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Transactivation of Dianthin Transgene Expression by African Cassava Mosaic Virus AC2

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We have recently described a novel strategy for engineering resistance to African cassava mosaic virus (ACMV) in transgenic *Nicotiana benthamiana* plants using a virus-inducible promoter to control the expression of a plant ribosome-inactivating protein (RIP) transgene (Y. Hong *et al., Virology* 220, 119–127, 1996). Here, we have used a potato virus X (PVX) vector to express the ACMV transactivator protein, AC2, *in planta.* We confirm that amplification of RIP activity in transgenic plants is mediated by AC2; disruption of AC2 expression by either the introduction of an in-frame stop codon or the deletion of 5′-terminal or 3′-terminal coding sequences reduced RIP expression to the basal level associated with PVX-infected plants. AC2 expression from the PVX vector induced necrosis in nontransformed plants as well as in plants containing the RIP transgene, suggesting that the protein can functionally interact with PVX and/or host factors. The potential of this system to provide a direct and sensitive assay to investigate AC2 function *in planta* is discussed.

Ribosome-inactivating proteins (RIPs) are cytotoxins that occur in a wide variety of higher plants (*1, 2*). They disrupt protein synthesis by depurinating eukaryotic large ribosomal subunit rRNA at a specific adenosine residue near the 3′ terminus. The exact role of RIPs remains unclear, but it has been suggested that their release into the cytosol during pathogen attack causes local cell suicide that provides a natural defense mechanism. Consistent with this view, the induction of RIP activity has recently been observed in virus-infected sugar beet plants (*3*). Although RIPs exhibit antiviral activity against a wide range of plant and animal viruses when applied exogenously (*4*), their extreme cytotoxicity could limit their use as constitutively expressed antiviral agents *in vivo* (*5, 6*). To circumvent this problem, we have recently developed a strategy for engineering virus resistance in transgenic plants using a virus-inducible promoter from African cassava mosaic virus (ACMV) to regulate expression of dianthin, a potent RIP isolated from carnations (*Dianthus caryophyllus*) (*7*). This was based on the observation that ACMV coat protein expression is transactivated by the product of gene *AC2* (*7, 8*). We demonstrated that *Nicotiana benthamiana* plants transformed with the dianthin coding sequence under the control of the ACMV coat protein promoter were less susceptible to ACMV infection. Plants accumulated less viral DNA, systemic symptoms were extremely mild, and plants recovered from infection. Transgenic plants produced atypical necrotic lesions on inoculated leaves that was attributed to virus-induced dianthin activity. However, because ACMV *AC2* mutants are unable to systemically infect plants (*9*), we were unable to confirm the specific role of AC2 protein in transactivation of dianthin activity *in planta.* Here, we have investigated the contribution of the *AC2* coding sequence when removed from the context of the ACMV genome and expressed from a potato virus X (PVX)-based vector.

To determine whether the *AC2* gene product can directly induce dianthin expression when supplied *in trans,* we have introduced the wild-type *AC2* coding sequence and mutant derivatives as *Cla*I–*Eco*RV fragments into the PVX vector pP2C2S (previously pPC2S (*10*)) to produce PVX-AC2, PVX-mAC2, PVX-bdAC2, and PVX-adAC2 (Fig. 1). The vector permits a high level of foreign gene expression from one copy of the duplicated coat protein promoter. Coding sequences of wild-type *AC2* (in PVX-AC2) and an *AC2* mutant containing a stop codon after 39 amino acids (in PVX-mAC2) were PCR-amplified from pCLVAC3-1 and pCLVAC2-2, respectively (*9*), using the primers ATTACTTGaTATCTAAAGACCC (nucleotides 1352–1373; introduced *Eco*RV site underlined) and GGCATTAAtcgATGCAATCTTC (complementary to nucleotides 1761–1782 and including the *AC2* initiation codon; introduced *Cla*I site underlined). Deletion of the C-terminal 28 amino acids (in PVX-bdAC2) and the N-terminal 66 amino acids (in PVX-adAC2) of the AC2 protein was accomplished by PCR amplification of fragments from pCLVAC3-1 (*9*) using the above complementary-sense

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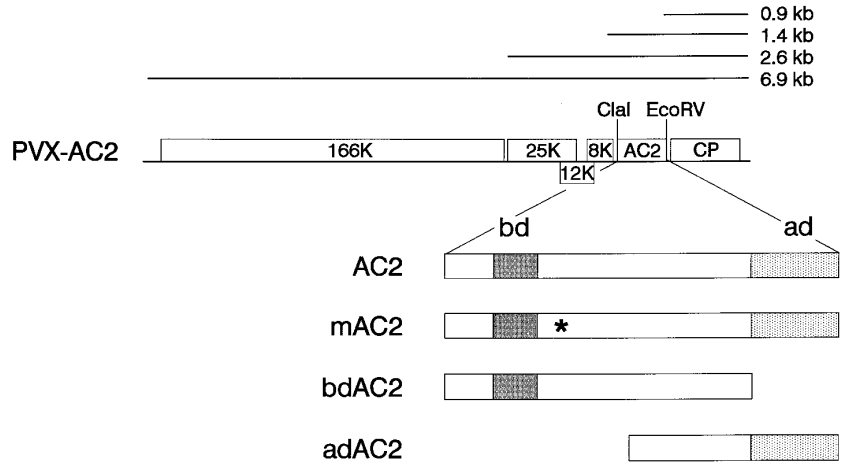

FIG. 1. PVX-based expression cassettes used for the induction of transgene expression. The *AC2* coding region was cloned downstream of one copy of the duplicated coat protein subgenomic RNA promoter in PVX-AC2. The coordinates of viral genomic (6.9 kb) and subgenomic mRNAs (2.6, 1.4, and 0.9 kb) are indicated above the PVX-AC2 map. The positions of basic and acidic domains (bd and ad, respectively) in AC2, an introduced stop codon (*) in the *AC2* mutant mAC2, and the deleted sequences in bdAC2 and adAC2 are indicated.

primer and the primer GAATCCCCAGCgataTCtTaAGGTTG (nucleotides 1437–1462; introduced *Eco*RV site underlined) or the above virion-sense primer and the primer GCAATGAATcGatgGTATACCTGGG (complementary to nucleotides 1560–1584 and including an in-frame initiation codon; introduced *Cla*I site underlined), respectively. The integrity of all PCR-amplified fragments was confirmed by sequence analysis using a T7 sequencing kit (Pharmacia).

Transgenic *N. benthamiana* plants from line 1030 (AV1-Pro-DIA), containing the dianthin coding sequence under the control of the ACMV DNA A virion-sense promoter, and the control line 1032 (AV1Pro), lacking the dianthin coding sequence (*7*), were mechanically inoculated with RNA transcripts generated *in vitro* from each PVX-based construct as described (*11*). AV1Pro-DIA plants inoculated with recombinant PVX-mAC2 transcripts containing a disrupted *AC2* coding sequence developed chlorotic lesions on the inoculated leaves 3–5 days postinoculation, and curling and yellow mottle symptoms on systemically infected leaves 1–2 days later (Fig. 2, right), that were indistinguishable from those associated with the PVX vector alone. In contrast, AV1Pro-DIA plants inoculated with PVX-AC2 transcripts containing the intact *AC2* coding sequence developed necrotic lesions on the inoculated leaves about 1 day after the appearance of chlorotic lesions, and extensive veinal necrosis appeared soon after on systemically infected leaves (Fig. 2, left). PVX transcripts with the potential to express truncated versions of AC2 protein, in which either the N-terminal basic domain (PVX-adAC2) or the C-terminal acidic domain (PVX-bdAC2) had been removed, were unable to induce the necrotic response in AV1Pro-DIA plants either when inoculated separately or co-inoculated. As the *AC2* coding sequence overlaps the 5′-terminal sequence of *AC3* within the ACMV genome, it is possible that a truncated version of AC3 protein is expressed from PVX-AC2. However, plants infected with PVX-mAC2, containing a single point mutation that disrupts the *AC2* coding sequence without affecting *AC3,* did not develop necrosis, suggesting that a truncated AC3 protein does not make a significant contribution to the phenotype. Surprisingly, nontransformed *N. benthamiana* as well as plants from the control line AV1Pro developed necrotic symptoms when inoculated with PVX-AC2 transcripts that were similar to those induced in AV1Pro-DIA plants. However, all plants developed typical PVX symptoms when inoculated with PVX-mAC2. The results demonstrate that AC2 expression is responsible for inducing necrosis and that the presence of the dianthin transgene is not a prerequisite for this phenotype.

To ensure that *AC2* expression was occurring from the PVX vectors, PVX-specific RNA was analyzed by Northern blotting (Fig. 3). Total RNAs were isolated using a guanidium hydrochloride method (*12*), and 5-μg aliquots were fractionated on an agarose gel containing 2.2 *M* formaldehyde (*13*), transferred to Hybond-N (Amersham International), and hybridized with oligolabeled probes (*14*) specific for either PVX or *AC2* sequences (Fig. 3). Plants infected with PVX-AC2 produced virus-specific transcripts corresponding to genomic RNA (6.9 kb) and the three anticipated subgenomic RNAs (2.6, 1.4, and 0.9 kb) (Figs. 1 and 2A). Transcripts of a similar size (allowing for differences resulting from deletions in the *AC2* coding sequence) were associated with PVX-mAC2, PVX-bdAC2, and PVX-adAC2 infections. The PVX-AC2 2.6- and 1.4-kb RNAs were noticeably larger than the equivalent RNAs associated with PVX vector infection (2.2 and 1.0 kb). The presence of a 1.4-kb RNA from which AC2 protein is expressed in PVX-AC2 was verified by probing for *AC2*-specific sequences (Fig. 3B).

We did not previously attempt to demonstrate dianthin

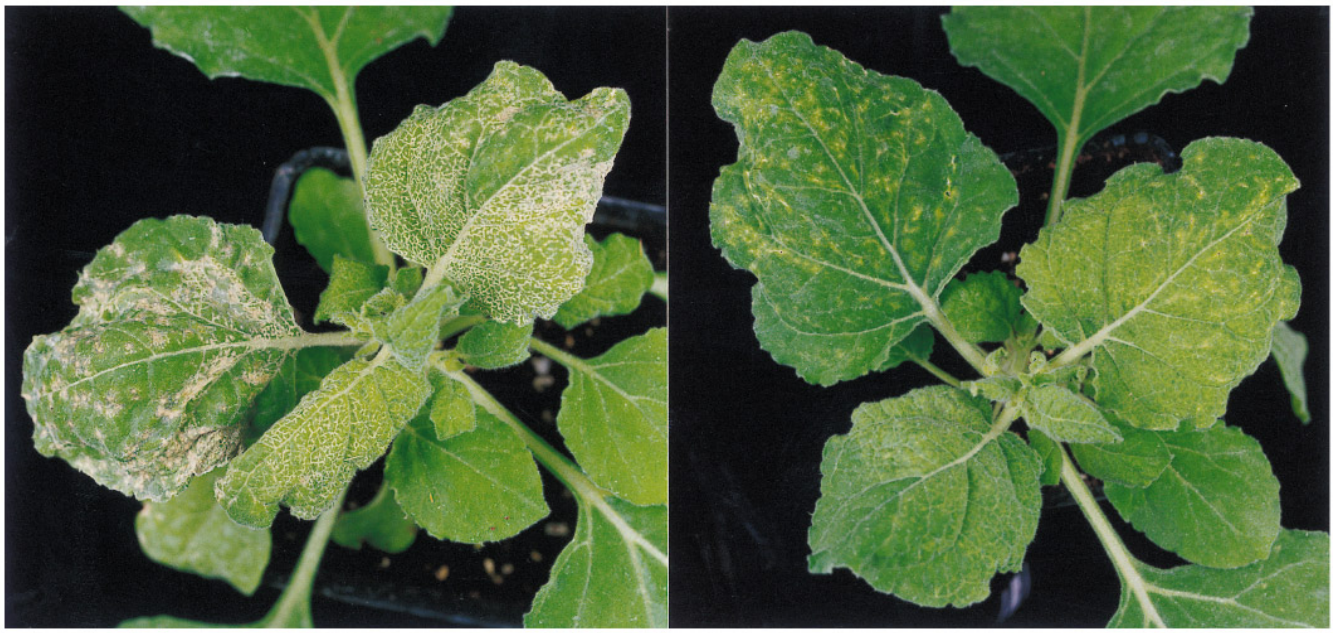

FIG. 2. Systemic symptoms produced in AV1Pro-DIA transgenic *N. benthamiana* infected with PVX-AC2 (left) and PVX-mAC2 (right). Plants were photographed 7 days postinoculation.

activity associated with ACMV infection of AV1Pro-DIA plants due to the highly sporadic nature of systemic infection (*7*). However, the ability of PVX-AC2 to produce a severe infection in these plants provided the opportunity to confirm that expression of functionally active dianthin was being transactivated by AC2 protein in AV1Pro-DIA plants. Catalytic *N*-glycosidase activity that results in depurination of a specific adenosine residue near the 3′ terminus of the rRNA was monitored by cleavage of the 3′-terminal fragment following treatment with aniline. *N. benthamiana* RNA was extracted as described above, 12-μg aliquots were treated with 1 *M* aniline (ACS reagent, Aldrich), and the products were resolved on an agarose gel containing 50% formamide (*15*). RNAs were detected using a probe for the 3′ terminus of tobacco 25S rRNA (*16*). A fragment diagnostic of *N*-glycosidase activity, approximately 400 ribonucleotides in length, was associated with extracts from AV1Pro-DIA plants infected with PVX-AC2 (Fig. 4). Such a fragment was absent in extracts from mock-inoculated plants, implying that if a basal level of constitutive dianthin expression occurs from the ACMV virion-sense promoter in AV1Pro-DIA plants it remains below the level of detection. The diagnostic fragment also occurred in extracts from AV1Pro-DIA plants infected with PVX, although the amount of fragment was approximately 15 times lower than that associated with PVX-AC2 infection, as estimated by absorbance scanning of autoradiographs using a Chromoscan 3 (Joyce Loebl). This low level of RIP activity is insufficient to induce necrosis in PVX-infected plants. As the fragment did not occur in extracts from PVX-infected AV1-Pro control plants (Fig. 4), RIP activity does not result from virus-induced expression of an endogenous host gene, as has recently been observed in infected sugar beet (*3*). It remains to be established if induction of transgene expression is due specifically to the action of

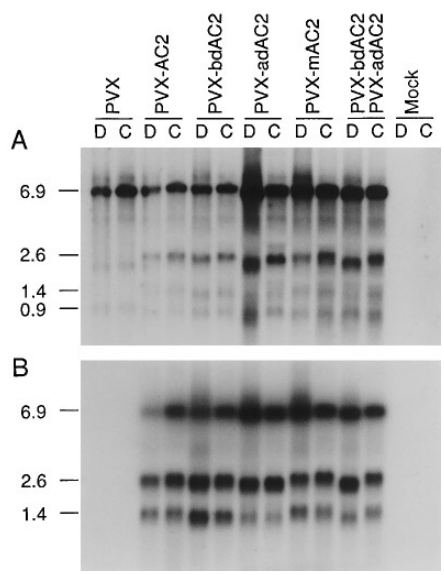

FIG. 3. Northern blot analysis of PVX mRNA extracted from transgenic *N. benthamiana* plants transformed with AV1Pro (control, C) and AV1-Pro-DIA (D). Leaves were harvested from mock-inoculated plants or from infected plants 7 days after inoculation with RNA transcripts produced *in vitro* from the PVX expression vector pP2C2S (PVX) and its *AC2*-containing derivatives (PVX-bdAC2, PVX-adAC2, and PVX-mAC2). Equal aliquots (5 μg) of RNA were loaded in each lane of an agarose gel containing 2.2 *M* formaldehyde. Blots were probed for sequences specific to PVX (A) and *AC2* (B). The positions and sizes (kb) of PVX-AC2 transcripts are indicated.

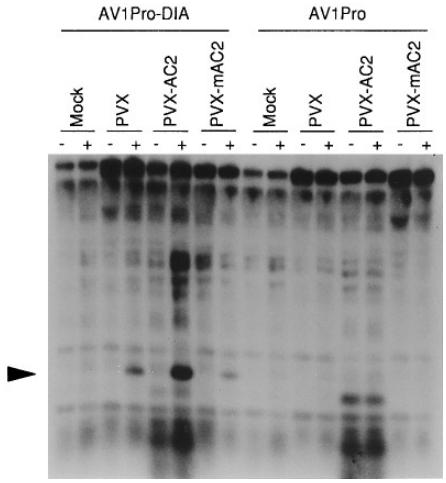

FIG. 4. Northern blot analysis of dianthin activity in transgenic *N. benthamiana* plants transformed with AV1Pro-DIA and AV1Pro. Leaves were harvested from mock-inoculated plants or from infected plants 7 days after inoculation with RNA transcripts produced *in vitro* from the PVX expression vector pP2C2S (PVX) and its *AC2*-containing derivatives (PVX-AC2 and PVX-mAC2). Equal aliquots (12 μg) of RNA were either untreated (−) or treated with aniline (+) before loading onto an agarose gel containing 50% formamide. The blot was probed using a fragment corresponding to the 3′ terminus of tobacco 25S rRNA. The fragment diagnostic of *N*-glycosidase activity, approximately 400 ribonucleotides in length, is indicated by an arrow.

a particular PVX gene or more indirectly, as a consequence of general disruption to the cell during virus infection. Truncation of the *AC2* coding sequence in PVX-mAC2 (Fig. 4), PVX-bdAC2, and PVX-adAC2 (data not shown) reduced RIP activity to the level associated with PVX infection, confirming the role of AC2 in transactivation of dianthin transgene expression. RIP activity could not be detected in AV1Pro and nontransformed plants infected with PVX-AC2 that exhibited necrotic symptoms.

AC2-induced dianthin activity in AV1Pro-DIA plants did not prevent systemic infection by PVX, and virus accumulation was largely unaffected in these plants immediately after the onset of infection (Fig. 3). Expression of AC2 and PVX coat protein should occur simultaneously from subgenomic RNAs transcribed from essentially identical promoters in the PVX-AC2 vector, and coat protein is required for PVX cell-to-cell movement (*10*). However, AC2 must first relocate from the cytoplasm, the site of PVX replication, to the nucleus in order to transactivate dianthin expression. Clearly, by the time dianthin expression is induced, PVX has had the opportunity to spread to adjacent cells. In the context of the ACMV genome, *AC2* is considered to be an "early" gene controlling the expression of gene products required for virion assembly and spread of the infection, functions that occur later in the infection cycle. Hence, expression of AC2 protein from the ACMV genome is sufficiently early for induced dianthin expression to have a significant effect on virus proliferation (*7*).

The approach that we have developed using a stably integrated inducible promoter not only has potential for engineering virus resistance in plants (*7*) but, in combination with a PVX gene delivery system, may provide a method for studying ACMV *AC2* function *in planta.* Previous studies have shown that the AC2 protein transcriptionally transactivates expression of the coat protein and DNA B genes (*7, 8, 17, 18*). Our results demonstrate that *cis*-acting elements in ACMV involved in transactivation are located within the virus-specific region of the AV1Pro-DIA transgene that includes the common region and sequences downstream as far as the start of the coat protein coding sequence (*7*). Alignment of AC2 proteins from different geminiviruses has indicated conserved basic and acidic domains as well as a putative zinc-binding motif, which are general properties of transcription factors. Although no function has yet been assigned to these domains, our data demonstrate that truncated AC2 proteins lacking either the basic or the acidic domain are unable to transactivate transgene expression and induce necrosis. Numerous studies on transcription factors from yeast, animals, plants, and viruses have shown that basic domains and zinc-binding motifs bind DNA whereas acidic domains activate gene transcription (*19, 20*). Recently, nonspecific DNA-binding activity of potato yellow mosaic virus AC2 protein and tomato yellow leaf curl virus C2 protein (the AC2 homologue) was demonstrated *in vitro* (*21, 22*). In preliminary experiments, we have been unable to restore AC2 function by co-inoculating AV1Pro-DIA plants with PVX-bdAC2 and PVX-adAC2. However, expression of mutants bearing more subtle modifications from the PVX vector should facilitate the investigation of AC2 functional elements *in planta.*

The ability of PVX-AC2 to induce necrosis in all plant lines tested, not necessarily those carrying the dianthin transgene, indicates that AC2 protein has a significant influence on cellular metabolism by interacting with PVX and/or host factors. Hence, during the course of ACMV infection, it is possible that *AC2* functionally regulates host gene expression in addition to its role in the transcriptional regulation of viral genes. If this is the case, the rapid development of necrosis associated with expression of AC2 protein from the PVX vector may explain our inability to regenerate transgenic plants that constitutively express this gene product (unpublished data). However, the ability to express potentially high levels of protein from the PVX vector could provide an alternative and convenient means to investigate the proposed influence of *AC2* on host gene expression.

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REFERENCES

1. Stirpe, F., Barbieri, L., Battelli, M. G., Soria, M., and Lappi, D. A., *Biotechnology* 10, 405–412 (1992).
2. Hartley, M. R., Chaddock, J. A., and Bonness, M. S., *Trends Plant Sci.* 1, 254–260 (1996).
3. Girbes, T., de Torre, C., Iglesias, R., and Ferreras, J. M., *Nature* 379, 777–778 (1996).
4. Irvin, J. D., *In* "Antiviral Proteins in Higher Plants" (M. Chessin, D. DeBorde, and A. Zipf, Eds.), pp. 65–94. CRC Press, Boca Raton, FL, 1994.
5. Lodge, J. K., Kaniewski, W. K., and Tumer, N. E., *Proc. Natl. Acad. Sci. USA* 90, 7089–7093 (1993).
6. Lam, Y-H., Wong, Y-S., Wang, B., Wong, R. N-S., Yeung, H-W., and Shaw, P-C., *Plant Sci.* 114, 111–117 (1996).
7. Hong, Y., Saunders, K., Hartley, M. R., and Stanley, J., *Virology* 220, 119–127 (1996).
8. Haley, A., Zhan, X., Richardson, K., Head, K., and Morris, B., *Virology* 188, 905–909 (1992).
9. Etessami, P., Saunders, K., Watts, J., and Stanley, J., *J. Gen. Virol.* 72, 1005–1012 (1991).
10. Baulcombe, D. C., Chapman, S., and Santa Cruz, S., *Plant J.* 7, 1045–1053 (1995).
11. Chapman, S., Kavanagh, T., and Baulcombe, D. C., *Plant J.* 2, 549–557 (1992).
12. Herrera-Estrella, L., and Simpson, J., *In* "Plant Molecular Biology: A Practical Approach" (C. H. Shaw, Ed.), pp. 131–160. IRL Press, Oxford, 1988.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T., *In* "Molecular Cloning: A Laboratory Manual" 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
14. Feinberg, A. P., and Vogelstein, B., *Anal. Biochem.* 132, 6–13 (1983).
15. May, M. J., Hartley, M. R., Roberts, L. M., Krieg, P. A., Osbourn, R. W., and Lord, J. M., *EMBO J.* 8, 301–308 (1989).
16. Taylor, S., Massiah, A., Lomonossoff, G., Roberts, L. M., Lord, J. M., and Hartley, M., *Plant J.* 5, 827–835 (1994).
17. Sunter, G., and Bisaro, D., *Virology* 180, 416–419 (1991).
18. Sunter, G., and Bisaro, D., *Plant Cell* 4, 1321–1331 (1992).
19. Johnson, P. F., and McKnight, S. L., *Annu. Rev. Biochem.* 58, 799–839 (1989).
20. Struhl, K., *Annu. Rev. Biochem.* 58, 1051–1077 (1989).
21. Sung, Y. K., and Coutts, R. H. A., *FEBS Lett.* 383, 51–54 (1996).
22. Noris, E., Jupin, I., Accotto, G. P., and Gronenborn, B., *Virology* 217, 607–612 (1996).