Replication of the DNA A component of African cassava mosaic virus in a heterologous system

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The capacity for autonomous replication of the DNA A of African cassava mosaic virus (ACMV), a member of the bipartite geminiviruses infecting dicotyledonous plants, has been compared in host and non-host cells. A derivative of the ACMV DNA A was transfected into tobacco and maize protoplasts. Although ACMV is not able to infect maize, replication of the DNA A in maize protoplasts was observed to occur. The efficiency of replication was 10 to 20% of that seen in tobacco protoplasts. In both plant systems, replication was detected after the onset of cell division. ACMV replication in maize cells was compared to that of wheat dwarf virus and found to be 10 to 20% of that observed with the monocotyledon-specific virus. Insertion of 1165 bp of non-viral DNA into the ACMV DNA A prevented replication in maize but not in tobacco.

Introduction

The genome of African cassava mosaic virus (ACMV) consists of two ssDNA molecules, DNA A [2779 nucleotides (nt)] and DNA B (2724 nt) (Stanley & Gay, 1983). The genes coding for viral functions such as replication, encapsidation and viral spread are distributed bi-directionally on both DNAs, and thus both are required for infection (reviewed by Davies & Stanley, 1989; Lazarowitz, 1992). DNA A alone is capable of autonomous replication in dividing host protoplasts since it provides all the functions needed for self-amplification (Townsend et al., 1986). The gene coding for the coat protein, located on DNA A, can be removed or replaced by non-viral DNA without abolishing replication and infection in the presence of DNA B (Townsend et al., 1986; Ward et al., 1988). This virus belongs to the subgroup of bipartite geminiviruses that are whitefly-transmitted and infect dicotyledonous plants (dicots).

Wheat dwarf virus (WDV) is a representative of the second subgroup of geminiviruses which are transmitted by leafhoppers and infect monocotyledonous plants (monocots). WDV has a 2749 nt long circular genome organized in a similar way to the DNA A of the bipartite geminiviruses. Removing or replacing the WDV coat protein gene has no influence on replication, but viral spread and development of systemic symptoms on inoculated plants is abolished (Woolston et al., 1989). The replication-associated proteins of ACMV and WDV are of similar Mr, and for amino acid sequences show 36% identity and 56% similarity (Schalk et al., 1989). In ACMV, the protein is encoded by a single open reading frame (ORF) (AC1), whereas in WDV two overlapping ORFs (C1 and C2), located at a similar position within the replicon, must be joined via RNA splicing to produce the replicase-like protein.

It is known that non-viral, monocot-specific introns are inefficiently spliced in dicots (Keith & Chua, 1986), whereas dicot-specific introns can be as efficiently spliced in monocots as in dicots (Peterhans et al., 1990; Goodall & Filipowicz, 1991). Thus, it has been suggested that the RNA maturation step required by monocot-infecting geminiviruses might be involved in determination of the host range (Schalk et al., 1989; Mullineaux et al., 1990). Indeed, in replication studies of digitaria streak virus (DSV), another monocot-specific geminivirus, in transgenic tobacco the complementary pre-mRNA for the replicase-like protein was produced but no splicing occurred and hence no replication was detected (Mullineaux et al., 1990).

In addition to the fact that splicing of viral sequences depends on host machinery, other host-specific functions may be indispensable for viral replication. Since splicing seems to impair replication of monopartite geminiviruses in dicot cells, heterologous replication can only be studied independently of splicing specificities. An ob-
vious experiment is the examination of the replication ability of the DNA A component of a dicot-specific geminivirus in monocot cells. This would allow two questions to be addressed: is the origin of replication of the dicot-infecting virus recognized in a monocot cell environment and is it possible that a geminivirus unable to infect a particular plant species systemically can still replicate within inoculated cells of this plant (subliminal infection) or does, in the chosen plant–virus combination, inability of infection parallel inability of replication? In order to address these questions, we have studied the efficiency of replication of the DNA A component of ACMV in *Nicotiana tabacum* and *Zea mays*. Our results indicate that non-host cells support the replication of heterologous viral replicons. However, the efficiency of this process is considerably reduced compared to that in host cells.

**Methods**

**Constructs.** The partial dimer of the DNA A component (pUNpd, Fig. 1) was generated by cloning a head-to-tail dimer of the viral DNA A monomer of pCLV020 (Etessami et al., 1988; courtesy of J. Stanley) as *ClaI* fragments into pCB1 (Peterhans et al., 1990) and resulted in pCBCLVdim. After a partial *DraI* digestion the *DraI–MscI* fragment spanning two-thirds of the second viral genome had been deleted to give the plasmid pUNpd (Fig. 1). The 1165 bp *EcoRV* fragment of pABD1 (Paszkowski & Saul, 1984) harbouring the neomycin phosphotransferase II (*NptII*) gene was inserted into the *SmaI* site of pLT98 (Dixoa et al., 1983). This allowed the transfer of the *NptII* gene as a *SalI* fragment into the *XhoI* site of pUNpd and resulted in pPDkan (Fig. 1). The construct containing a partial dimer of WDV (pWDVneo2) was kindly provided by B. Gronenborn and was a derivative of pWDVneo1 (Maizeit et al., 1991).

The methods used for DNA manipulations and cloning in *Escherichia coli* were as described by Mamiatis et al. (1982). Restriction endonucleases, DNA ligase and DNA polymerase Klenow fragment were purchased from Boehringer, Pharmacia and New England Biolabs, respectively, and used as recommended by the manufacturers.

**Preparation and transformation of tobacco protoplasts.** Mesophyll protoplasts of *N. tabacum* cv. Petit Havana line SR1 (Maliga et al., 1973) were isolated following the modified protocol of Nagy & Maliga (1976) and transformed using the chemical method of direct gene transfer as described by Negrutiu et al. (1987).

Circular DNA (5 µg) was introduced into 10⁶ protoplasts in the absence of carrier DNA. After transformation, the protoplasts were cultured in 4 ml liquid medium (1:1) (described in Nagy & Maliga, 1976; Potrykus & Shillito, 1986) at 25 °C with a photoperiod of 16 h light and 8 h darkness. The time points for protoplast harvest were 0 days (2 h post-transformation), 4 days and 6 days post-transformation.

**Preparation and transformation of maize protoplasts.** Maize protoplasts were released from embryogenic calli previously initiated from anther culture of the genotype Bai 17. The protocol used for protoplast isolation and transformation was as published by Zhang et al. (1990). In contrast to tobacco, maize protoplasts do not divide in liquid medium and must be cultured in medium solidified by agarose. Protoplasts (10⁶) were plated in 4 ml of culture medium. Harvest time points were 0 days (2 h), 7 days and 14 days post-transformation.

**Tobacco DNA isolation.** The culture containing tobacco protoplasts was transferred to tubes, diluted to 10 ml with wash solution W5 (Nagy & Maliga, 1976) and collected by centrifugation (450 r.p.m.) for 5 min. The protoplast pellets were transferred to Eppendorf tubes and frozen in liquid nitrogen. The frozen cells were homogenized using a glass rod in 400 µl of extraction buffer (10 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 10 mM-EDTA, 1% sarcosyl) and extracted with phenol saturated with TE (10 mM-Tris–HCl pH 8.0, 1 mM-EDTA). After the extraction, the DNA was precipitated from the aqueous phase with 2.5 vol. of ethanol and resuspended in TE containing RNase A at a final concentration of 200 µg/ml. After RNase digestion for 15 min at 37 °C, proteinase K and SDS were added to final concentrations of 50 µg/ml and 0.02%, respectively. Incubation was continued for 1 h at 37 °C. After an additional phenol extraction and ethanol precipitation, the DNA was dissolved in TE.

**Maize DNA isolation.** Segments of agarose (4 ml) containing the maize cells were melted at 70 °C in order to release the embedded cells. Homogenization buffer (50 mM-Tris–HCl pH 8.0, 50 mM-EDTA) at the same temperature was added to a maximum of 12 ml and the cells were harvested as described for tobacco. The cells were homogenized using a glass rod in 200 µl homogenization buffer containing sand. SDS was then added to a final concentration of 0.2% and the samples were incubated at 70 °C for 20 min. Potassium acetate was added to a final concentration of 2 M followed by incubation on ice for 10 min. The precipitate was sedimented by full-speed centrifugation in a table-top centrifuge for 10 min, and the DNA was ethanol-precipitated from the supernatant and resuspended in TE.

**Southern blot hybridization.** Non-restricted, total plant DNA was separated on a 1% agarose gel, transferred to Hybond-N nylon membrane (Amersham) and hybridized under previously described conditions (Peterhans et al., 1990) either to a DNA A-specific probe of ACMV, WDV or to the vector plasmid (pCB1).
Results

The direct introduction of DNA into plant cells is commonly achieved by transformation of protoplasts. Transformed protoplasts from different species respond differently in tissue culture and need specific culture periods for the regeneration of the cell wall and subsequent initiation of cell division. Replication of geminiviruses is dependent on replication of the host DNA (Etessami et al., 1991), which can be monitored by the time and frequency of cell division (Townsend et al., 1986). Therefore, treated cells harvested from different plant systems represent different developmental stages of cell culture. For example, tobacco protoplasts start to divide after 2 to 3 days, whereas maize protoplasts need approximately 7 days. The time points for cell harvest were adjusted to coincide with similar stages of culture development, namely 3 and 6 days for tobacco and 7 and 14 days for maize, corresponding to the onset of cell division and of microcalli formation with four to eight cells. In the experiments performed in maize, we included a partial dimer of the monocot-specific WDV which is capable of autonomous replication in monocot cells but owing to the replacement of the coat protein by the NptII gene this WDV derivative lost its ability to spread systemically in inoculated plants (Matzeit et al., 1991; Woolston et al., 1989). This construct was used as a standard control which allowed us to assay the replication efficiency and the viability of the plant cells independently from the variation in transformation frequency.

Replication in Nicotiana tabacum

Efficient replication of viral DNA was observed following transformation of tobacco protoplasts with the partial dimer of the DNA A component of ACMV (pUNpd). No replication was detected at 0 days, but after the onset of cell division all expected replicative forms, such as supercoiled, linear and open circular, were present. Analysis of the newly appearing viral dsDNA forms with methylation-sensitive restriction endonucleases (data not shown) as well as the lack of reciprocal products of homologous recombination demonstrated that accumulation of free replicons is indeed due to de novo synthesis. The supercoiled form was the most abundant (Fig. 2, lanes 2, 3). Variation in the accumulation levels of ssDNA is commonly observed for monocot- and dicot-specific geminiviruses after transformation of coat protein mutants into plant cells. Autonomous replication was also observed after the
of linear and open circular forms, was detected on day 14 (Fig. 3a, lane 3). Rehybridization of the same filter with the plasmid vector (pCB1) showed decreasing amounts of inoculum DNA. No reciprocal recombination products were found, even after prolonged exposure, thus confirming that the viral dsDNA forms are due to replication and not a product of recombination (Fig. 3b, lanes 1 to 3). However, replication of the ACMV replicon in maize was 10 to 20% of that in tobacco and was correspondingly less efficient than the replication of the partial dimer of WDV in maize (Fig. 4). The consistently high replication of WDV indicated that variation in protoplast transformation frequency or variation in the viability of maize cultures can not account for the lower replication of ACMV. Replication of the enlarged replicon (pPDkan) was not detected (data not shown).

Discussion

Establishment of infection by geminiviruses requires molecular cooperation between host and viral proteins and the success of this interaction may be a major factor in determining the viral host range. Geminiviruses consist of two main subgroups divided according to their host range and insect transmission vector. It has been proposed that their different genome organizations could be involved in determination of the host species. For example, monopartite, monocot-infecting geminiviruses (e.g. WDV, DSV) need an mRNA splicing step to produce the replication-associated C1::C2 protein (Schalk et al., 1989; Mullineaux et al., 1990), whereas AC1 from bipartite, dicot-infecting geminiviruses (e.g. ACMV) is encoded by a continuous ORF. Examples of exceptions to this classification have been described recently. One of them, tobacco yellow dwarf virus, although infecting dicots, possesses features typical of the monocot-infecting subgroup including the requirement for mRNA splicing for production of its functional C1::C2 protein (Morris et al., 1992).

It has been suggested that within the replication complex of host factors and the viral AC1 protein, the latter might be responsible for the recognition of the viral origin of replication and also for the initial nicking of the supercoiled DNA to start replication via a rolling circle (Fontes et al., 1992; Lazarowitz et al., 1992 and citations therein). We have shown that the AC1 protein of ACMV can interact with the non-host replication machinery of maize leading to replication. The reduced replication efficiency to between 10 and 20% suggests that the proteins of the heterologous host involved in the replication complex can still assemble with the viral AC1 protein but in a suboptimal manner. Furthermore, insertion of non-viral DNA into the replicon (pPDkan)
reduced the replication efficiency in tobacco to 30% of the level of the replicon without an insert. Assuming a similar rate of reduction in maize, replication of this construct should have been observed. The lack of detectable replication might suggest narrow size constraints of the ACMV replicon in heterologous systems.

Although the AC1 protein is the only viral protein that is essential for viral replication it could be speculated that other viral proteins that modulate viral replication in host cells could affect the optimal assembly or functionality of the replication complex in a heterologous system. A possible candidate is the AC3 protein of ACMV, which is encoded in the complementary-sense orientation on the DNA A component and can act as a modulator of replication (Etessami et al., 1991). Mutants with lesions of this ORF are still able to infect the host plant, but with delayed symptom development due to a reduced level of replication. Dot blot analysis of tissue extracts showed that the AC3 mutant replicated to only 10 to 20% of the levels of the wild-type. Also in the heterologous system it may well be that the contribution of the AC3 protein to efficient replication is somewhat impaired leading to a reduction of replication.

In summary, we conclude that replication complexes of virus and non-host factors can form in a heterologous system and result in replication. Further experiments on homologous gene replacement between the two viral replicons should reveal the viral proteins responsible for the host specificity of replication.

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References


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