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A Geminivirus Induces Expression of a Host DNA Synthesis Protein in Terminally Differentiated Plant Cells

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Geminiviruses are plant DNA viruses that replicate through DNA intermediates in plant nuclei. The viral components required for replication are known, but no host factors have yet been identified. We used immunolocalization to show that the replication proteins of the geminivirus tomato golden mosaic virus (TGMV) are located in nuclei of terminally differentiated cells that have left the cell cycle. In addition, TGMV infection resulted in a significant accumulation of the host DNA synthesis protein proliferating cell nuclear antigen (PCNA). PCNA, an accessory factor for DNA polymerase δ, was not present at detectable levels in healthy differentiated cells. The TGMV replication protein AL1 was sufficient to induce accumulation of PCNA in terminally differentiated cells of transgenic plants. Analysis of the mechanism(s) whereby AL1 induces the accumulation of host replication machinery in quiescent plant cells will provide a unique opportunity to study plant DNA synthesis.

INTRODUCTION

Geminiviruses are a family of plant viruses characterized by a twin icosahedral morphology and single-stranded DNA genomes (for review, see Lazarowitz, 1992; Timmermans et al., 1994). They replicate their small, circular genomes through double-stranded intermediates in plant nuclei using a rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991; Heyraud et al., 1993; Stanley, 1995). Geminiviruses encode only a few proteins for their replication (Elmer et al., 1988a; Hanley-Bowdoin et al., 1990; Sunter et al., 1990). There is no evidence that these viral proteins function as DNA polymerases, suggesting that geminiviruses must depend on plant DNA replication machinery. This reliance on host enzymes is similar to that seen during adenovirus, polyomavirus, and papillomavirus replication in animal cells. Analysis of these viruses and their replication proteins has contributed significantly to our knowledge of DNA replication and cell cycle regulation in animal cells (Sherr, 1994; Waga and Stillman, 1994). Geminiviruses have the same potential for plant systems and are unique in this capacity because most other known plant viruses replicate through RNA intermediates.

The geminivirus tomato golden mosaic virus (TGMV) has a bipartite genome consisting of two ~2.6-kb DNA components, designated A and B (Figure 1; Bisaro et al., 1982). The genome components are arranged similarly with divergent transcription units separated by a 5′ intergenic region that includes the ~200-bp common region (Hamilton et al., 1984). The common region, which is highly conserved between the two genome components, contains the viral origin of replication (Revington et al., 1989; Lazarowitz et al., 1992) and transcriptional elements (Hanley-Bowdoin et al., 1989; Sunter and Bisaro, 1989; Eagle et al., 1994). TGMV encodes seven open reading frames. Two of these specify the AL1 and AL3 proteins; these proteins are involved in viral replication. The AL1 protein is essential and sufficient for viral DNA replication in the presence of host factors (Elmer et al., 1988a; Hanley-Bowdoin et al., 1990). AL1 shows limited sequence identity to site-specific endonucleases that mediate initiation of rolling circle replication (Ilyina and Koonin, 1992). Recent experiments have shown that TGMV AL1 functions as an endonuclease to cleave specifically the replication origin within a conserved hairpin motif (B.M. Orozco and L. Hanley-Bowdoin, unpublished results). TGMV AL1 also functions as an origin recognition protein by specifically binding to a 13-bp motif, 5′-GGTAGTAAGGTAG, on the left side of the common region (Fontes et al., 1992). Interaction between AL1 and its recognition sequence is essential for replication and is virus specific (Fontes et al., 1994a, 1994b). The same DNA sequence also acts as a negative transcriptional element in the autoregulation of AL1 expression (Eagle et al., 1994). The AL3 protein is not required for TGMV replication but greatly enhances the levels of viral DNA accumulation by an unknown mechanism (Elmer et al., 1988a; Sunter et al., 1990).

Geminiviruses rely on their plant hosts for all of the other enzymes and factors required for their replication. Plants contain multiple DNA polymerases that catalyze replication

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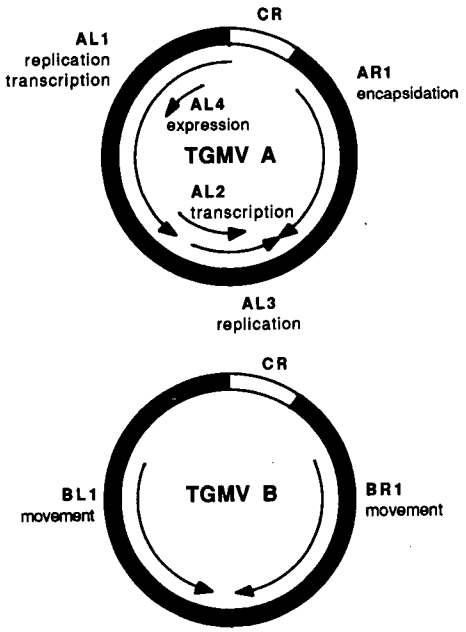

Figure 1. Genome Organization of TGMV.

The AL1, AL2, AL3, AL4, AR1, BL1, and BR1 open reading frames and their orientations are indicated by arrows. The functions of the various proteins encoded by TGMV are also given. The AL1 and AL3 open reading frames encode the only TGMV proteins involved in viral replication. The open boxes (CR) represent the common region, which includes the origin of replication.

and repair of their nuclear, plastid, and mitochondrial genomes (for review, see Aves and Bryant, 1993). Activities related to animal DNA polymerases α (Polα) and β have been identified in plant nuclei and are thought to be involved in replication and repair, respectively. There is no direct evidence for the involvement of the plant Polα-like enzyme in nuclear DNA replication, but its activity was found to increase 100-fold in germinating maize roots (Coello et al., 1992). A DNA polymerase activity related to animal DNA polymerase δ (Polδ; Richard et al., 1991) and auxillary activities required for DNA replication, including primase, helicase, topoisomerase, ligase, and single-stranded DNA binding activities, have also been found in plants (for review, see Aves and Bryant, 1993). Plant genomes contain DNA sequences similar to those of proliferating cell nuclear antigen (PCNA; Suzuka et al., 1989; Kodama et al., 1991). In animal and fungal cells, PCNA associates with Polδ to promote processivity of the enzyme (Bravo et al., 1987; Brown and Campbell, 1993). Rice PCNA can confer processivity to human Polδ (Matsumoto et al., 1994) and, thus, is functionally equivalent to animal PCNA. Calf thymus PCNA increases the processivity of two DNA polymerases from wheat embryos (Laquel et al., 1993), providing further evidence for δ- and ε-like DNA polymerases in plants. PCNA is involved in both replicative and repair DNA synthesis, but only actively dividing plant cells contain high levels of the protein (Kosugi et al., 1991; Citterio et al., 1992; Daidoji et al., 1992).

The host factors required for geminivirus replication have not been elucidated, and the mechanism whereby geminiviruses become established in plant nuclei is not known. During development, plant cells leave the cell division cycle and contain no detectable levels of DNA replication enzymes or PCNA upon differentiation (Coello et al., 1992; Daidoji et al., 1992). In mature dicotyledonous plants, DNA replication and cell division are restricted to undifferentiated, meristematic cells in the growing tips of shoots and roots or in the cambium of the stem (Martinez et al., 1992; Staiger and Doonan, 1993). These observations raise two important questions about how geminiviruses recruit host replication factors. Is replication restricted to meristematic cells with their complement of replication machinery? Alternatively, are geminiviruses able to modify their plant hosts by inducing the synthesis of replication enzymes in terminally differentiated cells, as has been observed for mammalian DNA tumor viruses (Nevins, 1992)? Some transient replication experiments have shown that cell division is a prerequisite for the appearance of replicative forms of geminivirus DNA (Townsend et al., 1986; Briddon et al., 1989; Matzeit et al., 1991; Boulton et al., 1993), whereas other experiments have detected viral replication in the absence of cell division (Brough et al., 1992; Timmermans et al., 1992). Flow cytometry analysis has suggested that there is a correlation between geminivirus replication and the S phase of the plant cell cycle (Accotto et al., 1993). However, these experiments included nuclei from both meristematic and differentiated cells, thereby precluding analysis of a requirement for meristematic cells for geminivirus replication. Ultrastructural studies have detected geminivirus particles in nuclei of terminally differentiated cells (Rushing et al., 1987), but it is not known whether the virus actually replicated in these cells or whether viral particles and/or DNA accumulated after movement from meristematic cells.

To reconcile these conflicting results, it is necessary to determine whether geminivirus replication occurs in meristematic or differentiated cells in intact plants. In this study, we used immunolocalization of the TGMV replication proteins AL1 and AL3 and of a host factor associated with DNA synthesis, PCNA, to identify and characterize the cell types that support geminivirus replication in TGMV-infected and transgenic plants.

RESULTS

AL1 and AL3 Are Localized to Nuclei of Infected Cells

Although geminiviruses are dependent on their host plants for much of their replication machinery, there is little information regarding the relationship between geminivirus and plant

DNA replication. Ultrastructural studies have shown viral particles in nuclei, chloroplasts, and differentiated sieve tube elements, which lack nuclei at maturity (Esau, 1969; Rushing et al., 1987; Groning et al., 1990). To begin to study the cellular basis of geminivirus replication, we asked whether the TGMV replication proteins AL1 and AL3 are located in a subcellular compartment where plant DNA replication occurs, that is, the nucleus, the plastid, and/or the mitochondrion. In Figure 2, antibodies specific to AL1 or AL3 and secondary antibodies conjugated to the fluorochrome Cascade Blue were incubated with sectioned tissue from TGMV- and mock-infected *Nicotiana benthamiana* plants. The infected tissue was from symptomatic plants 12 to 16 days postinoculation. The mock-infected leaf tissue was from expanded leaves of equivalent age as determined by their size and position on the plant.

AL1 and AL3 antigens were both detected in nuclei of infected leaf cells (Figures 2B and 2C). No material that cross-reacted with either anti-AL1 (Figures 2A and 2D) or anti-AL3 antibodies (data not shown) was detected in mock-infected leaf tissue. Xylem, which is dead at maturity and lacks nuclei, autofluoresced light blue and was distinguishable from the medium blue Cascade Blue labeling. Similar patterns of labeling and nuclear morphology were seen in plant tissue bombarded or agroinoculated with TGMV, suggesting that the changes were not an artifact of the inoculation procedure. These results established that AL1 and AL3 are concentrated in the nucleus, one of the three subcellular compartments in plant cells competent for DNA replication. The data in Figure 2 do not exclude the possibility that the AL1 and AL3 proteins are also present in other subcellular compartments at levels below the limits of detection of the assay. In Figure 2L, AL3 labeling can also be seen in the cytoplasm adjacent to the cell wall of stem cells. This observation is consistent with the results of biochemical experiments that detected the AL3 protein in cytoplasmic and organelle fractions but not in cell wall fractions from TGMV-infected *N. benthamiana* plants (Pedersen and Hanley-Bowdoin, 1994).

Many of the TGMV-infected cells that cross-reacted with anti-AL1 or anti-AL3 antibodies displayed abnormal nuclear morphology. Most of the positively labeled cells did not show visible signs of necrosis, but their nuclei were hypertrophic and located near the center of the cell (Figures 2B and 2C). In contrast, uninfected nuclei were elongated and typically found adjacent to the cell wall (Figures 2A and 2D). Frequently, the nucleoli of infected cells were not distinct structures. In those nuclei with discernible nucleoli, labeling of AL1 and AL3 was observed throughout the nucleus and the nucleolus. In some cases, punctate nuclear staining was observed with both anti-AL1 and anti-AL3 primary antibodies (Figures 2E to 2G). It is not clear whether the punctate staining represented viral aggregates or DNA replication complexes (Bravo and MacDonald-Bravo, 1987; Cardoso et al., 1993). Distorted nuclei with fibrillar bodies and viral aggregates have also been observed in electron micrographs of TGMV-infected cells (Rushing et al., 1987).

AL1 and AL3 Are in Terminally Differentiated Cells of Infected Plants

Geminivirus replication has been studied primarily in transient transfection systems that have used actively dividing suspension cell cultures (Matzeit et al., 1991; Brough et al., 1992; Timmermans et al., 1992; Boulton et al., 1993; Fontes et al., 1994a) or in tissue explants that were cultured under callus-inducing conditions (Elmer et al., 1988b; Lazarowitz et al., 1992; Stenger et al., 1992). There is no information regarding viral DNA replication in intact plants, and it is not known whether viral replication is confined to actively dividing cell populations or occurs in terminally differentiated cells of geminivirus-infected plants. We addressed these possibilities by determining which cell types in infected leaf and stem tissues show expression of the AL1 and AL3 open reading frames.

In tobacco leaves, cell divisions are scattered and occur throughout the blade until the leaf is 50% of its final length (Poethig and Sussex, 1985). In Figure 2, leaf sections were from fully expanded leaves and, thus, did not contain dividing cells. AL1 and AL3 antigens were present in nuclei of differentiated leaf cells including epidermal, mesophyll, and phloem-associated cells (Figure 2). The distribution of AL1 and AL3 in the nuclei of contiguous cells in infected tissue did not resemble the random pattern of dividing cells found in developing tobacco leaves 1 to 2 cm in length (Poethig and Sussex, 1985). These results suggest that TGMV replicates in terminally differentiated cells in leaf tissue.

Stem tissue is composed predominantly of differentiated cells that constitute the vascular cylinder and surrounding parenchyma cells. A single cell layer, the cambium, remains active for cell division. Cambial cells divide to produce terminally differentiated cells of the xylem or phloem on either side of the cambium. The AL1 antigen was seen in groups of fully differentiated cells throughout the outer cortex, xylem parenchyma, phloem, and pith of *N. benthamiana* stem tissue (Figures 2K and 2L). It is not possible to identify with certainty the cells constituting the cambium in the stem sections, and we have not seen patterns of viral protein labeling that correspond to the cambium. As shown in Figure 2K, AL1 labeling was more intense in the large cortical cells than in the smaller xylem parenchyma cells. Infected nuclei, with their hypertrophied morphology and central location in the cell, were readily visible (Figures 2K and 2L) in contiguous cells of the stem cortex. No cross-reacting material was detected in comparable sections of mock-inoculated stem tissue; therefore, the nuclei were difficult to visualize (Figure 2J). The nuclei of small, undifferentiated cells immediately adjacent to the xylem displayed green autofluorescence in both the control and infected stem tissues that was distinct from Cascade Blue fluorescence (Figures 2J and 2K). These results showed that TGMV replication occurs in differentiated cells and not in dividing cells of stem tissue and were consistent with those obtained with leaf sections.

The symptoms of TGMV infection in *N. benthamiana* are

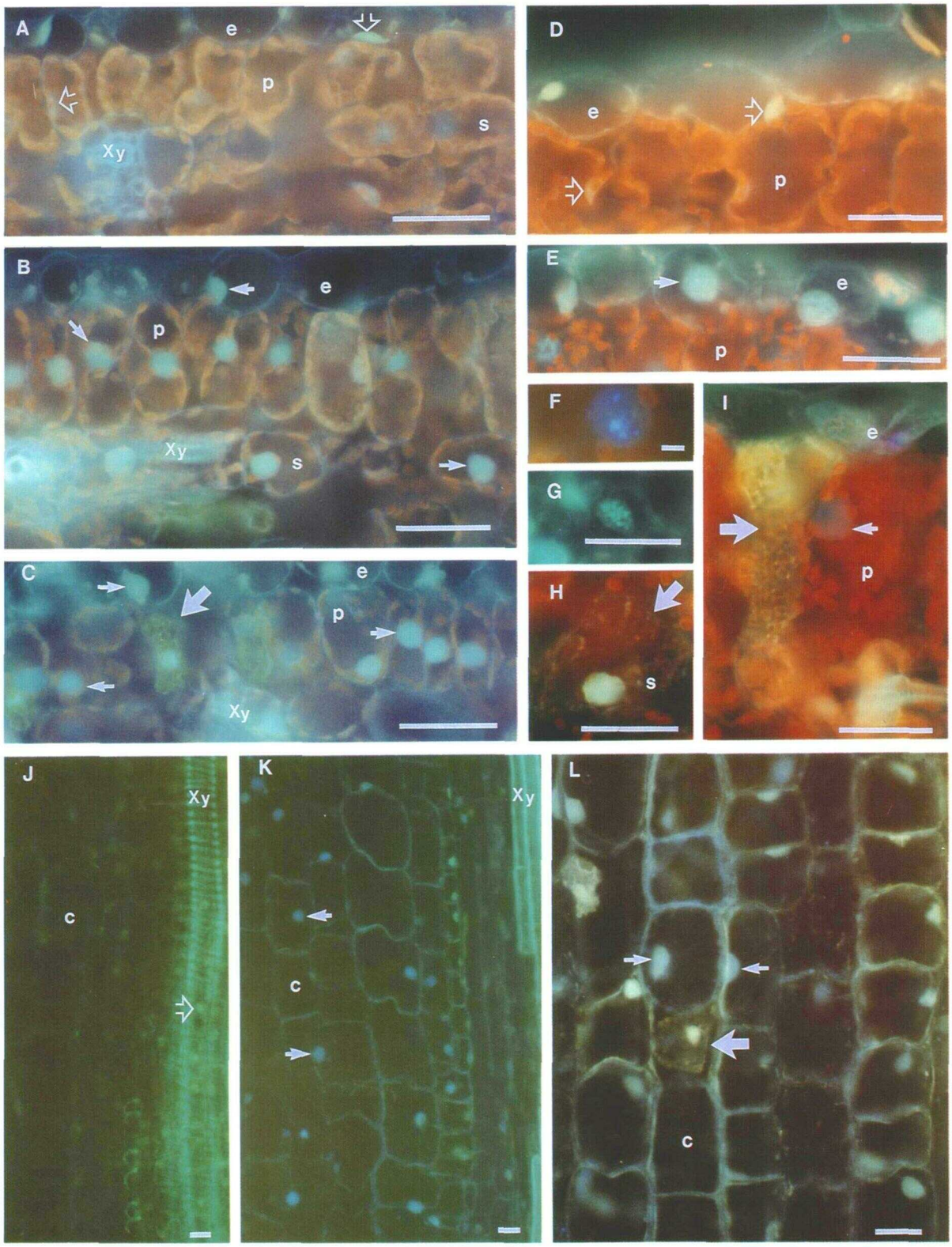

Figure 2. Localization of TGMV AL1 and AL3 Proteins in Nuclei of Differentiated Leaf and Stem Cells.

severe and include mottled chlorosis, stunted growth, and leaf curling. Surprisingly, most cells labeled with the anti-AL1 and anti-AL3 antibodies had intact plasmalemma and chloroplasts. The most prominent morphological changes were in the size, shape, and location of their nuclei (Figures 2B, 2C, and 2E). In some cases, labeled cells were severely necrotic, as indicated by a uniform loss of chlorophyll autofluorescence throughout the cell (Figure 2I). Cells showing different degrees of necrosis were seen in leaf tissue, as demonstrated by the necrotic cells in Figures 2C, 2H, and 2I. Necrotic cells, identified by detachment of the plasma membrane from the cell wall (Figure 2L), were also found in stem tissue but were less common. These studies indicated that after TGMV infection, *N. benthamiana* leaf cells supporting viral DNA replication can become necrotic. However, it is not clear whether the limited pattern of necrosis reflects cells at later stages of infection or whether only some infected cells become necrotic.

PCNA Is in Nuclei of Terminally Differentiated Cells of Infected Plants

Earlier studies have suggested that geminivirus replication may be associated with dividing plant cells (Briddon et al., 1989; Matzeit et al., 1991; Boulton et al., 1993) and coordinated with the plant cell cycle (Accotto et al., 1993). These results appear to conflict with our observations that TGMV replication proteins can be detected only in terminally differentiated cells that have left the cell cycle and do not contain detectable levels of replication enzymes (Coello et al., 1992; Staiger and Doonan, 1993). To address this inconsistency, we have been investigating whether TGMV-infected cells contain proteins that are associated with DNA replication and are not present at detectable levels in terminally differentiated cells (Coello et al., 1992). In the experiments reported here, we asked whether TGMV-infected tissues contain PCNA, which is the processivity factor for DNA Polδ. Several studies have established that the PCNA protein and PCNA gene transcription can be detected only in actively dividing plant cells (Kosugi et al., 1991; Citterio et al., 1992; Daidoji et al., 1992).

PCNA is a highly conserved protein (Suzuka et al., 1991). We asked whether antibodies raised against human PCNA, which is >60% identical to PCNA proteins from five plant species, can cross-react with *N. benthamiana* PCNA. Total soluble protein from mid-log phase *N. benthamiana* suspension cells was resolved by SDS-PAGE and analyzed by immunoblotting using a monoclonal antibody, PC10, raised against human PCNA (Waseem and Lane, 1990). A single 33-kD polypeptide was detected in the plant cell extract (Figure 3, lane 4). This protein coelectrophoresed with recombinant human PCNA (Figure 3, lane 3). The PC10 antibody did not cross-react with purified, recombinant AL1 (Figure 3, lane 1) or the His-AL3 (lane

Figure 2. (continued).

TGMV-infected and mock-inoculated leaf tissues of *N. benthamiana* were fixed 16 days after agroinoculation unless otherwise stated. Sections were incubated with primary polyclonal antibodies raised against AL1 or AL3 and labeled with secondary antibodies conjugated to Cascade Blue, which fluoresces medium blue. Small solid arrows show nuclear localization of the TGMV protein. Differences in chlorophyll autofluorescence (red orange versus brown orange) in **(A)** to **(C)** versus **(D)** to **(I)** are due to different barrier filters. Xylem (Xy) is dead at maturity, lacks nuclei, and autofluoresces light blue. Open arrows show uninfected nuclei that fluoresce yellow or yellow green. Epidermal (e), palisade mesophyll (p), spongy mesophyll (s), and cortical (c) cells are marked.

(A) Cross-section of mock-inoculated leaf incubated with anti-AL1 antibodies; control for **(B)** and **(C)**. The open arrows show the nuclei of an epidermal and a mesophyll cell.

(B) Cross-section of a TGMV-infected leaf labeled with anti-AL1 antibodies. The arrows point to three of many swollen AL1-positive nuclei in epidermal and mesophyll cells.

(C) Cross-section of the same leaf as shown in **(B)** but labeled with anti-AL3 antibodies. The small arrows show epidermal and mesophyll cells with AL3-containing nuclei. The heavy arrow shows a necrotic cell.

(D) Cross-section of a mock-inoculated leaf incubated with anti-AL1 antibodies; control for **(E)** to **(I)**. The open arrows show nuclei of an epidermal and a mesophyll cell. Autofluorescence of other nuclei can also be seen.

(E) Cross-section of a TGMV-infected leaf labeled with anti-AL1 antibodies. The arrow shows one of several hypertrophic epidermal nuclei with punctate staining.

(F) High magnification of a mesophyll nucleus 6 days after bombardment and labeled with anti-AL1 antibodies. The nucleus is spherical and shows particulate staining.

(G) Cortical nucleus in a TGMV-infected stem labeled with anti-AL3 antibodies. The nucleus shows punctate staining.

(H) Cross-section of a TGMV-infected leaf labeled with anti-AL3 antibodies. The heavy arrow marks a necrotic spongy mesophyll cell.

(I) Cross-section of a TGMV-infected leaf labeled with anti-AL3 antibodies. The small arrow shows a nucleus containing AL3 next to a symptomatic palisade mesophyll cell (heavy arrow).

(J) Longitudinal section of a mock-inoculated stem incubated with anti-AL3 antibodies. The open arrow shows an uninfected, greenish nucleus in xylem parenchyma.

(K) Longitudinal section of a TGMV-infected stem incubated with anti-AL1 antibodies. The arrows show two of many contiguous cortical nuclei that contain AL1.

(L) Longitudinal section of a TGMV-infected stem incubated with anti-AL3 antibodies. The small arrows indicate two of several AL3-positive nuclei. The parietal cytoplasm also shows AL3 labeling. The heavy arrow shows a necrotic cortical cell.

Bars = 10 μm except for **(F)**, in which bar = 1 μm.

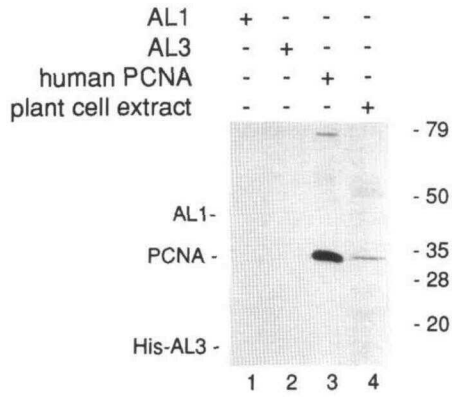

Figure 3. Protein Gel Blot Analysis of Whole-Cell Extract Proteins Prepared from *N. benthamiana* Suspension Cells.

Bacterially produced AL1 (lane 1), His–AL3 (lane 2), and PCNA (lane 3) (10 ng each) and plant cell extract proteins (lane 4; 100 μg) were resolved by SDS-PAGE and assayed by immunoblotting using anti-PCNA antibodies (PC10) and rabbit anti–mouse antibodies conjugated to horseradish peroxidase as the primary and secondary antibodies, respectively. The positions of the molecular mass standards are indicated at right in kilodaltons. (+) indicates that a component was part of the extract separated on the protein gel; (–) indicates that a component was lacking.

2) protein. These results showed that the anti-PCNA antibody specifically recognizes *N. benthamiana* PCNA.

We then used the anti-PCNA antibody in immunolocalization experiments to examine TGMV- and mock-infected tissues at identical stages of development for the presence of PCNA (Figure 4). The leaf tissue was from fully expanded leaves that no longer contained actively dividing cells (Poethig and Sussex, 1985). The PCNA antigen was found in nuclei of cells from TGMV-infected leaf tissue (Figures 4B, 4F, and 4G). No PCNA labeling was seen in mock-infected leaf tissue from fully expanded leaves (Figure 4A) or from leaves ~10 to 20% of their final length. Analogous results were obtained for stem sections of nondividing, differentiated cortical tissue. The anti-PCNA antibody labeled nuclei of infected stem tissue (Figures 4D, 4E, and 4H) but did not label mock-inoculated stem tissue (Figure 4C). We were unable to detect PCNA in soluble protein extracts from TGMV-infected tissue (data not shown) using a variety of protein extraction and immunological techniques. This was probably due to problems with extraction of PCNA protein from infected tissue or with dilution of the PCNA antigen in the extracts by proteins from uninfected cells.

One interpretation of our results is that the accumulation of PCNA in TGMV-infected tissue is related to a generalized wound response resulting from viral infection. To address this possibility, we asked whether *N. benthamiana* plants infected with the potyvirus potato virus X (PVX; Hemenway et al., 1988) also contain PCNA. PVX is an RNA virus that encodes its own RNA-dependent RNA polymerase and replicates in the cytoplasm. Sections from symptomatic PVX-infected leaves that had been prepared and analyzed identically to TGMV-infected plants showed no nuclear staining when incubated with the anti-PCNA antibody (data not shown). These results demonstrated that induction of PCNA accumulation is not due to virus infection in general, but rather is specific to geminivirus infection.

The distribution of PCNA in TGMV-infected tissue was similar to the labeling patterns for AL1 and AL3. In leaf tissue, nuclei of a variety of differentiated cell types, including epidermal, palisade mesophyll, and spongy mesophyll cells, were labeled with the anti-PCNA antibody (Figures 4B, 4F, and 4G). In stem tissue, PCNA was detected in fully expanded cells throughout the outer cortex and pith. Labeled cells in both tissues were generally clustered (Figures 4B and 4D), possibly reflecting simultaneous invasion of viral DNA into contiguous cells during the infection process. The PCNA-positive cells in leaves were highly vacuolated and had well-developed chloroplasts, both characteristic of differentiated cells. However, their nuclei were hypertrophic and centrally located, similar to nuclei containing AL1 and AL3 antigens. PCNA labeling was uniform throughout the nucleus and was not excluded from the nucleolus (Figures 4E, 4F, and 4H).

The similarities between PCNA, AL1, and AL3 labeling patterns may reflect expression of PCNA and the viral replication proteins in the same cells. This hypothesis was addressed by using anti-PCNA and anti-AL1 antibodies simultaneously to probe leaf tissue (Figure 5). For these experiments, the anti-PCNA and anti-AL1 primary antibodies were detected using secondary antibodies conjugated to Cascade Blue and BODIPY-FL, respectively. This approach colocalized PCNA and AL1 to nuclei of a number of differentiated cell types (data not shown). Colocalization of PCNA and AL1 in parenchyma cells near the leaf midvein is shown in Figures 5B and 5C, respectively. These results established that PCNA and AL1 can occur together in terminally differentiated cells.

PCNA Is in Terminally Differentiated Cells of Plants Transgenic for AL1

DNA tumor viruses induce their hosts to produce the enzymes necessary for viral DNA replication, usually through the action of a single or a few viral proteins (Chellappan et al., 1992; Fanning and Knippers, 1992; Nevins, 1992). Frequently, these viral proteins also function in the initiation of viral replication and/or control of viral gene expression (Fanning and Knippers, 1992). Earlier studies have established that AL1 is involved in both of these processes during the TGMV life cycle (Eagle et al., 1994; Fontes et al., 1994a, 1994b), making it a good candidate for the geminivirus protein responsible for PCNA accumulation in TGMV-infected plants. To address this, we asked whether TGMV AL1 can induce the accumulation of PCNA in transgenic *N. benthamiana* plants that contain the AL1 coding sequence under the control of the constitutive 35S promoter from cauliflower mosaic virus and the 3′ end of the nopaline synthase gene (Hanley-Bowdoin et al., 1990).

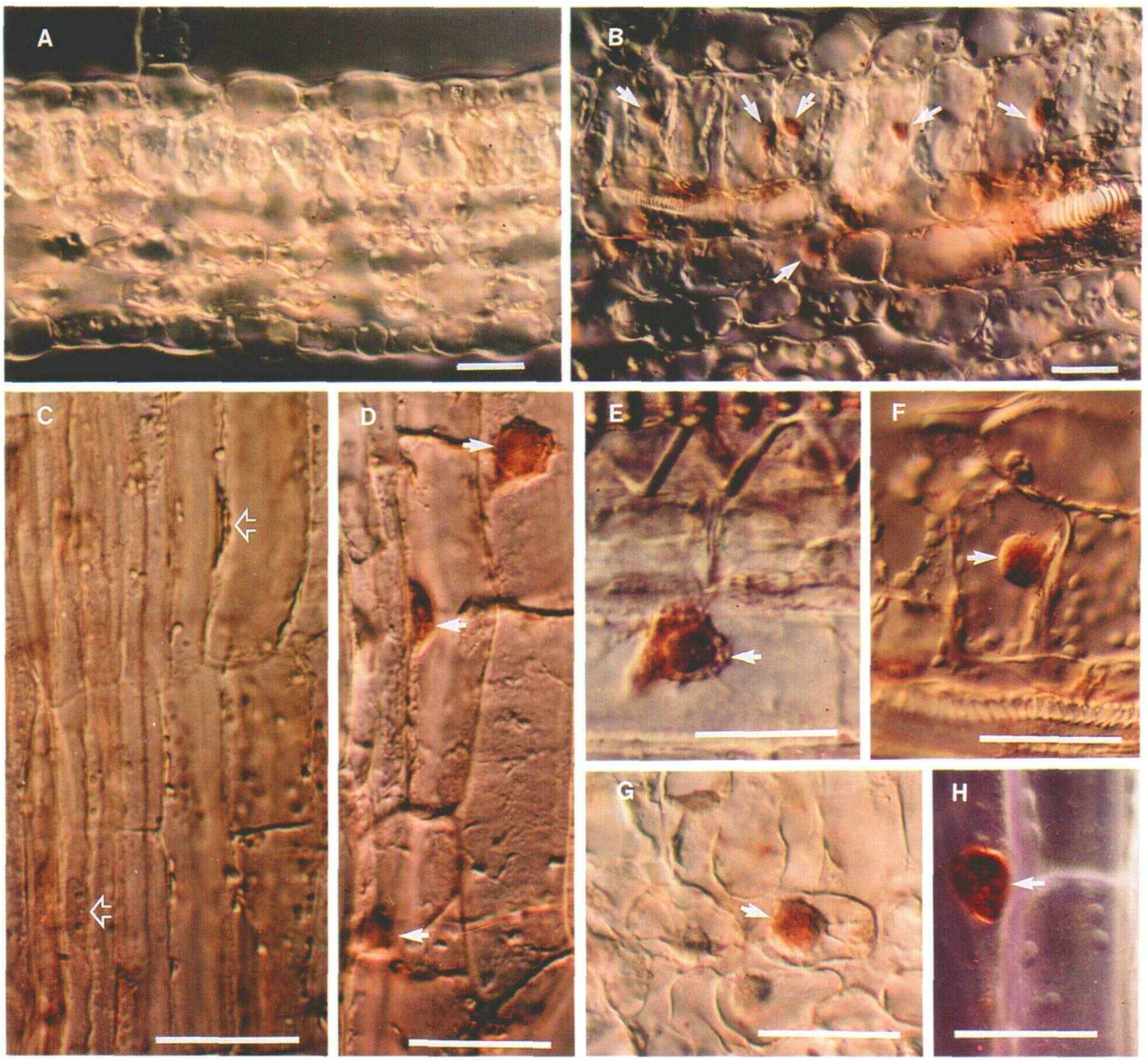

Figure 4. Localization of PCNA in Differentiated Stem and Leaf Cells of TGMV-Infected Plants.

Sections were from plant material 10 days after agroinoculation unless otherwise stated. PCNA was labeled with PC10 antibodies and detected using an ABC horseradish peroxidase method.

(A) Cross-section of a mock-inoculated leaf. There is no PCNA staining, and nuclei are difficult to see.

(B) Cross-section of a TGMV-infected leaf. The arrows show PCNA-containing nuclei in mesophyll cells.

(C) Longitudinal section of a mock-inoculated stem. The open arrows show uninfected nuclei.

(D) Longitudinal section of a TGMV-infected stem. PCNA-containing nuclei are found in three cortical cells (arrows).

(E) Longitudinal section of a TGMV-infected stem. The arrow shows a distorted nucleus containing PCNA.

(F) Cross-section of a TGMV-infected leaf. The arrow shows a swollen, PCNA-containing nucleus in a palisade mesophyll cell.

(G) Cross-section of a TGMV-infected leaf 18 days after agroinoculation. The arrow shows a spongy mesophyll nucleus with PCNA labeling.

(H) Longitudinal section of a stem showing a PCNA-containing nucleus (arrow) in a cortical cell with well-developed chloroplasts.

Bars = 10 μm for **(A)** to **(H)**.

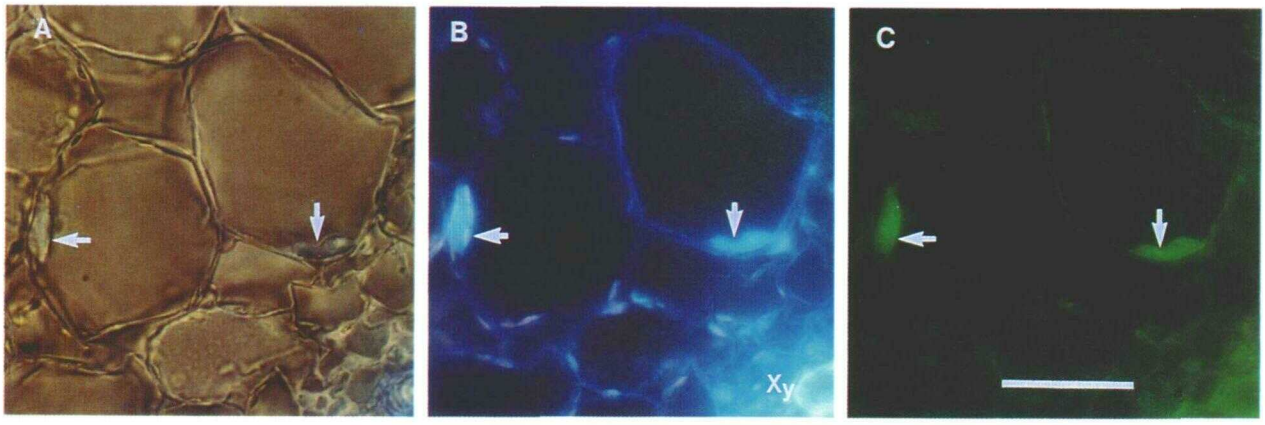

Figure 5. Colocalization of PCNA and AL1.

Cross-sections of TGMV-infected *N. benthamiana* leaf tissue were incubated with mouse anti-PCNA monoclonal and rabbit anti-AL1 polyclonal antibodies followed by incubation with anti-mouse biotinylated secondary antibodies and Cascade Blue-conjugated streptavidin and anti-rabbit BODIPY-FL-conjugated secondary antibodies. Two nuclei from midvein parenchyma cells show colocalization.
(A) Combined bright-field microscopy and UV fluorescence of a TGMV-infected leaf cross-section.
(B) Same cross-section as shown in **(A)** but with PCNA localization (Cascade Blue fluorescence).
(C) Same cross-section as shown in **(A)** but with AL1 localization (BODIPY-FL).
The arrows show labeled nuclei. Xy, nonspecific xylem autofluorescence. Bar in **(C)** = 10 μm.

Tissue from transgenic plant line pMON455-9985, which was shown previously to express functional AL1 protein (Hanley-Bowdoin et al., 1990), was excised from fully expanded leaves and probed with anti-PCNA and anti-AL1 antibodies. PCNA and AL1 were detected in nuclei of a variety of differentiated cell types from transgenic tissue (Figure 6), albeit not as strongly as in TGMV-infected nuclei. The pattern of AL1 and PCNA labeling in transgenic plant tissues was sporadic. This contrasted with the pattern of labeling in virus-infected plants, which showed contiguous cells with AL1 or PCNA antigens. This most likely reflected variations in transgene expression from the cauliflower mosaic virus 35S promoter. The proportion of cells labeled with AL1 antibodies was equivalent to the proportion of cells labeled with PCNA, suggesting that those cells expressing AL1 also expressed PCNA. The morphology of nuclei in cells expressing AL1 was similar to that of cells expressing PCNA (compare Figures 6B and 6C). Recent experiments have colocalized AL1 and PCNA to the same cells (data not shown).

PCNA was seen only in meristematic tissue of healthy, wild-type plants (Figure 7). We did not detect PCNA in healthy leaves that were fully expanded, corresponding in development to the TGMV-infected leaves, or in immature leaves that were ~2 cm in length. PCNA-labeled cells of healthy floral meristems were small, isodiametric, and uniform in size, lacked chloroplasts, and had round nuclei that, due to the small size of the cell, occupied a large proportion of the cell volume. This morphology was distinct from labeled cells of transgenic (Figure 6) and virus-infected (Figure 4) plants, which were much bigger, had large central vacuoles, and, in photosynthetic tissues, contained chloroplasts. No PCNA antigen was detected in differentiated leaf cells from *N. benthamiana* plants transformed with three tandem copies of TGMV B DNA (pMON337 line 3427; Elmer et al., 1988a; data not shown), thereby establishing that increased PCNA accumulation in the AL1 transgenic plants was not a general consequence of plant transformation. The presence of AL1 and PCNA in terminally differentiated leaf cells of transgenic plants expressing AL1 established that AL1 can induce PCNA accumulation in intact plants independent of other viral proteins and the geminivirus infection process.

DISCUSSION

Although DNA replication and cell division are central to the growth and development of all living organisms, little is known about the molecular mechanisms that mediate and coordinate these processes in plants. This lack of information is striking in view of the unique character of plant development. In animal systems, studies of DNA tumor viruses have facilitated the identification and functional analysis of host replication and cell cycle regulatory factors. This is best demonstrated by experiments on the replication of simian virus 40 (SV40) and its integration into the mammalian cell cycle (Hamel et al., 1992; Waga et al., 1994). It has been difficult to apply a similar strategy to the analysis of plant DNA replication and cell division because the majority of plant viruses, including some DNA viruses, replicate through RNA intermediates. Geminivirus replication has been studied extensively in plant tissue culture, but there has been no information regarding viral

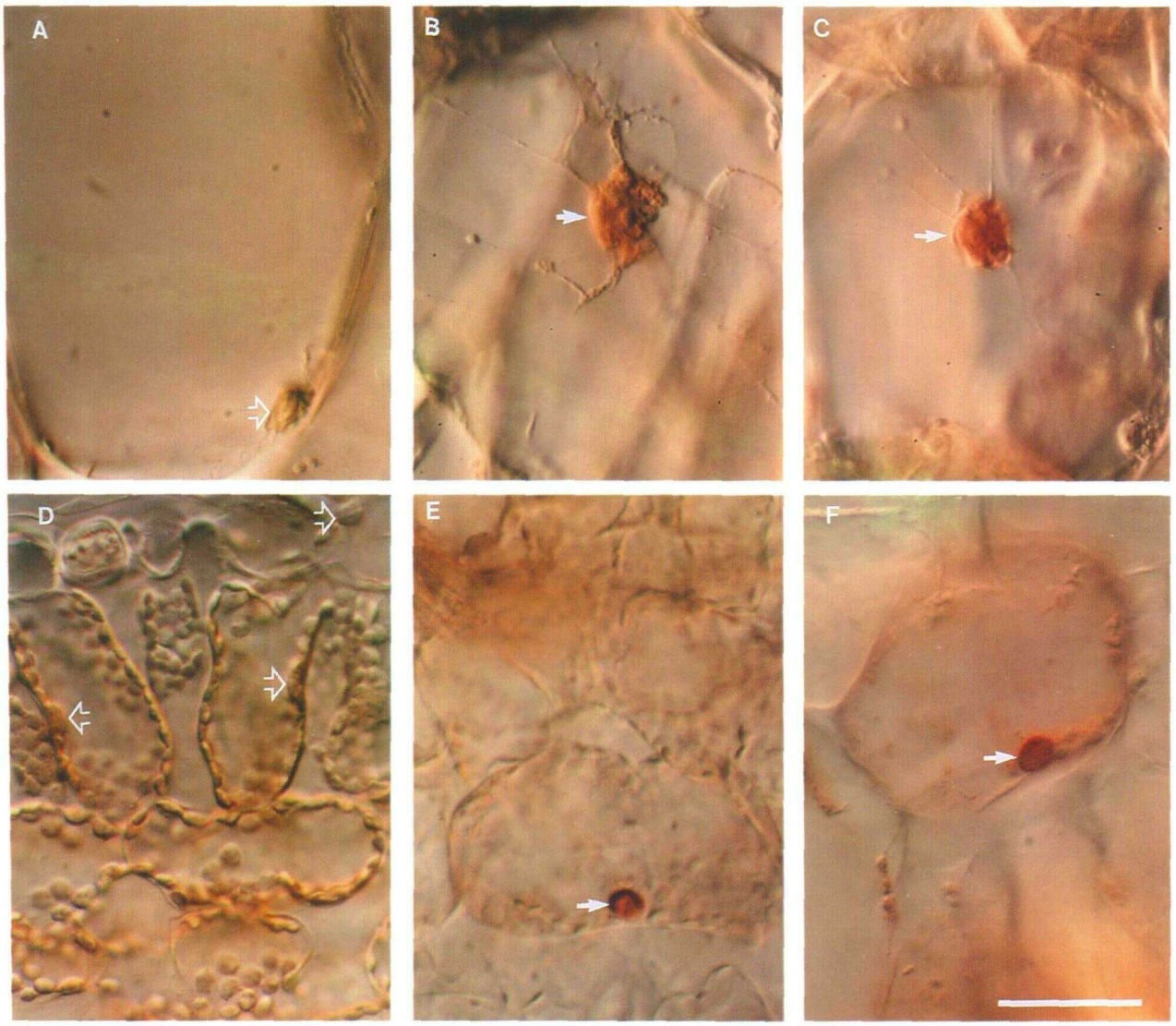

Figure 6. AL1 and PCNA Localization in Plants Transformed with the AL1 Gene.

Labeling was performed as given for Figure 4 using either *PC10* or *anti-AL1* primary antibodies as indicated.

(A) Cross-section of a leaf midvein parenchyma cell from a control, nontransgenic plant incubated with anti-PCNA antibodies. The open arrow shows a wild-type unlabeled nucleus.

(B) Cross-section of a leaf midvein parenchyma cell from a transgenic plant expressing AL1 incubated with anti-PCNA antibodies. The arrow shows a PCNA-containing nucleus.

(C) Cross-section of a leaf midvein parenchyma cell from a transgenic plant expressing AL1 incubated with anti-AL1 antibodies. The arrow shows an AL1-containing nucleus.

(D) Cross-section of a control, nontransgenic leaf incubated with anti-PCNA antibodies. The open arrows show elongated nuclei adjacent to the walls in leaf palisade mesophyll and epidermal cells.

(E) Cross-section of a leaf from a transgenic plant expressing AL1 incubated with anti-PCNA antibodies. The arrow shows a nucleus of a spongy mesophyll cell with PCNA staining.

(F) Cross-section of a leaf midvein parenchyma cell from a transgenic plant expressing AL1 incubated with anti-PCNA antibodies. The arrow shows a PCNA-containing nucleus.

Bar in **(F)** = 10 μm for **(A)** to **(F)**.

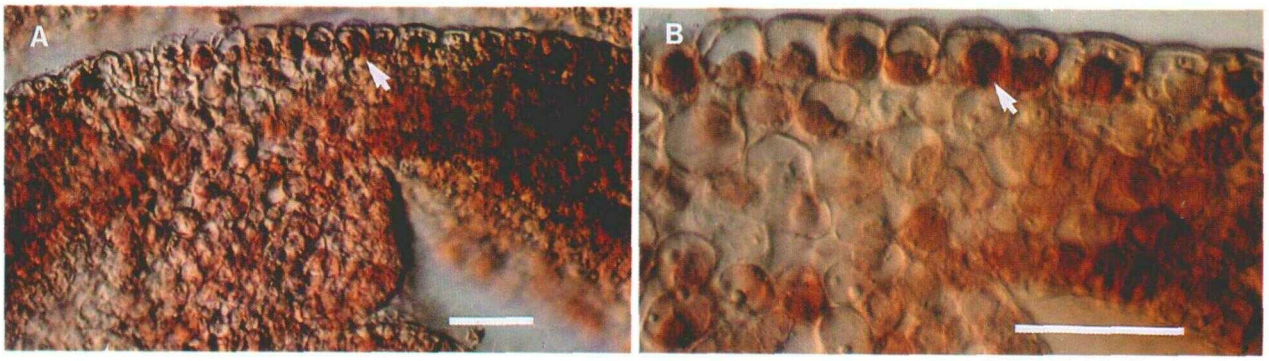

Figure 7. PCNA in *N. benthamiana* Floral Meristems from Healthy Plants.

Tissue was stained as given for Figure 4.
(A) Low magnification of meristematic cells. The arrow shows a PCNA-containing nucleus.
(B) Same cells as shown in **(A)** but at a higher magnification. Arrow indicates the nucleus shown in **(A)**.
Bars in **(A)** and **(B)** = 10 μm.

replication in plants. In this research, we used immunolocalization to study geminivirus replication in plants. We showed that geminivirus replication proteins are located in the nuclei of terminally differentiated cells in intact plants and that geminivirus infection induces the accumulation of the host DNA synthesis protein, PCNA, in the same cells. We also showed that the geminivirus replication protein, AL1, is sufficient to induce the accumulation of PCNA in terminally differentiated cells of transgenic plants. Together, these results strongly suggest that geminiviruses can induce the accumulation of DNA synthesis machinery in quiescent cells of intact plants and that AL1 plays a key role in the induction process, analogous to the DNA tumor antigen proteins of mammalian viruses (Nevins, 1992).

Immunolocalization experiments demonstrated that the AL1 and AL3 replication proteins of the geminivirus TGMV are located in the nuclei of infected cells in intact plants (Figure 2). The nuclear localization of AL1 is consistent with its essential roles as the origin recognition protein (Fontes et al., 1994a, 1994b) and the nicking enzyme for initiation of rolling circle replication (B.M. Orozco and L. Hanley-Bowdoin, unpublished results). AL3 is not required for replication but greatly enhances the level of viral DNA accumulation (Elmer et al., 1988a; Sunter et al., 1990). The nuclear location of AL3 suggests that it may also participate directly in replication or in stabilization of nascent DNA. Immunolocalization experiments further established that AL1 and AL3 are present in a variety of terminally differentiated cells of leaf and stem tissues (Figure 2). TGMV particles have also been found in differentiated leaf cells (Rushing et al., 1987). TGMV infection of *N. benthamiana* may be unusual because of the large number of cell types that contain viral proteins and particles. In general, geminiviruses are thought to be confined to phloem parenchyma and companion cells and to developing and mature sieve elements of vascular tissue (Hull, 1989; Horns and Jeske, 1991).

TGMV-infected cells and transgenic cells expressing AL1 displayed an altered morphology, with their nuclei becoming spherical. Differentiated cells containing the PCNA antigen also showed a change in nuclear structure. TGMV-infected nuclei occupied a much higher proportion of the total cell volume than nuclei from transgenic plants expressing AL1 (compare Figures 4F and 4G with 6E and 6F), indicating that other factors in addition to AL1 are necessary to cause all of the alterations in nuclear morphology associated with geminivirus infection (Rushing et al., 1987). It is interesting that the nuclei of healthy, differentiated cells were elongated and found adjacent to the cell wall (Figures 6A and 6D), whereas nuclei of meristematic cells were round, located toward the cell center (Figure 7B), and more closely resembled the TGMV-infected nuclei (Figures 4F and 4G). Nuclear rounding and migration have also been observed in alfalfa cortical cells after induction of nodule formation by *Rhizobium* and preceding cell division (Cooper and Long, 1994). Some infected cells showed punctate labeling of AL1, AL3, and PCNA. The punctate labeling may represent replication complexes (Hozak et al., 1994). PCNA has been associated with DNA replication foci in mammalian cells (Bravo and MacDonald-Bravo, 1987; Cardoso et al., 1993). Alternatively, the punctate labeling could reflect fixation artifacts or the stage of viral infection. Different PCNA labeling patterns have been seen depending on the method of fixation (Waseem and Lane, 1990). Fibrillar spheres and inclusion bodies characteristic of geminivirus infections (Rushing et al., 1987) might also distort labeling patterns of nuclear antigens.

Localization of TGMV AL1, TGMV AL3, and viral particles to terminally differentiated cells of plants raises an important

question: Is geminivirus replication confined to cells competent for cell division or can geminiviruses induce the accumulation of host factors necessary for viral DNA replication? One possibility is that geminiviruses replicate in actively dividing plant cells, which provide the requisite host replication factors, and then move into differentiated cells. Cell-to-cell movement of DNA and the geminivirus movement protein BL1 in the absence of viral replication was recently shown by microinjection of mature leaf cells (Noueiry et al., 1994). However, our results and those of Horns and Jeske (1991), who did not find geminivirus DNA in actively dividing plant cells, cannot be explained by this hypothesis. Recent studies have shown that plasmodesmata, the intercellular connections through which plant viruses move cell to cell, have distinct structural properties in different cell types (for review, see Lucas and Wolf, 1993). The plasmodesmata of meristematic cells may not be compatible with geminivirus movement proteins, thereby preventing geminiviruses from moving into or out of meristematic cell populations in plants.

A second possibility is that geminiviruses replicate in terminally differentiated plant cells induced to accumulate host DNA replication factors. This hypothesis is supported by the localization of AL1 and AL3 to terminally differentiated cells and by the accumulation of the host DNA synthesis factor, PCNA, in the same cells. Unlike animals, most cells in mature plants have the capacity to dedifferentiate, resume cell division, and form new plants. Differentiated plant cells normally require wounding or hormone application to reenter the cell cycle (Gorst et al., 1991; Kosugi et al., 1991; Hemerly et al., 1993). The detection of PCNA in differentiated cells of transgenic plants expressing AL1 indicated that the AL1 protein can provide the stimulus necessary to induce the dedifferentiation process. Two lines of evidence suggest that AL1 is likely to act at the level of cell cycle control. First, high levels of PCNA, which were the same as those detected in TGMV-infected plants, have been found only in actively dividing cells of plants (Kosugi et al., 1991; Citterio et al., 1992; Daidoji et al., 1992). Second, geminivirus-infected cells contain $>10^4$ viral genome copies per cell (Timmermans et al., 1992), indicating that they must be able to support efficient DNA replication, as occurs during S phase of the cell cycle. However, because PCNA is also associated with DNA repair (Shivji et al., 1992), our data do not eliminate the possibility that the primary effect of AL1 is only on PCNA accumulation. Future experiments will determine whether AL1 also induces the expression of other cell cycle–related host factors.

Some of the morphological changes associated with dedifferentiation of plant cells, that is, nuclear rounding and migration to the cell center, were observed in TGMV-infected and AL1-transformed plants. However, a decrease in cell size, a disappearance of the central vacuole, and an increase in cytoplasmic contents, which are also characteristic of undifferentiated plant cells (Gorst et al., 1991), were not seen (compare Figures 4G and 7B). AL1 may only be able to trigger the early stages of the dedifferentiation process leading to the accumulation of host replication factors, and other signals may be required for full dedifferentiation. Alternatively, AL1 may actively interfere with the progression through G_2 phase and full dedifferentiation, thereby locking infected plant cells into S phase. This idea is consistent with the facts that cell division could not be detected in infected leaf cells and that transgenic tobacco cells expressing AL1 have significantly increased doubling times in suspension culture (T.J. Pedersen and L. Hanley-Bowdoin, unpublished results). Progression through G_2 into M phase requires the activation of cell cycle–dependent protein kinases, such as cdc2. The SV40 large T antigen interferes with G_2 transit by preventing the activation of cyclin B–cdc2 complexes during lytic infection (Scarano et al., 1994).

The ability of TGMV AL1 to induce the accumulation of a host protein associated with DNA replication in terminally differentiated plant cells suggested that it is a plant homolog of mammalian tumor antigens. Three very different mammalian DNA viruses encode proteins that modify cell cycle controls by interfering with the action of the host tumor suppressor proteins, pRb and p53 (Hamel et al., 1992; Nevins, 1992; Sherr, 1994). Binding of pRb by the SV40 large T antigen, adenovirus E1A, and the human papillomavirus E7 proteins results in the release of host transcription factors (Chellappan et al., 1992; Morris et al., 1994; Mudrak et al., 1994), which then induce expression of proteins required for DNA replication (Nevins, 1992). The adenovirus E1A protein can also activate transcription of the PCNA gene directly (Morris et al., 1994). TGMV AL1 may use similar mechanisms to induce synthesis of plant DNA replication proteins. The sequence-specific DNA binding activity of AL1 could be involved in direct transcriptional regulation of host genes. Alternatively, AL1 may interact with a plant tumor suppressor protein(s) and modify cell cycle regulation in terminally differentiated cells of infected plants. The feasibility of this idea is strengthened by the recent identification in Arabidopsis of cyclin D homologs showing conservation of the pRb interaction motif (Soni et al., 1995).

Some components of the plant cell cycle have been identified by homology with fungal and animal proteins and by differential cDNA screening of synchronized suspension cells (for review, see Staiger and Doonan, 1993; Doerner, 1994). However, there is only limited functional information on plant cell cycle proteins, and this information is primarily from yeast complementation experiments and plant expression studies (Colasanti et al., 1993; Bergounioux et al., 1994; Fobert et al., 1994). There is no direct evidence for the existence of tumor suppressor proteins in plants. Plant homologs to pRb or to the related p107 tumor suppressor protein have not been found. A protein related to the tumor suppressor protein p53 has been detected (Georgieva et al., 1994), but its function has not been demonstrated. The approaches based on homology and complementation of animal and fungal systems have provided valuable information regarding plant cell cycle components but are unlikely to uncover plant-specific features. Analysis of

the mechanism(s) whereby AL1 induces the synthesis of a host DNA synthesis protein now provides a unique opportunity to study plant cell cycle regulation directly.

METHODS

Plant Materials

Nicotiana benthamiana plants were grown in a controlled environmental chamber at 25°C, 65% humidity, and a 14-hr-light/10-hr-dark photoperiod. Plants were agroinoculated with tomato golden mosaic virus (TGMV) using mixed cultures of *Agrobacterium tumefaciens* containing the T-DNA plasmids pMON337 (1.5 tandem copies of TGMV A) and pMON393 (1.5 tandem copies of TGMV B), as described by Elmer et al. (1988b). Tissues were harvested from infected plants exhibiting chlorosis and leaf curling at 12 to 18 days postinfection and from corresponding plants mock-inoculated with water. Alternatively, plants at the 4 to 6 leaf stage were bombarded using a Bio-Rad Biolistic PDS-1000/He system, according to the manufacturer's directions. Plasmid DNA containing partial tandem copies of the TGMV A (pTG1.3A) or TGMV B (pTG1.4B) components (Fontes et al., 1994a) was purified using a Qiagen plasmid purification kit (Qiagen, Inc., Chatsworth, CA). DNA (5 μg of each plasmid) was coated onto 1.0-μm microprojectiles and bombarded into plants at a pressure of 1300 psi. Aluminum foil was used to shield all but one expanded leaf on each plant. Tissues were harvested from systemically infected plants and control plants at 6 days postbombardment.

N. benthamiana plants transformed with the TGMV AL1 protein (pMON455 line 9985; Hanley-Bowdoin et al., 1990) or TGMV B (pMON393 line 3427; Elmer et al., 1988a) were germinated on media containing 0.43% Murashige and Skoog salts (GIBCO BRL), 3% sucrose, 0.01% inositol, 0.0001% thiamine-HCl, 0.02% KH_2PO_4, 0.8% agar supplemented with 300 mg/L kanamycin monosulfate and were transferred to soil at 3 weeks. Fully expanded leaves from 6-week-old plants were processed identically to TGMV-infected tissues. Symptomatic potato virus X (PVX)–infected *N. benthamiana* plants were generated as described by Hemenway et al. (1988) and harvested at 5 days postinoculation. Vegetative and floral meristematic tissues were harvested from 6-week-old healthy plants (*N. benthamiana*) and processed as described in the following sections.

Fixation and Sectioning of Plant Tissues

Leaves and stems were cut into 0.5- to 1.0-cm pieces and fixed for 48 hr at room temperature in 10 mM PBS, pH 7.2, containing 4% formaldehyde prepared from powdered paraformaldehyde (Fisher). Tissues were then stored in the fixative at 4°C until sectioned. Fixed tissues were embedded in 5% low-gelling-temperature agarose (Type XI; Sigma) in 10 mM PBS, pH 7.2, before sectioning. Thick tissue sections (50 to 60 μm) were cut using a Vibratome 1000 (Technical Products International, Inc., St. Louis, MO). Tissue sections were then transferred to 10 mM PBS, pH 7.2, 0.1% BSA and stored at 4°C.

Immunolocalization of Viral and Host Replication Proteins

All incubations were done at room temperature. Tissue sections were blocked in 0.5% goat IgG (Sigma) in 10 mM PBS, pH 7.4, 0.1% BSA (PBS-BSA) for 1 hr. After a 5-min rinse in PBS-BSA, sections were incubated for 1 hr with either an AL1-specific (Hanley-Bowdoin et al., 1990) or an AL3-specific (Pedersen and Hanley-Bowdoin, 1994) rabbit polyclonal antiserum diluted 1:250 in PBS-BSA. After three 10-min rinses in PBS-BSA, sections were incubated for 1 hr with a Cascade Blue–conjugated goat anti–rabbit secondary antibody (Molecular Probes, Eugene, OR) diluted 1:250 in PBS-BSA. After three 10-min rinses in PBS-BSA, the sections were mounted on Fisher ProbeOn Plus slides in 10 mM PBS, pH 7.4, that contained 90% glycerol. Visualization of the AL1- and AL3-specific signals was achieved using a Nikon Optiphot-2 microscope with epifluorescence (Southern Micro Instruments, Atlanta, GA). Color photographs were produced using a Nikon UFX camera system and Kodak Gold ASA 400 film.

Proliferating cell nuclear antigen (PCNA) was localized in Vibratome sections of TGMV-infected, transgenic AL1 and healthy meristematic tissues using a Vectastain Elite ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions with the following modifications. Sections were pretreated with 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity and remove chlorophyll. After incubation for 1 hr with 1:100 diluted mouse monoclonal antibodies raised against human PCNA (PC10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), sections were incubated with biotinylated secondary antibodies and then with the avidin-biotinylated enzyme complex for 1 hr each. The 3-amino-9-ethylcarbazole substrate (Vector Laboratories) was used at a development time of 20 min. Identical staining parameters were used for negative tissue controls. Sections were mounted as described previously and imaged using a Nikon Optiphot microscope and Nomarski differential interference contrast optics. Photographs were produced using a Nikon UFX camera system and Kodak Royal Gold ASA 25 film.

For colocalization of TGMV AL1 and host PCNA, fluorescent immunolocalization was performed as described previously, but a biotinylated goat anti–mouse secondary antibody followed by Cascade Blue–conjugated streptavidin was used for PCNA, and a BODIPY-FL–conjugated goat anti–rabbit secondary antibody (Molecular Probes) was used for AL1.

Protein Analysis

Whole-cell extract proteins were prepared from exponentially growing suspension cell cultures of *N. benthamiana*. Cells were collected on Whatman No. 1 filter paper, weighed, and transferred to a chilled mortar. Cells were ground under liquid N_2 to a fine powder and suspended in whole-cell extract buffer (40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 M sucrose, 10 mM β-mercaptoethanol, 0.8 mM phenylmethylsulfonyl fluoride, 0.01 mM benzamidine, 0.05 mM ε-aminocaproic acid, 5 μg/mL leupeptin) at 2 mL/g fresh weight. Cell debris was removed by filtration through Miracloth (Calbiochem, San Diego, CA). The filtrate was adjusted to 500 mM NaCl, mixed for 1 hr at 4°C, and centrifuged at 125,000*g* (1 hr at 4°C) to remove insoluble material. Soluble proteins were resolved by SDS-PAGE (12.5% polyacrylamide), transferred to nitrocellulose, and analyzed by immunoblotting (Pedersen and Hanley-Bowdoin, 1994). Protein blots were blocked in TBS (20 mM Tris-HCl, pH 7.4, 137 mM NaCl) and 5% nonfat dry milk (TBSM). Blots were incubated with the anti-PC10 antibody diluted 1:2000 in TBSM. Proteins were visualized after incubation with a sheep anti–mouse antibody conjugated to horseradish peroxidase (diluted 1:5000 in TBS and 0.1% Tween 20) using an enhanced chemiluminescence detection system (Amersham).

The recombinant AL1 protein was produced in *Escherichia coli* BL21(DE3) transformed with the bacterial expression cassette

pMON1539, containing the AL1 open reading frame under the control of the T7 promoter (Hanley-Bowdoin et al., 1990). The AL1 protein was purified from the refractile body fraction using SDS-PAGE as described previously (Hanley-Bowdoin et al., 1990). The recombinant His–AL3 protein was produced from *E. coli* transformed with the bacterial expression cassette pNSB195, containing an AL3 open reading frame fusion with 10 histidine codons at its 5′ end (Pedersen and Hanley-Bowdoin, 1994). The recombinant His–AL3 protein was purified from the refractile body fraction by Ni^{+}-affinity chromatography in the presence of 6 M guanidine-HCl (Pedersen and Hanley-Bowdoin, 1994). Recombinant human PCNA was expressed in *E. coli* and purified by Q-Sepharose and phenyl-Sepharose chromatography (Fien and Stillman, 1992).

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