 70% ethanol and photographed. The samples were also examined by light microscopy as described by Thordal-Christensen et al. (1997).

GFP images

Seedlings of transgenic *N. benthamiana* plant line 16c expressing GFP (Brigneti et al., 1998) were mechanically co-inoculated with

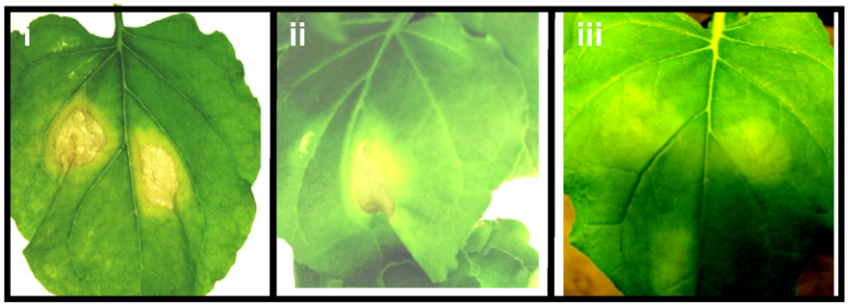

Fig. 6. ToLCJV-A[ID] mediated induction of local necrotic response in *N. benthamiana*. Plants were infiltrated with *A. tumefaciens* C58C1 carrying pBToX1.4 for DNA A (i), pBToβ02 (ii), and both pBToX1.4β02 (iii). The clones were obtained as described by Kon et al. (2006). Necrosis was induced only in plants after agroinfiltration with ToLCJV-A[ID] DNA A alone or with DNAβ02 but not DNAβ02 alone. Leaves were photographed 18 days post-agroinfiltration.

PVX-GFP and PVX-V2 and its derivatives. GFP fluorescence was determined using a 100 W long-wave UV lamp (Black Ray Model B 100A, UV Products, Upland, CA). Plants were photographed with a digital camera (Camedai C-3030, Olympus, Japan) using a yellow filter (Wratten No. 8, Kodak, Japan). Photographic images were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

RNA analysis

Total RNA was isolated from virus-infected plants using TRIzoL reagent (Invitrogen Corporation, CA). For northern analysis of GFP, total RNA was separated on a 1.8% formaldehyde gel, transferred to a Hybond-N+ membrane (GE Healthcare Bio-Science), and hybridized and detected using a DIG RNA labeling kit (Roche Diagnostics). For siRNA blotting, low molecular weight RNA was enriched from total RNA by eliminating high molecular weight RNA using 5% polyethylene glycol/0.05 M NaCl, and was then separated on a 15% polyacrylamide/7 M urea gel and transferred to a Hybond-N+ membrane. Hybridization and detection of siRNA were performed as described by Kon et al. (2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.10.012.

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