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Review

Geminivirus DNA replication

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Abstract. Geminiviruses are DNA viruses which infect plants. They have a small genome and encode only a few proteins. Therefore, their DNA replication cycle relies largely on the use of cellular DNA replication proteins. The strategy used by geminiviruses to replicate their single-stranded DNA (ssDNA) genome consists of a first stage of conversion of ssDNA into double-stranded DNA (dsDNA) intermediates and, then, the use of dsDNA as a template to amplify viral dsDNA and to produce mature ssDNA genomes by a rolling-circle replication mechanism. In addition, the accumulating evidence indicates that viral DNA replication is somehow coupled to the cell cycle regulatory network of the infected cell. For these reasons, geminiviruses are excellent model systems to understand the regulation of DNA replication and cell cycle in plant cells. Recent years have witnessed significant progress in the identification of cis-acting signals and their interaction with trans-acting factors that contribute to geminivirus origin function. These and other aspects of the geminivirus DNA replication cycle will be reviewed.

Key words. DNA replication; rolling circle; geminivirus; plant; protein-DNA complex; transcription.

Introduction and scope

The Geminiviridae family includes a large number of viruses that infect plants and produce in many cases very significant reductions in economically important crops of both monocotyledonous and dicotyledonous plants. They are, basically, characterized by two distinctive features: (i) the morphology of the virion particle which is geminate, ~18–30 nm in size, and has the appearance of two quasi-icosahedral moieties with a total of 22 pentameric capsomers, and (ii) the nature of their genetic material, consisting of one or two single-stranded DNA (ssDNA) molecules, 2.5–3.0 kb in length [1, 2]. The geminivirus replicative cycle relies entirely on DNA intermediates and occurs within the nucleus of the infected cell. Recent and rapid advances in the elucidation of the molecular and cellular biology of geminiviruses make them excellent model systems to study DNA replication and cell cycle events in plants. In addition, insights into their replication cycle and the molecular mechanisms of virus-cell interactions should help in the rational design of novel strategies for disease control.

Different aspects of the biology of geminiviruses have been the subject of comprehensive reviews [3, 4; see also 5–7, for early references]. Their potential use as plant expression vectors [8–10] as well as their refined intra- and intercellular movement strategy [3, 11] have been also the subject of more specialized reviews. Finally, results from the perspective of their mechanism of DNA replication have been partially covered [3, 4, 12–15]. However, this aspect together with the likely connection between geminivirus DNA replication and the regulatory circuitry controlling cell cycle progression and maintenance of proliferative capacity are very fast mov-

ing and expanding fields. Thus, the scope of this review article is to discuss these topics, specially emphasizing the molecular characteristics which are shared by different geminiviruses and those which seem to have evolved uniquely in the different genera. It is worth noting that some results obtained for geminiviruses would likely apply to other, less-studied ssDNA viral replicons from plant [16] and animal [17] origin.

Geminiviridae family members differ in their genomic organization

The differences in the genetic organization of geminiviruses as well as their host range and insect vectors serve as criteria for recognizing three different genera. Mastreviruses [maize streak virus (MSV) as type member] are transmitted by a variety of leafhopper (Cicadellidae) species, have a monopartite genome and, generally, infect monocotyledonous species (wheat dwarf virus, WDV; *Digitaria* streak virus, DSV; see [1, 2] for a comprehensive list). Interestingly, two members have been isolated, so far, that infect dicotyledonous plants, namely, tobacco yellow dwarf virus (TYDV) and bean yellow dwarf virus (BYDV). The *Mastrevirus* genome contains two intergenic regions, one large (LIR) and another small (SIR) located at opposite sides of the viral genome (fig. 1). Two features are unique to this genus: (i) the presence of an ~80 nt-long DNA sequence annealed to a region within the SIR, which is present already inside the viral particle, and (ii) the

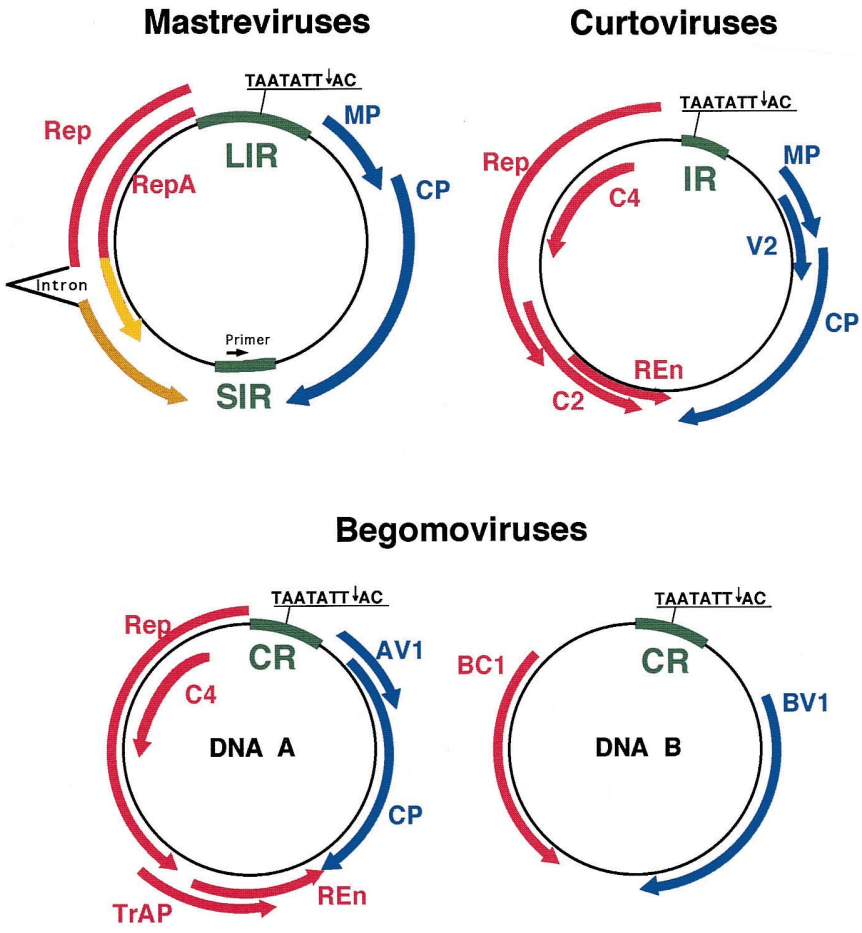

Figure 1. Genome organization of the three geminivirus genera. The genetic organization of the dsDNA forms are represented. The sequences regulating DNA replication and transcriptional activity are located in the intergenic regions. Mastreviruses contain a large (LIR) and a small (SIR) intergenic region, to which a small DNA molecule (primer) is associated. The invariant TAATATT↓AC sequence located in the LIR (mastreviruses), IR (curtoviruses) and CR (begomoviruses) containing the initiation site (↓) of rolling-circle DNA replication is shown. Arrows indicate the viral proteins which have been named according to either their function, if known, or their genetic location and direction of transcription: MP, movement protein; CP, capsid protein; Rep, replication protein; TrAP, transcriptional activator; REn, replication enhancer. The RepA protein is also shown in mastreviruses.

occurrence of a splicing event of the complementary-sense (c-sense) transcript. Four proteins are generally considered to be encoded by the *Mastrevirus* genome: the movement protein (MP) and the capsid protein (CP), on the viral-sense (v-sense) strand, and the RepA protein, exclusive of this genus, and the Rep protein, on the c-sense strand.

Curtoviruses (beet curly top virus (BCTV) as type member) are also transmitted by leafhoppers, have a monopartite genome, although with a genetic organization different from that mastreviruses, and infect dicotyledonous plants (fig. 1). They apparently occupy an intermediate phylogenetic position [18]. In addition to MP and CP, their genome encodes a V2 protein on the v-sense strand, whereas on the c-sense strand, four open reading frames (ORFs) exist, namely Rep, C2, REn (replication enhancer) and C4.

Begomoviruses [bean golden mosaic virus (BGMV) as type member] are transmitted by the whitefly *Bemisia tabacci*, have bipartite genomes (A and B components), except in a few cases [19], and infect dicotyledonous plants (fig. 1). Other members frequently used in DNA replication studies are tomato golden mosaic virus (TGMV), squash leaf curl virus (SqLCV) and tomato yellow leaf curl virus (TYLCV). They have the most complex genome among all geminiviruses. The A component encodes for the CP, on the v-sense strand, and a set of four proteins, Rep, TrAP (a transcriptional activator), REn and C4, on the c-sense strand. The B component encodes proteins directly involved in movement—BC1 and BV1—on the c-sense and v-sense strands, respectively.

The expression of viral proteins occurs from the circular dsDNA intermediates, the transcriptionally active DNA forms, which contain two sets of genes whose transcription occurs in a divergent way, separated by intergenic regions where the cis-acting signals regulating viral replication and transcription are located. Within this intergenic region, all geminiviruses sequenced to date possess a characteristic inverted repeat, variable in sequence and length, separated by a sequence which contains an invariant 9-nt stretch (TAATATTAC). In general, genes encoded on the c-sense are involved in DNA replication, in regulation of transcription and, most likely, in interfering with cellular processes needed for the replicative cycle, whereas genes encoded on the v-sense have movement and structural functions.

The viral replicative cycle: an overview

The geminivirus replication cycle can be subdivided in several functionally distinct stages characterized by specific events (fig. 2). Early during the infection process, viral particles are injected by the insect vector, presumably uncoated, and the viral genome is transported into the host cell nucleus by mechanisms whose molecular details are largely unknown [3, 4]. Once within the nucleus, amplification of the viral genome, which involves an efficient DNA replication process, occurs in three distinct stages [20–25]. First, stage A involves the conversion of the genomic circular ssDNA [(c)ssDNA] into supercoiled covalently closed circular dsDNA intermediates or replicative form I [(ccc)dsDNA(RFI)]. Second, or stage B, is the amplification of the dsDNA intermediates by a rolling-circle mechanism. Third, or stage C, is the production and encapsidation of mature genomic circular ssDNA into viral particles. This strategy is remarkably similar to that of many prokaryotic replicons [26, 27]. Although direct evidence for each of the steps of the geminivirus DNA replication cycle awaits the development of an in vitro DNA replication system, some of the characteristics defining the molecular events that take place will be discussed below.

Stage A: conversion of (c)ssDNA into (ccc)dsDNA(RFI)

Initiation of (−)strand DNA replication

The conversion of the viral DNA, also named the (+)strand, into a dsDNA intermediate requires the activation of the so-called (−)strand origin of DNA replication by a priming event. In the case of mastreviruses, but not in curto- and begomoviruses, a small ssDNA molecule annealed to the (+)strand is encapsidated [28–32]. The observation that this small complementary DNA molecule is capable of being extended in vitro supports the view that it can act as a primer at this early stage of viral DNA replication. Its 5′ end contains a few ribonucleotide monophosphates (rNMPs) which may be the consequence of initiation by the activity of a DNA primase. Its size, ~80 nt in all cases studied, is a mystery, and it suggests that the priming step is followed by the use of the newly synthesized RNA primer as a substrate for a DNA polymerase complex with a limited processivity. Alternatively, a mechanism involving a regulated arrest of DNA synthesis may also operate. Both DNA primase and DNA polymerase activities which can fulfill the requirements to complete the initiation of (−)strand DNA replication have been identified in several plant systems, especially from monocot origin, such as wheat [33] and maize [34]. Before a definite demonstration that the priming step involves the synthesis of an RNA primer by a DNA primase, it should be kept in mind that several priming mechanisms have been identified in prokaryotic replicons to initiate (−)strand DNA replication (reviewed in [26])

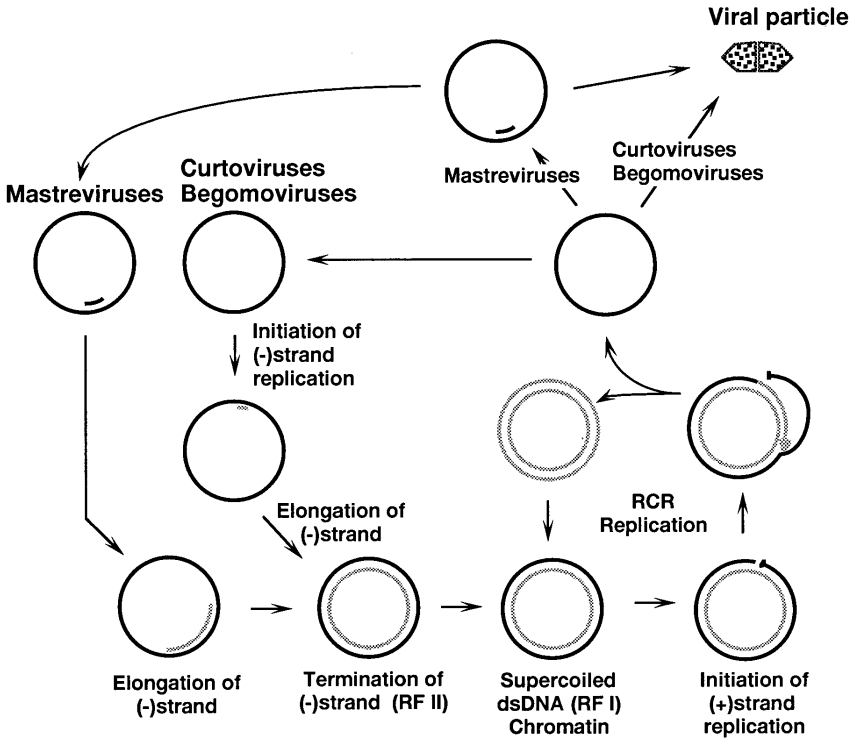

Figure 2. Summary of the geminivirus replicative cycle. Proposed stages of the geminivirus DNA replication cycle. Stage A: conversion of circular ssDNA into covalently closed circular dsDNA intermediates (RF I). Stage B: rolling-circle replication (RCR). Stage C: production of circular ssDNA genomes for encapsidation. See text for details.

which depend on the activity of a primosome (e.g. phage ϕX174), a DNA primase alone (e.g. phage G4) or the host RNA polymerase (e.g. phage M13). This is important given that in the related plant *Nanovirus* genus there is no evidence for rNMPs at the 5′ end of the virion-associated small DNA molecule [35]. Thus, although a primosome-like priming mechanism for initiation at the (−)strand origin likely takes place, detailed studies on this step are required to delineate the process at the biochemical level.

Neither the mechanism controlling the selection mechanism of the site where DNA replication initiates at the (−)strand origin nor the cis-acting signals involved are known. The fact that the small complementary DNA is annealed at the SIR of the *Mastrevirus* genome strongly suggested that this region contains the (−)strand origin, a conclusion reinforced by the absolute requirement of this genomic region for efficient MSV amplification [36, 37]. The situation seems to be quite different in other geminiviruses whose genome lacks the small complementary DNA molecule. Although (−)strand DNA replication has been shown to be RNA-primed in African cassava mosaic virus (ACMV; [25]), the 5′ end of the newly synthesized (−)strand DNA molecule lies within a region spanning the map nucleotide positions 2581 to 221 [25]. Therefore, the intergenic region (IR) contains the cis-acting signals defining the (−)strand origin in begomoviruses and most probably also in curtoviruses. This conclusion is reinforced by the ability of constructs containing the IR, which also contains the (+)strand origin, as discussed later, to support efficient begomovirus DNA replication [38]. It will be necessary in the future to identify precisely the minimal sequences required to activate the (−)strand origin both within the SIR of mastreviruses and within the IR of curto- and begomoviruses.

Elongation: production of (ccc)dsDNA

As in the case of the priming step, elongation of primed ssDNA circles and synthesis of full-length circular covalently closed dsDNA, also named replicative form II (RFII), relies entirely on host enzymes. The replicative complex which carries out this step should contain a processive DNA polymerase most likely in association with accessory factors. Candidates to be present in such a complex are the α-like and/or δ-like DNA poly-

merases which have been identified in several plant species such as wheat [39–42], maize [43–46] and pea [47]. A challenge for the future is to identify which DNA polymerase activities act during this stage of the geminiviral DNA replication cycle.

An additional complication comes from analysis of geminivirus DNA replication by two-dimensional agarose gel electrophoresis. Saunders et al. [25] found that a previously unidentified replicative intermediate, most likely consisting of full-length circles partially ss-DNA and partially dsDNA, accumulate during infection with ACMV. The mechanism by which such an intermediate is formed, its significance and whether this step is common to other geminiviruses is not presently known.

Production of supercoiled covalently closed circular dsDNA (RFI)

Early studies on the topological forms of circular ds-DNA isolated from TGMV-infected plants revealed that one of the prominent replicative forms was supercoiled viral DNA [48]. About 16 superhelical turns were estimated to be present per TGMV DNA circle [20]. It is not likely that conversion of RFII, the relaxed covalently closed circular intermediate, into supercoiled ds-DNA circles (RFI) occurs by a mechanism relying on a DNA gyrase, as in prokaryotic systems [49]. Rather, the mechanism most probably operating during geminivirus DNA replication depends on the association of RFII molecules with histones from the host cell. Thus, nucleosome formation would produce a geminiviral minichromosome, much in the same way as in the simian virus 40 (SV40; [50]) or the cauliflower mosaic virus (CaMV; [51]). Nucleosome-like particles have been identified in replicative forms of *Abutilon* mosaic virus (AbMV; [52]). Micrococcal nuclease digestion analysis of viral replication products revealed the appearance of DNA fragments consistent with the idea that geminivirus DNA is packed into minichromosomes [53]. One possibility is that nucleosome association does not take place once a full-length complementary DNA strand is synthesized but, rather, that it is coupled to the DNA replication process as in the case of SV40 [54–57].

Stage B: conversion of supercoiled (ccc)dsDNA into dsDNA and ssDNA by rolling-circle replication (RCR)

The viral RFI serves as template for further DNA replication steps as well as for viral transcription. Viral transcription, RNA processing and the effects of viral proteins on viral gene expression have been recently reviewed [4, 15]. This is the first replicative stage where some viral proteins participate in viral DNA replication. Viral proteins are either absolutely required at this stage or modulate the product yield. Before discussing the RCR mechanism, I will summarize the properties of the geminivirus proteins with a function at some stage of the viral DNA replication cycle. Some of them, e.g. RepA, are unique to mastreviruses; others, such as REn, are encoded by members of the other two genera, and others, such as Rep, are common to all geminiviruses, although they may play some specific roles in different genera.

Viral proteins with a function during viral DNA replication

RepA (mastreviruses). In mastreviruses, two c-sense transcripts with the same 5′ end have been detected [58]. One striking feature, characteristic of this genus, is that one of the messenger RNAs (mRNAs) is originated from the other after a splicing event which removes a small (~ 80–90 nt) intron [59]. The unspliced mRNA, which accounts for most of all c-sense transcripts, contains an ORF capable of encoding RepA, an ~ 31-kDa protein (fig. 3). Although its presence in infected cells has not been demonstrated yet, several lines of evidence strongly support the idea that it may be required at different stages during the viral replicative cycle. First, the unspliced mRNAs exhibit some heterogeneity at their 3′ ends, and in some cases, the truncated mRNAs cannot be spliced and thus can be translated into the RepA protein [58, 60]. Second, RepA is required for viral-sense transcription responsible for the production of MP and CP, and it has been proposed to negatively regulate viral DNA replication [61]. Third, the C-terminal half of MSV RepA contains a unique domain with trans-activation ability in yeast [62]. Fourth, the C-terminal domain of RepA mediates its binding to cellular GRAB (for geminivirus RepA-binding) proteins [63], a recently identified family of cellular factors containing the NAC (for the no-apical-meristem, ATAF and CUC2 genes) domain [64, 65], which when overexpressed, strongly inhibits WDV DNA replication in cultured wheat cells [63]. Fifth, binding to the retinoblastoma (Rb) protein, which regulates the transition from G1 to the S phase of the cell cycle, seems to be a strategy developed by all geminiviruses and is very likely mediated by RepA in mastreviruses and by Rep in the other two genera (see [66] and below). Sixth, the conservation through evolution of the splicing event of the c-sense transcript in all mastreviruses also suggests a requirement for RepA protein.

RepA shares ~ 75% of its primary sequence with Rep, e.g. the N-terminal 210 amino acids in the case of WDV (fig. 3). Therefore, in this region, the same motifs are found in both *Mastrevirus* RepA and Rep. The roles of

these common motifs in DNA binding, nicking and joining, and oligomerization will be discussed below in the section on Rep protein. Also in this region, an LxCxE amino acid (single letter code) motif which mediates its binding to human Rb protein was identified [67]. This observation was later confirmed with plant Rb proteins for WDV RepA [68, 69], MSV RepA [62] and BYDV RepA (L. Liu and J. Stanley, personal communication). The LxCxE Rb binding motif is conserved in all mastrevirus RepA proteins [67] except in the available sequences for sugarcane streak virus (SSV) and *Miscanthus* streak virus (MiSV). Interestingly, the SSV isolate analyzed in the initial study [67], which lacks an LxCxE motif, is not infectious [70]. All these studies reinforce the idea that mastrevirus RepA protein may have a functional role during the viral DNA replication cycle through its coupling to the cell cycle regulatory machinery of the infected cell, most likely to generate a cellular environment favorable for viral DNA replication (see also discussion below).

Rep (all geminiviruses). All geminiviruses encode a Rep protein, the replication initiator protein, also named C1:C2 in mastreviruses, C1 or L1 in curtoviruses and AC1 or AL1 in begomoviruses. Rep is an ~40-kDa multifunctional, highly conserved protein in all members, which is necessary and sufficient for viral DNA replication [59, 71–73]. In mastreviruses, Rep is translated from the spliced c-sense mRNA [59, 60]. Geminivirus Rep protein is a sequence-specific DNA-binding protein with site-specific nicking and joining activity, required for initiation and termination of rolling-circle DNA replication, that contains a nucleosite triphosphate (NTP) binding motif, homo- and heterooligomerizes and, in some cases, interacts with Rb. In addition, it regulates viral gene expression (reviewed in [4]).

Alignment of the primary sequence of geminivirus Rep with initiator proteins of prokaryotic rolling-circle replicons revealed the existence of three highly conserved motifs (RCR-I through -III) within the N-terminal ~120 amino acids [74, 75]. This conservation is maintained with initiator proteins of parvoviruses, animal circoviruses and plant nanoviruses (fig. 4) which all use rolling-circle mechanisms of DNA replication. The function of motif RCR-I (FLTYPxC) is not known. Motif RCR-II (HLHxxxQ) is likely involved in the coordination of a divalent cation (Mg^{2+} or Mn^{2+}) through the invariant histidine residues. Motif RCR-III (VxDYxxK) is required for the initiation (nicking) activity through the invariant tyrosine residue [76]. In addition, the conserved lysine residue is essential for viral DNA replication [77]. Initial studies with bacterially expressed WDV Rep [78] and TYLCV Rep [79] demonstrated that it has a site-specific nicking activity within the invariant 9-nt sequence of the loop (TAATATTAC). This activity is directly responsible for the initiation of (+)strand DNA replication (discussed

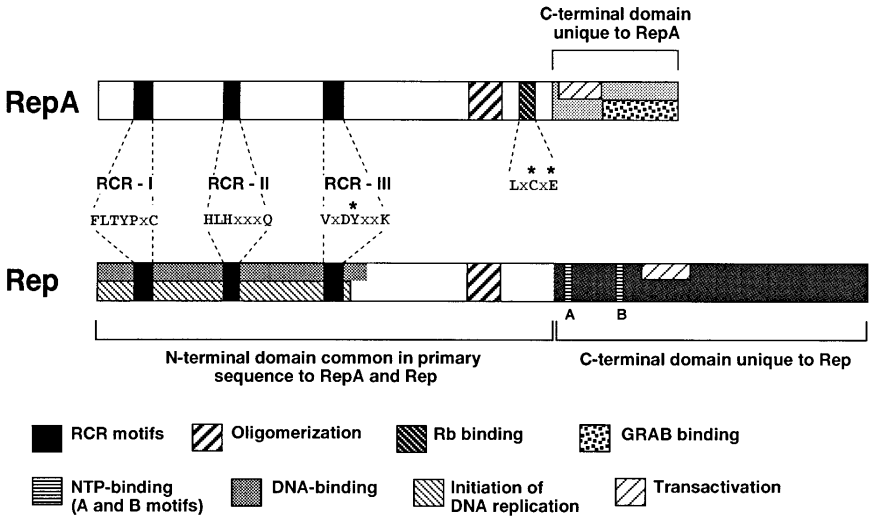

Figure 3. Domain organization of RepA (mastreviruses) and Rep (all geminiviruses) proteins. The different domains (boxes) have been identified by amino acid sequence homology, deletion analysis and point mutational analysis (asterisks in conserved motifs). Boxes represent a composite gathering information available for WDV and MSV RepA, and for WDV, MSV, TGMV and TYLCV Rep. Thus the location of each motif within the protein is approximate. Note that the boundaries of the DNA binding and oligomerization domains, determined by deletion analysis, do not match in proteins from different viruses. Also note that *Mastrevirus* (such as WDV and MSV) Rep has a LxCxE motif which is lacking in *Begomovirus* (such as TGMV) Rep. The details of each protein domain are discussed in the text.

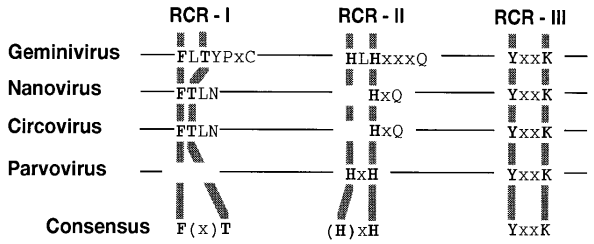

Figure 4. Conserved motifs involved in RCR of eukaryotic ssDNA replicons. Summary of the three highly conserved motifs (RCR-I to -III) present in the Rep proteins encoded by eukaryotic rolling-circle replicons: plant geminiviruses and nanoviruses, and animal circoviruses and parvoviruses.

below) since the same sequence was previously identified by genetic analysis to be the site where DNA replication starts in vivo [23, 80]. The N-terminal domain of Rep, which contains the three conserved RCR motifs, is necessary and sufficient for the nicking activity of Rep [78]. In this respect, mastrevirus RepA protein behaves in a similar way since it also contains the three RCR conserved motifs [78]. The same domain is responsible for the joining activity of Rep [78, 79], which most likely plays a role at the termination step of rolling-circle DNA replication (see below).

Geminivirus Rep contains an NTP binding domain, with typical A- and B-motifs [81], which contributes to its adenosine triphosphatase (ATPase) activity [82]. Point mutations within the conserved NTP binding domain abolish the intrinsic ATPase activity of Rep [82] and drastically reduce viral DNA accumulation [82, 83]. However, Rep ATPase activity is not required for the cleavage reaction using oligonucleotides containing the nicking site [78]. Therefore, the exact role of this ATPase activity still has to be elucidated. Since it is DNA-independent, it is unlikely that the NTP binding domain contributes to a helicase activity of Rep.

Studies with TGMV Rep have shown that it can form oligomers in solution [84]. The oligomerization domain has been mapped between residues 134 and 181 in TGMV Rep [85] and between residues 175 and 187 in MSV Rep [62]. Recent electron microscopic studies have served to visualize directly WDV Rep oligomers bound to DNA and suggest that a large protein core, most likely of six to eight monomers, is present in the nucleoprotein complex [86]. Rep-Rep interaction has been proposed to be a prerequisite for DNA binding but not for nicking, since Rep monomers can support the in vitro cleavage and joining reactions [85].

Geminivirus Rep also interacts with other viral proteins. In begomoviruses, Rep has been shown to form stable complexes with REn (or AL3) protein [87], while in mastreviruses it can interact with RepA [62; E. Ramirez-Parra and C. Gutierrez, unpublished results]. None of these interactions have been shown to occur in vivo, although in the case of TGMV, the fact that replication of DNA molecules containing point mutations in the Rep binding site can proceed only in the presence of REn protein provided in trans is highly suggestive that a Rep-REn interaction is required at some point during viral DNA replication, perhaps by stabilizing an initiation complex (see below).

Initial studies demonstrated that TGMV Rep has DNA binding activity [38, 88]. The DNA binding domain has been mapped to the N-terminal region (116 amino acids in TYLCV Rep [89] and 89 amino acids in BCTV [90, 91]. Based on amino acid homologies, it is likely that the DNA binding domain of *Mastrevirus* Rep also resides in its N-terminal moiety. The functional consequences of the interaction between Rep and specific DNA sequences within the viral regulatory region will be discussed below in the context of the (+)strand origin activation. TGMV Rep has also been shown to exhibit strong ssDNA binding activity whose potential role during viral DNA replication is not presently understood [92].

Begomovirus Rep interacts in yeast two-hybrids with a maize protein related to human Rb (RRB1; [93]). Quite interestingly, this Rep protein lacks an LxCxE Rb binding motif, and therefore it will be important to identify the amino acid motif which mediates interaction with Rb in *Begomovirus* and *Curtovirus* Rep (see [66] for details). Interaction between *Mastrevirus* Rep and human Rb has been also reported [61]. However, more recent studies using stringent yeast two-hybrid conditions and in vitro binding experiments with purified proteins have not confirmed an interaction between plant Rb and Rep of several mastreviruses such as MSV [62], WDV (Q. Xie and C. Gutierrez, unpublished) and BYDV (L. Liu and J. Stanley, personal communication). It is worth mentioning that although *Mastrevirus* Rep contains an LxCxE motif, it lies very close to the splicing site and, therefore, just downstream of the motif, the Rep and RepA proteins have different primary sequences. Secondary structure predictions suggest that they also may have a different folded structure around this region. Thus, it may occur that the LxCxE motif in *Mastrevirus* Rep is hidden (or not accessible) by the C-terminal domain of Rep. Deletion studies with MSV Rep [62] and WDV Rep (E. Ramirez-Parra and C. Gutierrez, unpublished) reinforce this idea. Whether the differences in binding to Rb in an LxCxE-dependent (*Mastrevirus* RepA) or LxCxE-independent (*Begomovirus* and, likely, *Curtovirus* Rep) represent different evolutionary trends in the strategy developed by geminiviruses to impinge on the Rb pathway is an attractive possibility which has to be substantiated experimentally.

TrAP (begomoviruses). This is an ~15-kDa transcriptional activator protein, also named AC2 or AL2, unique to begomoviruses since it is absent in mastreviruses and a related protein in curtoviruses, the C2 protein, seems to play a different role [94]. TrAP is required to activate the expression of both CP [95, 96] and BV1 [97]. The mechanism of this transcriptional regulation seems to be cell type-specific since it activates the CP promoter in mesophyll cells and derepresses the CP promoter in phloem tissue [98]. TrAP mutants accumulate reduced amounts of viral ssDNA [99]. A direct role of TrAP in the switch from replication stage B to stage C cannot be excluded (discussed in [15]). TrAP might also participate in viral DNA replication as a sequence-independent dsDNA- and ssDNA-binding protein [100, 101].

REn (curto- and begomoviruses). REn is an ~16-kDa protein, also named C3 or L3 in curtoviruses and AC3 or AL3 in begomoviruses, which does not have a counterpart in the *Mastrevirus* genus. Its role during the geminiviral replicative cycle has not yet been elucidated. Although a functional REn protein is not required for infectivity, mutational studies have shown that viruses expressing mutant REn proteins exhibit delayed and greatly attenuated symptoms [71, 102]. REn mutations also produce a reduced level of accumulation of viral DNA in transient replication assays [99, 102, 103]. Such a stimulatory effect of viral DNA replication justifies the proposed name of REn [67]. Complementation studies revealed that REn can act on heterologous viruses [94, 104]. However, the mechanistic basis of REn activity may be different depending on the plant species [105]. REn is a highly hydrophobic protein which can be detected both soluble and associated with the organellar fraction [106]. As already mentioned, REn can interact with Rep [87]. The possible functional relevance of this interaction will be discussed below.

Other proteins (R2 and CP). Curtoviruses encode the R2 (or V2) protein (~12 kDa) which has been postulated to function in the switch from the rolling circle (stage B) to the production of ssDNA genomes (stage C), since accumulation of ssDNA, with a concomitant increase in dsDNA forms, has been detected in R2 mutants [103].

CP (~28–30 kDa) has been shown to affect ssDNA accumulation [107], a role consistent with its abilty to bind to ssDNA [108].

Anatomy of the (+)strand DNA replication origin

Initiation at the (+)strand origin of DNA replication conforms to the so-called model of simple genomes [109] in which this step depends on the interaction between the initiator protein and specific DNA sequences within the cis-acting element, the replicator. Consequently, many efforts are being made to define the minimal (+)strand origin, to identify the Rep binding sites and to elucidate the mechanism of initiation.

All geminiviruses contain an intergenic region where the cis-acting signals required for initiation of rolling-circle DNA replication (and transcription) are located. Within it, the invariant 9-nt sequence (TAATATT↓AC) contains the site (↓) where the initiation of (+)strand DNA replication has been mapped in vivo [23, 80, 110, 111] and in vitro [78, 79]. The invariant loop sequence has a crucial role for viral DNA replication [38, 112, 113]. The structure of the stem is also important since point mutations which destroy base pairing are deleterious for viral replication, whereas those contributing to the maintenance of the stem-loop structure restore replication ability [114].

In addition, the replicator contains other DNA sequences which exhibit specific characteristics in different geminivirus genera. Phylogenetic sequence analysis of the geminivirus intergenic regions revealed the existence of iterative sequence motifs whose organization is highly conserved and, apparently, genus-specific [115]. Two major evolutionary trends have been proposed for (+)strand origin architecture: one for the *Mastrevirus* genus and another for the *Curtovirus* and *Begomovirus* genera. In the next paragraphs, I will summarize recent results, some of which suggest remarkable differences between the two types of (+)strand origin organization with potential consequences for the mechanisms of origin activation.

***Mastrevirus* (+)strand origin.** Initial studies demonstrated that both in WDV [116] and MSV [117] the stem-loop is part of the (+)strand origin. Further deletion analyses have shown that mastreviruses require a large (~300 bp) cis-acting element for efficient viral DNA replication in cultured cells (fig. 5; [86]). This element is built up by at least three major components: a central core, ~200 bp in size (in WDV between positions −178/−169 and +28, taking the A residue at the initiation site as position +1), which is essential for viral DNA replication; the core is flanked by two regions which stimulate replication, the auxiliary regions 5′-aux and 3′-aux, ~70 and ~25 bp in size, respectively [86].

The 3′-aux region (spanning from +28 to +50/+63 in WDV) contains two phased A/T tracts, part of the region conferring a static DNA curvature to the WDV LIR [118]. Interestingly, the MSV LIR also exhibits a static bent DNA structure [119]. The LIRs of other *Mastrevirus* members also contain several phased A/T tracts, although their bent structure has not been experimentally addressed. In the light of (i) this conservation of phased A/T tracts in relatively similar positions of *Mastrevirus* LIRs, (ii) their location relative to the v-sense TATA box (fig. 5) and (iii) the known effects of

DNA curvature on transcriptional regulation [120, 121], it would be interesting to determine whether they affect v-sense transcription. In fact, the nonstructural proteins of WDV have been implicated in regulation of v-sense promoter [61, 116], a significant difference with other geminivirus genera where v-sense transcription does not depend on Rep [96]. Transient replication assays have shown that the curvature at the LIR has only a very marginal contribution to WDV DNA replication in cultured cells [86, 122]. The 5′-aux (between positions -242 and $-178/-169$ in WDV) does not contain any sequence features identified so far which might explain their stimulatory effect. Based on the proximity of Rep binding sites (see below), 5′-aux (and perhaps also 3′-aux) might contribute to the stabilization and/or activity of an initiation complex formed by Rep.

Not all the DNA sequences within the minimal cis-acting core are required for viral DNA replication since internal deletions do not have a significant influence on viral DNA accumulation in cultured cells [116; A. P. Sanz-Burgos and C. Gutierrez, unpublished]. However, the 3′ domain of the core, containing the stem-loop, and its 5′ domain are absolutely required [86]. Interestingly, these regions of the core element contain the iteron sequences identified by sequence analysis [115]. In addition, a GC-rich element is present near the stem-loop in MSV [123], which may have an indirect role in DNA replication.

Electron microscopy studies have allowed direct visualization of a Rep-DNA complex within the core element [86]. The spherical nucleoprotein structure appears with a homogeneous structure. Its large size is consistent with the participation of several Rep monomers in the complex, in agreement with the ability of *Mastrevirus* Rep monomers to interact in a yeast two-hybrid assay [62]; E. Ramirez-Parra and C. Gutierrez, unpublished results). Since the DNA length is not reduced upon binding, complex formation should not involve extensive DNA wrapping around the protein core [86].

This WDV Rep-DNA complex maps 144 ± 18 bp upstream from the initiation site, between the start site for c-sense transcription and the TATA box ([86]; fig. 5). This position is similar to the location of TGMV Rep binding sites ([88]; see also below). Around this position, GT-rich sequence motifs exist. DNase I footprinting studies revealed that a large DNA region, containing two direct repeats separated by ~ 20 bp and including the TATA box for c-sense transcription, is protected from DNase I digestion [124]. Based on its location, this Rep-DNA complex has been termed the C-complex to distinguish it from another WDV Rep-DNA complex, the V-complex, which is formed near

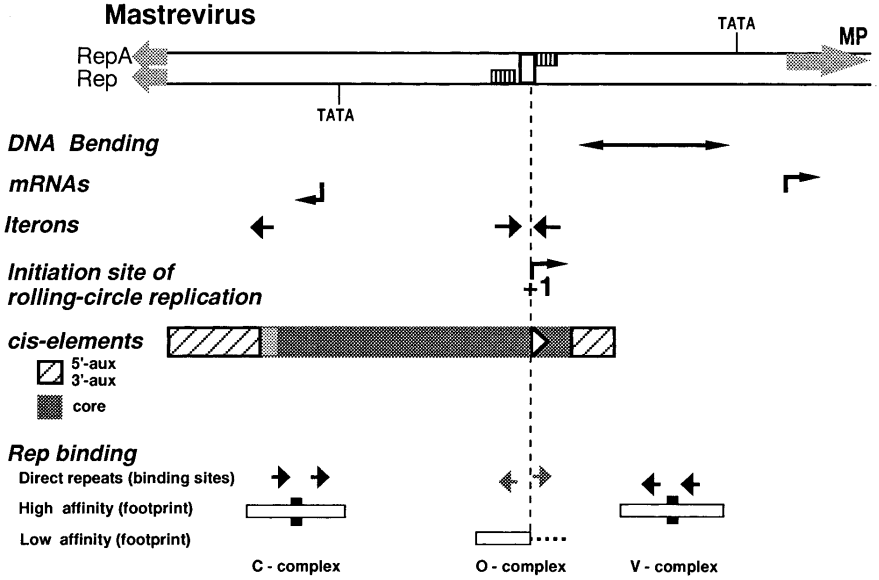

Figure 5. The (+)strand origin of DNA replication in mastreviruses. Domain organization and landmarks defining the (+)strand origin located within the mastrevirus LIR where the two divergent TATA boxes and the sequences which can be potentially involved in stem-loop extrusion (white box flanked by two striped boxes) are shown. The figure is largely based on studies with WDV. The location of iterons [115] and other features represent a consensus for which they may not be applicable to all mastreviruses. The position +1, where rolling-circle DNA replication starts, corresponds to the penultimate residue (A) of the invariant 9-nt sequence of the loop (white box in upper bar). The minimal cis-element (core, in gray), which contains the DNA replication start site, is flanked by auxiliary regions (aux, striped). The small direct repeats are putatively involved in Rep binding. The high-affinity Rep-DNA complexes (C- and V-complexes) have been mapped by electron microscopy (the small vertical bars point to the location of the complexes) and by DNase I footprinting (protected DNA regions are indicated by boxes), whereas the low-affinity (O-complex) complex has been identified by DNase I footprinting. Note that the 3′ boundary of this complex has not yet been defined.

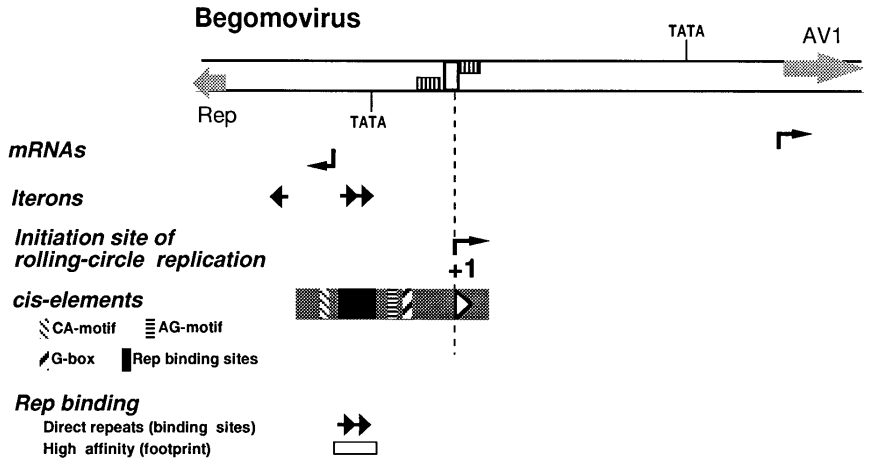

Figure 6. The (+)strand origin of DNA replication in begomoviruses. Domain organization and landmarks defining the (+)strand origin located within the begomovirus intergenic region. As in the case of mastreviruses, the figure represents a consensus of data from TGMV, BGMV and TYLCV. The start sites for mRNAs and for DNA replication, the TATA boxes and the potential stem-loop are depicted as in figure 5. The minimal cis-acting DNA replication element (gray) with motifs critical for function are also shown. Note that in begomoviruses, only one major Rep-DNA complex has been identified so far.

the TATA box for v-sense transcription [124]. Interestingly, the location of the V-complex is fully consistent with a potential role in upregulation of v-sense transcription [116], although it has been suggested that this effect is achieved by the RepA protein instead of Rep [61]. On the other hand, the position of the C-complex strongly suggests its participation in downregulating Rep (and RepA) gene expression in mastreviruses, a property which has been demonstrated for begomoviruses [125–129].

***Curtovirus* (+)strand origin.** An 82–97-bp-long element of BCTV intergenic region has been demonstrated to contain the cis-acting signals conferring replication activity [90]. In transient replication assays, chimeric BCTV genomes containing reciprocally exchanged regions of the CFH and the Logan genomes were competent for DNA replication only when the cis-acting signal and the trans-acting replication determinants were derived from the same strain, indicating that BCTV DNA replication is strain-specific [90]. Also in this genus, the direct repeats are responsible for the strain-specific replication [91].

***Begomovirus* (+)strand origin.** DNA replication studies in begomoviruses have indicated that in addition to the stem-loop, upstream sequence elements are also required to constitute a functional (+)strand origin (fig. 6), and that such region may contain the Rep binding site [38, 88]. In vitro DNA binding experiments with purified TGMV Rep showed that an ~52-bp sequence, located upstream from the stem-loop, directs Rep binding [88]. More refined studies have identified a 13-bp sequence as the TGMV Rep binding site which contains a directly repeated 5-bp sequence with a 3-bp spacer in between [130, 131]. The TGMV Rep binding site lies exactly between the TATA box controlling the expression of c-sense genes, including Rep, and the transcription start site. TGMV Rep [125–127] and ACMV Rep [128, 129] downregulate its own expression. Since the presence of the Rep binding site is required for downregulation [126], it is plausible that Rep interferes with the transcription machinery, as has been hypothesized for mastreviruses [86].

TGMV Rep binding, but not cleavage at the loop, requires dimerization [132]. The two repeats of the TGMV Rep binding site play different roles: the 3′ repeat is an essential component, whereas the 5′ repeat enhances viral replication [131]. Comparison of the TGMV Rep binding sites with putative binding sites in other *Begomoviruses* yields a consensus *Begomovirus* Rep binding site, GG-AGTAYYGG-AG (Y = pyrimidine). The GG [131] and the AG [85] dinucleotides are necessary for Rep binding and viral DNA replication. However, the sequence at the position occupied by the T does not seem to be critical for replication [85]. In some cases, the in vitro DNA-binding experiments do not fully correlate with the replication ability of mutant viruses [85].

DNase I footprinting studies using bacterially expressed and purified TLCV Rep protein revealed two protected regions, each ~17–18 bp long, containing the direct repeat element GGTGTCT as a core sequence, separated by a 9-bp nonprotected spacer [133]. The distance between the direct repeats is 20 bp in TLCV, an old-world geminivirus, whereas in TGMV and BGMV, both new-world viruses, it is only 3 bp, a feature whose significance, if any, is presently unknown. The stem-loop is not required for TLCV Rep binding, and complex formation in other regions downstream of the stem-loop has not been detected [133].

Begomoviruses frequently contain partial copies of the inverted repeat which have been proposed to have a role in viral replication and/or transcription [115]. However, this partial copy in TGMV binds Rep poorly, and it does not contribute to DNA replication and only slightly to transcription [85]. A third cis-acting element, downstream from the TATA box (the 'AG' motif), has been identified [85]. Two other motifs, the G-box and the TATA-box, seem to play a role in origin utilization [85]. Finally, a CA motif, located outside the minimal origin, enhances viral DNA replication [85].

Initiation at the (+)strand DNA replication origin

One of the most striking findings regarding the formation of nucleoprotein complexes by geminivirus initiator Rep protein within the replicator sequences is that the high-affinity Rep binding sites are located relatively far away from the stem-loop where DNA replication starts ($\sim$140 bp in mastreviruses and $\sim$50–60 bp in curto- and begomoviruses). Although in some cases, it seems that the presence of a stem-loop structure may not be needed for initiation, but rather for termination [36, 80, 110], the evidence pointing to the crucial role at initiation (although perhaps not exclusively) of the stem-loop structure is overwhelming [38, 80, 112–114]. Mutational analyses have demonstrated that base pairing, which contributes to the maintenance of the stem-loop, is critical for viral DNA replication [114].

Results obtained for mastreviruses and begomoviruses suggest that initiation of (+)strand DNA replication may require the formation of higher-order, perhaps looped, structures. Assembly of higher-order structures held by initiator proteins, in some cases in association with cellular factors, has precedent in prokaryotic rolling-circle replicons [134–136], a situation which favors the interaction of the initiator protein with its target site (reviewed in [27]). A model based on this idea and taking into account the well-characterized binding of Rep to its high-affinity site, upstream from the initiation site, and the interaction observed between Rep and REn, has been proposed for initiation in begomoviruses [14]. In this model, REn would recognize and bind, at the same time, to the stem-loop and to Rep bound to its binding site. This would provide a mechanism for bringing Rep closer to the nicking site at the invariant sequence of the loop. However, direct evidence for REn binding to the stem-loop and further interaction with Rep is still lacking.

Mastreviruses lack a protein homologous to REn and therefore a different situation should occur. Recent evidence showing that WDV Rep is able to bind with high affinity to a region $\sim$140 bp upstream from the initiation site (the C-complex) but also with lower affinity to the stem-loop structure (the O-complex) has provided a clue as to how mastreviruses might initiate (+)strand DNA replication, since formation of the O-complex is sufficient to carry out sequence-specific DNA cleavage at the loop [124], a reaction which may mimic initiation of rolling-circle DNA replication. Additional contacts between Rep bound to the stem-loop with its high-affinity site cannot be excluded and, in fact, might provide the framework to maintain a higher-order complex at the origin.

Whatever the mechanism to assemble an initiation complex, the initiation reaction involves the nucleophilic attack by the OH group of the conserved tyrosine residue in motif RCR-III of all geminivirus Rep, to the phosphodiester bond between the last T and A in the invariant loop sequence [76]. The product of this reaction is a dsDNA circle with a nick which provides the necessary 3′-OH, susceptible of being extended by cellular DNA polymerases, whereas the initiator protein seems to remain covalently attached to the 5′ ends [76].

Elongation and termination

Molecular details on these two steps are largely unknown. Cellular replication factors might interact with viral proteins and/or with DNA structures generated during initiation to achieve complete synthesis of the viral DNA strand in a process which should involve full displacement of the parental viral DNA strand.

The termination mechanism remains equally obscure from the mechanistic point of view. In this context, the data available for prokaryotic rolling-circle replicons [27] should provide an adequate framework to design future experiments to determine whether geminivirus RCR is a continuous, as for example in phage ΦX174 [137], or a noncontinuous process, as for example in the case of plasmid pC194 [138]. In the former case, two active tyrosine residues are required for switching from initiation to termination, whereas in the later, only one is needed. It is noteworthy, as previously discussed [13], that geminivirus Rep contains a single conserved tyrosine residue. Therefore, most likely, termination occurs through the second (noncontinuous) mechanism, probably using an acidic residue and a water molecule for the termination reaction.

Stage C: production of (c)ssDNA genomes for encapsidation

It is conceivable that at some late point during the viral replication cycle, the v-sense circular ssDNA molecules produced by RCR accumulate instead of being incorporated to the replicating pool. A role of viral proteins, e.g. CP, has been proposed, although the mechanism operating has not been fully elucidated (see above). This

process has to be coupled to the intra- and intercellular movement of viral genomes, a subject which is beyond the scope of this review. It has, however, received increasing attention in recent years [139–142]. In the case of mastreviruses, this stage also has to be coupled to the synthesis of the small DNA annealed to the v-sense strand before encapsidation.

Geminiviruses: prokaryotic replicons in eukaryotic cells?

From the previous paragraphs, one can easily reach the conclusion that a number of similarities can be drawn between the DNA replication strategy used by prokaryotic rolling-circle replicons (plasmids and phages) and geminiviruses. This poses interesting evolutionary questions about their origin. A revealing observation indicates that geminivirus DNA replication is supported by bacterial proteins, since after transformation of *Agrobacterium tumefaciens* with TLCV-derived plasmid constructs containing greater than the viral unit length, replicative intermediates accumulate efficiently [143]. Moreover, plasmid amplification depends on the presence of two viral origins and is reduced drastically by mutations in the Rep protein [143]. These results point to a prokaryotic episomal replicon as the ancestor of current geminiviruses. However, a number of features are unique to eukaryotes, and consequently geminiviruses have adapted to the peculiar environment of the eukaryotic cell, including advantages and restrictions, by gaining functions which are exclusive of eukaryotic replicons. We can identify at least three aspects which are relevant to this issue. One is the association of dsDNA replicative forms with histones from the infected cell to form a minichromosome ([53]; see discussion above). A second aspect takes advantage of mechanisms developed by eukaryotes to produce different mRNAs from a single transcript through splicing events. Mastreviruses are, so far, the only known geminiviruses which have adapted such a strategy to increase the number of proteins encoded by a small genome ([59, 144]; reviewed in [4]). A third feature distinctive of geminiviruses as eukaryotic replicons is that their DNA replication cycle seems to be coupled to the regulatory network controlling the cell cycle of the infected cell. This aspect of geminivirus biology, which has been instrumental in current research on cell cycle regulation in plants [66], will be discussed below in more detail.

Geminivirus DNA replication and cell cycle regulation

Completion of the geminivirus DNA replication cycle requires the participation of the cellular DNA replication machinery. These factors are frequently absent or functionally inactive in nonproliferating cells. Thus, geminivirus DNA replication must be restricted to the few cells with proliferative capacity or, alternatively, have evolved a strategy to activate specific sets of cellular genes required for the viral replication cycle. Studies in this direction to elucidate the mechanism used by geminiviruses to alter cellular gene expression have concentrated an increasing interest since they exhibit striking similarities to human oncogenic viruses, such as SV40 or adenoviruses, in the way they interfere with cell cycle, DNA replication and gene expression within the infected cell.

It has been shown that S-phase nuclei are enriched in viral dsDNA intermediates [145]. Studies with transgenic plants have demonstrated that TGMV Rep is able to induce the accumulation of proliferating cell nuclear antigen (PCNA) in terminally differentiated cells where, in the absence of Rep protein, it is undetectable [146]. A functional LxCxE Rb binding motif was identified in *Mastrevirus* RepA protein [67], which mediates its interaction with a plant Rb protein [68]. This, together with the observation that plant Rb interacts with plant cyclins D [93, 147], has served to reinforce the idea of a coupling between geminivirus DNA replication and cell cycle regulation. As already discussed, TGMV Rep, which lacks a LxCxE motif, also interacts with plant RRB1 protein [93]. If the strategy evolved by geminiviruses is similar to that of human oncoviruses, one (or more) geminivirus proteins would be able to interfere with cellular processes controlling cell cycle activation, i.e. reentry into the cell cycle, and the G1/S transition. This effect would be mediated by the geminivirus protein-dependent release of Rb-bound transcription factors involved in G1/S transition [67]. These transcription factors, most likely related to the animal E2F family, would trigger the expression of genes required for cell cycle progression through S phase as well as for DNA replication, much in the same way human viral oncoproteins do [148, 149], thus creating a cellular environment that permits geminivirus DNA replication. Quite interestingly, it has been demonstrated that in cells expressing MSV Rep protein, the S phase-specific histone H2b mRNA is undetectable [150]. This might be due to the fact that not all genes required for S-phase progression or expressed during S-phase are E2F-dependent. In this context, the recent identification and cloning of a plant E2F protein has revealed the presence of unique and conserved features with animal members of the family (E. Ramirez-Parra and C. Gutierrez, unpublished) and should help in future studies relating the effects of geminivirus proteins with the Rb/E2F growth regulatory pathway in plants [66]. In addition, it should be considered that the effects of geminivirus proteins may well be restricted to upregulate some S-phase functions required for the viral cycle rather than inducing cell cycle progression. An intriguing observation which

should be pursued in the light of recent discoveries is that the BCTV C4 protein, a major determinant of virus pathogenicity, seems to be responsible for a hyperplastic response at the infection site [152, 153], suggesting that such tumorlike overgrowths in the phloem parenchyma could be mediated by the interference of cell cycle regulation by the C4 protein. It is clear that more efforts are needed to elucidate, at the molecular level, the consequences of geminivirus infection on cell proliferation, growth and differentiation.

Future prospects

Studies on geminiviruses have received increasing attention in recent years. They have been mostly focused on understanding the cellular and molecular events involved in their replicative cycle and in the pathogenicity associated with geminivirus infection. These efforts have involved research on DNA replication, gene expression, intra- and intercellular movement, virus-host and virus-vector interactions, symptom development, strategies for disease control, evolution and variability, among others.

In the case of DNA replication, many questions are still only poorly understood. The functional organization of the (−)strand DNA replication origin certainly needs to receive more attention in the future to identify its structure and the cellular factors involved in its activation. Another poorly studied aspect is the association of viral dsDNA with histones and its potential consequences on viral gene expression and DNA replication. A reasonable amount of information has already been gathered on the modular architecture of the (+)strand DNA replication origin. Thus, the next step should be to obtain information which will shed light on its functional organization. A characteristic feature of the geminivirus replication cycle is its dependence on cellular functions. Thus, we need to identify the series of events which allow geminiviruses to take advantage of cellular factors to replicate their genome. In this context, geminiviruses are excellent model systems to uncover the components and regulation of DNA replication and cell cycle in plants. The consequences of geminivirus infection on cellular gene expression, cell cycle regulation and, perhaps, cellular DNA replication are also subjects of particular relevance. Thus, whether interference with the Rb/E2F pathway has an effect on G1/S gene expression and G1/S transition, either forcing the cell to enter S phase and further cell cycle phases, or just inducing (some) of the S-phase functions required for viral DNA replication, are extremely attractive questions in search of an answer.

It is clear that recent developments are important to set the ground for novel experimental approaches. At the same time, they are exciting because they are just opening up new doors and revealing unforeseen links between the geminivirus replicative cycle and cellular processes such as DNA replication, cell cycle regulation, growth and differentiation. We can look forward to a very promising future in this field offering attractive scientific challenges.

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