Expression of Cauliflower Mosaic Virus Proteins from an Integrated Form of the Viral Genome

By MARK YOUNG, 1* C. K. SHEWMAKER, 2 STEPHEN D. DAUBERT 1 AND R. J. SHEPHERD 2

1Department of Plant Pathology, University of California, Davis, California 95616, 2Calgene Inc., 1920 Fifth Street, Davis, California 95616 and 3Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40506, U.S.A.

(Accepted 20 August 1987)

SUMMARY

The expression of cauliflower mosaic virus proteins was examined in crown gall tissue transformed by integration of virus genome DNA into plant host chromosomes by Agrobacterium tumefaciens-mediated transfer. Galls upon plants of species that support infection by virus particles as well as upon non-host plants were analysed by immunoblotting using antisera specific to proteins encoded by viral genes I, IV and VI. The product of viral gene VI was present in all plants tested, but no other viral gene products could be detected in any host.

The caulimoviruses, of which cauliflower mosaic virus (CaMV) is the type member, have circular, double-stranded DNA genomes. Although the DNA sequence and coding capacity of the CaMV genome have been completely described (Dixon & Hohn, 1985; Gardner et al., 1981), the expression of the viral genes, especially their translation into proteins, is poorly understood. During viral infection an RNA transcript of slightly greater than genome length is produced (Covey et al., 1981; Guilley et al., 1982) (Fig. 1a). This transcript, the 35S RNA, carries the information for genes I to VI sequentially. The transcript initiates from the 35S promoter (located between genes VI and I on the viral DNA), proceeds completely around the circle, and continues 180 bp past the promoter region before terminating, thereby creating a terminal redundancy (Covey et al., 1981; Guilley et al., 1982). The information for viral gene VI, the last gene encoded on the 35S RNA, is also expressed separately on a second transcript, the 19S RNA, initiated from the 19S promoter which is between genes V and VI (Covey & Hull, 1981; Guilley et al., 1982; Nagy et al., 1985) (Fig. 1a). The CaMV promoters are functional when integrated into plant chromosomes (Shewmaker et al., 1985) and both have found wide application as strong constitutive promoters for the expression of a variety of heterologous genes (Koziel et al., 1984; Lloyd et al., 1986; Nagy et al., 1985). In this study we have investigated the expression of the homologous viral gene products from their own promoters in the integrated state.

The products of viral genes I (Young et al., 1987), II (aphid acquisition factor; Woolston et al., 1983), III (possible DNA-binding protein; Giband et al., 1986), IV (capsid protein; Daubert et al., 1982), V (replicase; Ziegler et al., 1985) and VI (Young et al., 1987) have all been characterized to some extent in infected plant tissues. Viral products usually increase during natural infection as the (unintegrated) viral genomes multiply within cells and then spread from cell to cell. In the system described here, however, virus particles cannot be synthesized. A linear permutation of the circular CaMV genome integrated by Agrobacterium tumefaciens-mediated transfer was the source of viral products in gall tissue (Fig. 1b). This permutation precluded autonomous viral replication. Previous studies with this construction, pTiCGN406 (Shewmaker et al., 1985), demonstrated that the viral promoters were transcriptionally active, yielding polyadenylated products from both the 19S promoter and the 35S promoter. The transcript
Short communication

Fig. 1. (a) CaMV transcript diagram showing genes I to VI. The 35S RNA transcript, 180 bp longer than genomic length, is shown around the periphery of the genetic map. The 19S transcript of gene VI is shown beyond that. Restriction endonuclease sites used in the construction of the antigen against which the antiserum to the gene VI product was raised are shown, as is the SalI site at which the genome was linearized for insertion into the T-DNA of the Agrobacterium Ti plasmid. (b) Diagram of pTiCGN406, the linear permutation of the CaMV genome inserted into Agrobacterium T-DNA. Promoters for the transcripts are indicated; RNAs are depicted as in (a). T-DNA is hatched. The kanamycin resistance gene APH(3')-I is inserted into the intergenic region between genes VI and I. The junction with the T-DNA vector is within truncated gene V; the transcript continues across that point, terminating at a site in the adjacent T-DNA.

produced from the 35S promoter spanned genes I to V and terminated in adjacent T-DNA sequences (Fig. 1b). The transcript from the 19S promoter encompassed gene VI, and appeared to be identical to the 19S transcript found in virus-infected cells (Fig. 1b).

The integration of the CaMV genome was accomplished using the entire genome of CaMV isolate 4-184 (Howarth et al., 1981), carrying in the intergenic region a kanamycin resistance gene [APH(3')-I] derived from Tn903. This construct, linearized at its unique SalI site located within viral gene V, was integrated into the T-DNA region of an Agrobacterium Ti plasmid creating pTiCGN406 (Shewmaker et al., 1985) (Fig. 1b). A. tumefaciens cultures containing pTiCGN406 were inoculated into the stems of Brassica campestris, Raphanus sativa, Nicotiana tabacum and Lycopersicon esculentum. Negative controls were galls on B. campestris that were induced by A348 (Comai et al., 1983), an A. tumefaciens strain containing the parental Ti plasmid used in the construction of pTiCGN406.

Immunoassays were used to test for the expression of viral proteins from the 19S and permuted 35S transcripts present in galls. Antiserum specific to the CaMV gene VI product was raised against a chimeric protein. The fusion protein was expressed in Escherichia coli from DNA sequences that encode a section of CaMV gene VI fused in phase to the β-galactosidase gene. The CaMV gene VI segment was recovered from a clone of full-length virus DNA (pCaMV 10; Gardner et al., 1981) by cleavage with HgiAl (see Fig. 1a; HgiAl cleaves the SstI site). A 1203 bp segment, from nucleotide positions 5822 to 7025 (nucleotide references are to the sequence of CM-1841; Gardner et al., 1981) was cloned into the SstI site of pUC13. This subclone was cleaved with EcoRI, which cut once in the vector and once in the gene VI insert segment (position 6105). This generated a DNA segment coding for an amino-terminal segment of CaMV gene VI (amino acid positions 19 to 110; bp 5822 to 6105) coding for 18% of the gene VI product. The EcoRI fragment was inserted into the EcoRI site of the E. coli expression vector pKB850.3 (Buckley, 1985) and designated pMY20. This placed the gene VI segment in phase with (i) Mr 116000 β-galactosidase protein (which formed the carboxy-terminal portion of the chimeric antigen) and (ii) a portion of the bacteriophage λ cro gene on the amino terminus of the chimeric protein. Chimeric protein expression was under the control of temperature-sensitive cI857 repressor. When pMY20 was transformed into E. coli strain MC1000 (Casadaban et al.,
Fig. 2. Immunoblot analysis of plant proteins transferred to nitrocellulose from polyacrylamide gel. Bound proteins were probed with antiserum specific for the CaMV gene VI protein. Proteins were from gall (lanes 1 to 5) or leaf (lanes 6 and 7) tissues. The control galls tested in lane 1 were produced on stems inoculated with A348. Lanes 2 to 5 represent analyses of galls resulting from pTiCGN406 inoculation. Tissue sources were *B. campestris* (lanes 1, 5 to 7), *L. esculentum* (lane 2), *N. tabacum* (lane 3) and *R. sativa* (lane 4). Turnip plants used for lane 6 were systemically infected with CM-1841 virus particles; lane 7 shows healthy plants. All samples contained 110 µg total plant protein. Protein mol. wt. standards were phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase.

1980), the fusion protein was induced by a temperature shift from 30 °C to 42 °C for 5 h. At 42 °C the chimeric antigen constituted 10% of the newly synthesized bacterial protein. This protein was recovered from preparative SDS–polyacrylamide gels by electroelution (Young *et al.*, 1987) and used to generate polyclonal antibodies in rabbits. A similar approach has been used to construct a chimeric antigen containing determinants encoded by viral gene I (Young *et al.*, 1987) to generate antiserum specific to the CaMV gene I product. Antiserum against the viral gene IV product (capsid protein) was raised using purified virus particles (Daubert *et al.*, 1982).

Samples for immunoblot analysis (0.5 g fresh weight) were ground in liquid nitrogen and mixed with 1.5 ml protein extraction buffer (150 mM-Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol). Samples were incubated at 100 °C for 5 min and clarified by centrifugation at 10000 g for 10 min. Immunoblots were performed as described by Bers & Garfin (1985) using antisera specific to various virus proteins. Aliquots of the above supernatant fraction (5 to 100 µg total plant protein as determined by the BCA assay) were analysed by 10% or 12% SDS–PAGE (Laemmli, 1970). After transfer to nitrocellulose, CaMV proteins were detected by incubating with antibodies specific to viral proteins and then visualized using goat anti-rabbit gammaglobulin linked to alkaline phosphatase (Bio-Rad) as a second antibody (Bers & Garfin, 1985).

Virus-infected plant tissue and crown gall tissue that contained the CaMV genome integrated into the host chromosome via pTiCGN406 were analysed for viral proteins by immunoblotting. Antiserum to the product of CaMV gene VI detected a peptide with an apparent mol. wt. of 62000 in CaMV-infected turnip leaves. Proteins of similar apparent mol. wt. were detected in pTiCGN406 galls (Fig. 2). No peptide with a similar mol. wt. was detected in healthy turnip leaves. This mol. wt. is in agreement with other reports of the size of the gene VI product (Dixon & Hohn, 1985; Harker *et al.*, 1987). The protein accumulated in all pTiCGN406-transformed gall tissues tested, which included galls on *L. esculentum* and *N. tabacum*, as well as on *R. sativa* and *B. campestris* (Fig. 2). As intact plants only the latter two (brassicaceous) hosts will support viral infection; virus does not multiply detectably in the two solanaceous species. The accumulation of the gene VI product was considerably greater in gall tissue derived from *B. campestris* (Fig. 2e), the host in which this isolate of CaMV was routinely propagated prior to its cloning, than
Fig. 3. Immunoblot analysis of total plant proteins transferred to nitrocellulose from 12% polyacrylamide gels. Bound proteins were probed with antiserum specific for CaMV gene I protein (lanes 1 to 7) or gene IV protein (lanes 8 to 14). Proteins were from gall (lanes 3 to 12) or leaf (lanes 1, 2, 13 and 14) tissue. The control galls tested in lanes 7 and 8 were produced on stems inoculated with A348. Lanes 3 to 6 and 9 to 12 represent analyses of galls resulting from pTiCGN406 inoculation. Tissue sources were *B. campestris* (lanes 1 to 3, 12 to 14), *R. sativa* (lanes 4 and 11), *L. esculentum* (lanes 5 and 10) and *N. tabacum* (lanes 6 and 9). Turnip plants used for lanes 2 and 13 were systemically infected with CM-1841 virus particles; lanes 1 and 14 show healthy plants. All samples contained 110 μg total plant protein, except lanes 2 and 13 which contained 5.5 μg total plant protein. Protein mol. wt. standards were the same as Fig. 2.

the other species. The levels of accumulation of the gene VI product were proportional to levels of the 19S RNA transcript previously observed in these galls (Shewmaker *et al*., 1985). No gene VI product was detected in tissues adjacent to the galls (data not shown). Thus, there was no indication of movement of this viral protein into other host tissues.

Solanaceous plants do not support a systemic infection of virus isolate 4-184 or the closely related isolate CM-1841. *Datura stramonium* (Daubert *et al*., 1984) or *N. bigelovii* (Schoelz *et al*., 1986) usually show no symptoms but, under some conditions, may develop a weak hypersensitive reaction, giving small necrotic lesions and no systemic spread. For each of these two hosts, the viral determinant that induces this response has been mapped to gene VI (Daubert *et al*., 1984; Schoelz *et al*., 1986).

A hypersensitive response to tobacco mosaic virus has been reported in *Nicotiana* callus (Beachy & Murakishi, 1971). No such obvious response was detected in tissues adjacent to the galls (data not shown). Thus, there was no indication of movement of this viral protein into other host tissues.

CaMV genomic constructions can be designed for integration into host chromosomes by *Agrobacterium* transfer so that a greater than full-length RNA copy of the viral DNA, initiated at the viral 35S promoter, can be transcribed just as from the circular template. Such a construct has given rise to circular viral DNA and infectious virus particles (Grimsley *et al*., 1986). The permutation of the viral genome used in this study, however, (Fig. 1b) cannot give rise to the terminally redundant 35S RNA, so no circular DNA can be made. Nonetheless, the genes for both the gene I product and gene IV product (the capsid protein) are transcribed onto a multicistronic polyadenylated RNA (Shewmaker *et al*., 1985) initiated from the 35S promoter (Fig. 1b). However, we could not detect either of these protein products in transformed galls using
specific antisera with the immunoblotting procedure described above which detects the gene VI product (Fig. 3). Analysis of virus-infected control samples, corresponding to 1/20 the amount of extract examined from galls, easily detects the gene I and IV protein products (Fig. 3, lanes 2 and 13). The amount of the 35S transcript produced in pTiCGN406 turnip galls [0.2% of poly(A) RNA] is 1/10 that observed in CaMV-infected tissue (Shewmaker et al., 1985); therefore the immunoblot analysis was sensitive enough to detect the gene I and IV protein products if they had been produced at this efficiency. The levels of the 35S transcript are significantly lower in pTiCGN406 tobacco [0-0005% of poly(A)RNA], tomato (0-002%) and radish (0-008%) galls. Therefore it is possible that the gene I and IV proteins might be present, but below detectable levels in these galls.

There are several possible reasons for the failure to detect gene I and IV proteins in these galls. Both gene products I and IV are associated with virus particles. It is possible that information for both of these proteins is expressed and translated in these galls, but that the protein products are unstable in the absence of virion particles; if the circular viral DNA is necessary for the formation of virus particles, then the virion-associated peptides may not accumulate in its absence. Alternatively, in pTiCGN406 the kanamycin resistance gene present upstream from viral gene I may be an alteration in the polycistronic transcript compared with the wild-type configuration. Dixon & Hohn (1984) have demonstrated that extra upstream coding regions do not hinder the production of the gene I product when they are followed by in-phase termination codons, as is the APH(3')-I gene. However, it is still possible that the insertion of the APH(3')-I gene has disrupted translation. In summary, the presence of the multi-gene transcript encoding viral gene I may be an alteration in the polycistronic transcript compared with the wild-type configuration. Dixon & Hohn (1984) have demonstrated that extra upstream coding regions do not hinder the production of the gene I product when they are followed by in-phase termination codons, as is the APH(3')-I gene. However, it is still possible that the insertion of the APH(3')-I gene has disrupted translation. In summary, the presence of the multi-gene transcript encoding viral genes I and IV does not give rise to detectable levels of the respective translation products in these galls, although in plants this does appear to happen during productive viral infection. The separate 19S gene VI transcript, however, does give rise to its translation product (which is not virion-associated; Young et al., 1987) in the absence of other viral gene products in this crown gall system. Further work on the CaMV translational strategy may resolve this apparent paradox.

We thank Dr George Bruening for providing advice and the laboratory space in which these experiments were carried out. M.Y. is a predoctoral fellow of the McKnight Foundation. This research was supported by the United States Department of Energy, Division of Biological Energy Research, grant number 85ER13353, and by the Agricultural Experiment Station of the University of California.

REFERENCES


Short communication


(Received 20 May 1987)