Research Note

Gene C2 of the Monopartite Geminivirus *Tomato yellow leaf curl virus*-China Encodes a Pathogenicity Determinant That Is Localized in the Nucleus

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Expression of the *Tomato yellow leaf curl virus*-China (TYLCV-C) C2 protein and green fluorescent protein (GFP) fused to the C2 protein (C2-GFP) in *Nicotiana benthamiana* from a *Potato virus X* (PVX) vector induced necrotic ringspots on inoculated leaves as well as necrotic vein banding and severe necrosis on systemically infected leaves. The localization of GFP fluorescence in plant cells infected with PVX/C2-GFP and in insect cells transfected with *Baculovirus* expressing C2-GFP indicates that the TYLCV-C C2 protein is capable of shuttling GFP into plant and insect cell nuclei. Our data demonstrate that the TYLCV-C C2 protein may contribute to viral pathogenicity in planta and is nuclear localized.

Additional keywords: *Begomovirus*, *Geminiviridae*, putative transcriptional activator protein.

A viral disease of tomato in China, which is transmitted by the whitefly *Bemisia tabaci* was first reported in 1965 (Ke et al. 1965) and has since become a serious threat to tomato production. Several distinct whitefly-transmitted viruses belonging to the genus *Begomovirus* in the family *Geminiviridae*, referred to collectively as *Tomato yellow leaf curl virus*, cause devastating damage to cultivated tomato worldwide (Picó et al. 1996). On the basis of sequence comparison, the causative agent of the disease in China was identified as a distinct begomovirus species, *Tomato yellow leaf curl virus*-China (TYLCV-C) (Liu et al. 1998). TYLCV-C has a single genomic component that is organized similarly to other monopartite begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl virus*-Sardinia (TYLCV-S), *Tomato leaf curl virus* (ToLCV), and *Ageratum yellow vein virus* (Dry et al. 1993; Kheyr-Pour et al. 1991; Navot et al. 1991; Tan et al. 1995) (viruses are named according to Fauquet et al. 2000). The cloned TYLCV-C genomic DNA is infectious in tomato and *Nicotiana benthamiana* (P. Tien, un-

published data). Little information is available concerning the TYLCV-C gene function, however, other than from comparisons with work on related monopartite begomoviruses.

Most begomoviruses, typified by *African cassava mosaic virus* (ACMV) and *Tomato golden mosaic virus* (TGMV), have bipartite genomes (DNA A, resembling the monopartite virus component, and DNA B). TGMV and ACMV transcriptional activator proteins (TrAP, also known as AL2 and AC2) regulate viral coat protein and movement protein expression (Brough et al. 1992; Groning et al. 1994; Haley et al. 1992; Hong et al. 1996; Sunter and Bisaro 1991; Sunter and Bisaro 1992; Sunter and Bisaro 1997). The monopartite begomovirus homolog C2 protein shares high amino acid sequence identity with TrAP and possesses a basic domain within its N-terminal half, an acidic domain at the C terminus that has been implicated in transcriptional activation for TGMV, and a zinc finger motif between the two. Similar to TrAP, the TYLCV C2 protein exhibits sequence-nonspecific single-stranded and, to a lesser extent, double-stranded DNA binding activity, which is attributed to the zinc finger motif for TGMV (Hartitz et al. 1999; Noris et al. 1996; Sung and Coutts 1996). There is evidence that the ToLCV C2 protein is also involved in the regulation of coat protein expression (Dry et al. 2000). Genetic analysis of TYLCV-S suggests that C2 protein plays a role in virus systemic spread in planta, although the mechanism remains to be established (Wartig et al. 1997).

The expression of ACMV TrAP from a PVX vector produced a necrotic response in plants, and the protein may play a role in circumventing the host defense mechanism by suppressing gene silencing (Hong et al. 1997; Voinnet et al. 1999). It is not known, however, whether the C2 protein functions in a similar manner. To investigate this possibility, the TYLCV-C C2 protein was expressed in *N. benthamiana* plants with a PVX vector. The wild-type C2 protein coding sequence (402 nucleotides) and a mutant derivative were amplified by polymerase chain reaction (PCR) and cloned as *Clal*-*EagI* fragments into the vector p45P46 (unpublished data), which is a modified version of pP2C2S (Baulcombe et al. 1995), to produce PVX/C2 and PVX/mC2, respectively. The mutant

contained a stop codon (TGA) immediately downstream of the initiation codon. It should be noted that the wild-type and mutant have the potential to encode a truncated version of the C3 protein. Additionally, the green fluorescent protein (GFP) coding sequence (738 nucleotides) was isolated from TXS.GFP-CP (Santa Cruz et al. 1996) as an *EagI*-*BspEI* fragment and fused in-frame to the wild-type and mutated C2 protein coding sequences to produce PVX/C2-GFP and PVX/mC2-GFP, respectively. The GFP coding sequence also was introduced as an *EagI*-*BspEI* fragment into p45P46 to produce PVX/GFP (Fig. 1). Sequences were confirmed by automatic sequencing of the recombinant plasmids.

RNA transcripts were produced by in vitro transcription from the recombinant PVX clones after linearization with *SpeI*. *N. benthamiana* plants inoculated with PVX/mC2 tran-

scripts developed chlorotic lesions on the inoculated leaves 3 to 5 days postinoculation (dpi), and curling and mosaic symptoms on systemically infected leaves, which were indistinguishable from those seen in plants infected with the parental PVX vector, at 6 to 7 dpi. Plants inoculated with PVX/mC2-GFP and PVX/GFP showed slightly milder chlorotic and mosaic symptoms, possibly as a result of the larger size of the replicating viral RNA. In contrast, plants inoculated with PVX/C2 produced necrotic ringspots on the inoculated leaves at 3 to 4 dpi as well as necrotic vein banding on systemically infected leaves (Fig. 2). Interestingly, plants inoculated with PVX/C2-GFP expressing the C2-GFP fusion protein developed similar local and systemic necrosis. The results demonstrate that expression of the C2 protein rather than the overlapping truncated C3 protein is responsible for the induction of the necrotic phenotype and that the C-terminal GFP fusion has little effect on this phenotype. To confirm this, the coding sequence (258 nucleotides) for the truncated C3 was amplified by PCR with a primer (5'-GCTGTCACATCGATGATTCACGCACAG-3'; the C3 start codon is in bold and the *Clal* site is underlined) and the 3' end of the primer used for PCR amplification of the C2 gene, and cloned as a *Clal*-*EagI* fragment into p45P46. Indeed, the truncated C3 protein did not induce

Fig. 1. *Potato virus X*-based expression cassettes. Expression of the inserted gene was under the control of one copy of the duplicated coat protein subgenomic RNA promoter. Stop codons (*) introduced in *mC2* and *mC2*-GFP are indicated.

Fig. 2. Phenotypes produced in *Nicotiana benthamiana* infected with *Potato virus X* (PVX)/mC2 and PVX/C2. **A**, Chlorotic lesion on the inoculated leaf and **B**, mosaic symptoms on a systemically infected leaf of a plant inoculated with PVX/mC2. **C**, Necrotic ringspots on the inoculated leaf and **D**, necrotic vein banding on a systemically infected leaf of a plant inoculated with PVX/C2. Plants were photographed 7 days postinoculation.

Fig. 3. Northern blot analysis of RNAs extracted 7 days postinoculation from mock-inoculated and virus-infected plants. Blots were hybridized with DIG-labeled probes specific to **A**, *Potato virus X*, **B**, AC2, **C**, and green fluorescent protein sequences. Genomic RNA positions are indicated.

necrosis when expressed from this PVX construct.

The presence of PVX RNAs in infected plants was investigated by Northern blot analysis. RNA was extracted from leaf tissues with the RNeasy plant mini kit (Qiagen, Hilden, Germany). RNA aliquots (2.5 µg) were fractionated on 1% formaldehyde agarose gel, transferred to a nylon membrane, hybridized with DIG-labeled probes specific for PVX, C2, or GFP sequences, and immunodetected with a DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany). The accumulation of genomic RNAs containing the appropriate C2 and GFP sequences was observed in systemically infected tissues (Fig. 3). To investigate gene expression from the PVX vectors, proteins were extracted from systemically infected tissues and fractionated on 12.5% polyacrylamide gel lacking sodium dodecyl sulfate. GFP was detected by UV illumination as described (Baulcombe et al. 1995). No fluorescence was observed in extracts from either mock-inoculated plants or plants infected with PVX, PVX/mC2-GFP, PVX/C2, or PVX/mC2 (Fig. 4). A fluorescent protein corresponding to GFP (approximately 27 kDa) was observed in extracts from plants infected with PVX/GFP, and a slower migrating fluorescent protein, consistent with the size of the C2-GFP fusion protein (approximately 40 kDa), was observed in extracts from plants infected with PVX/C2-GFP. The results indicate that functionally active GFP is expressed as a C-terminal fusion to the C2 protein, providing a convenient probe with which to investigate the cellular compartmentalization of the viral protein.

Systemically infected tissues from plants challenged with the various PVX-based constructs were examined by fluorescence microscopy (Fig. 5). Fluorescence was present throughout the cytoplasm of trichome and epidermal cells in plants infected with PVX/GFP (Fig. 5A, B, E, and F), but was localized predominantly in the nuclei of cells infected with PVX/C2-GFP (Fig. 5C, D, G, and H). This suggests that the C2 protein moiety relocates GFP to the nucleus. To substantiate this observation, C2-GFP was expressed transiently in insect Sf9 cells with a Bac-to-Bac Baculovirus expression system (Invitrogen Life Technologies, Groningen, The Netherlands). Once again, GFP fluorescence occurred throughout the

cytoplasm, but C2-GFP fluorescence was confined primarily to the nucleus (Fig. 6), which is consistent with nuclear localization of the C2 protein. A nuclear localization signal has not yet been determined, but the basic domain between amino acids 17 and 31, **KVQHRIAKKTT**RRRR (the majority of the basic amino acids indicated in bold are highly conserved in begomoviruses), may contribute to this function.

Our data indicate that expression of the TYLCV-C C2 protein induces a necrotic response in plants, suggesting that it may function in gene silencing suppression, as demonstrated for the TrAP of the bipartite begomovirus ACMV (Voinnet et al. 1999). It is noteworthy that TYLCV-C infection of *N. benthamiana* is not reported to be associated with tissue necrosis. The fact that the C2-GFP fusion protein also induces a necrotic response indicates that the C2 protein moiety remains functionally active in this context and suggests that the observed nuclear localization of the fusion protein reflects the C2 protein function. Nuclear localization would be expected if the C2 protein is to fulfill its proposed function in transcriptional transactivation.

Bipartite begomovirus TrAP is nuclear localized (Sanderfoot and Lazarowitz 1995). Although ACMV AC2 protein expression from a PVX vector also induced a necrotic response in infected *N. benthamiana* (Hong et al. 1997), the two phenotypes are remarkably distinct. ACMV AC2 protein produced necrotic lesions on inoculated leaves and veinal necrosis on systemically infected leaves, whereas TYLCV-C C2 protein produced necrotic ringspots on inoculated leaves and necrotic vein banding on systemically infected leaves. Be-

Fig. 4. Detection of green fluorescent protein fluorescence in plant extracts. Proteins were extracted 7 days postinoculation from a mock-inoculated plant or plants infected with the *Potato virus X*-based constructs indicated. Aliquots (100 µg) were fractionated by polyacrylamide gel electrophoresis and observed under UV illumination. Positions of size markers (kDa) are indicated.

Fig. 5. Fluorescence microscopy of green fluorescent protein (GFP) localization in plant cells of systemically infected leaves at 7 days postinoculation. Trichome cells infected with **A and B**, *Potato virus X* (PVX)/GFP and **C and D**, PVX/C2-GFP. Epidermal cells infected with **E and F**, PVX/GFP and **G and H**, PVX/C2-GFP. Samples were analyzed for **A, C, E, and G**, GFP fluorescence or by **B, D, F, and H**, 4',6'-diamidino-2-phenylindole staining. Bar = 5 µm.

Fig. 6. Green fluorescent protein (GFP) localization in insect Sf9 cells by fluorescence microscopy. Cells were transfected with **A**, empty Baculovirus vector or vectors expressing **B**, GFP and **C**, C2-GFP. Bar = 10 µm.

cause there is much variation in the extent of suppression of gene silencing by different viruses (Vionnet et al. 1999), this result might indicate that the ACMV AC2 protein triggers a more aggressive effect on plant defense mechanism, i.e., gene silencing than that triggered by the TYLCV-C C2 protein. Alternatively, the different plant responses might reflect functional differences between these gene homologs from monopartite and bipartite begomoviruses. Experiments to determine whether the TYLCV-C C2 protein also is able to suppress gene silencing are currently in progress. The analysis of C2 and AC2 mutants and recombinant proteins may identify the pathogenicity domains responsible for the induction of their distinct phenotypes.

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