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Differential methylation of the circular DNA in geminiviral minichromosomes

Kathrin Deuschle, Gabi Kepp, Holger Jeske *

Institut für Biomaterialien und biomolekulare Systeme, Abteilung für Molekularbiologie und Virologie der Pflanzen, Universität Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

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ABSTRACT

Geminiviral minichromosomes were purified to explore epigenetic modifications. The levels of methylation in their covalently closed circular DNA were examined with the help of methylation-dependent restriction (MdR). DNA with 12 superhelical turns was preferentially modified, indicating minichromosomes with 12 nucleosomes leaving an open gap. MdR digestion yielded a specific product of genomic length, which was cloned and Sanger-sequenced, or amplified following ligation-mediated rolling circle amplification and deep-sequenced (circomics). The conventional approach revealed a single cleavage product indicating specific methylations at the borders of the common region. The circomics approach identified considerably more MdR sites in a preferential distance to each other of ~200 nts, which is the DNA length in a nucleosome. They accumulated in regions of nucleosome-free gaps, but scattered also along the genomic components. These results may hint at a function in specific gene regulation, as well as in virus resistance.

1. Introduction

Geminiviruses (Jeske, 2009) are useful probes to explore the molecular biology of plants, comparable to the application of SV40 in animals. Due to the small size of their genomic components (2.5–3.0 kb), they rely considerably on host cell factors for replication, transcription and spread (Hanley-Bowdoin et al., 2013). Thus, they are excellent tools to decipher genetic and epigenetic functions (Pooggin, 2013). The corresponding fundamental knowledge is thought to be important in combating the devastating crop reductions exerted by geminiviruses worldwide (Moffat, 1999), with resistance breeding based on transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), and further molecular mechanisms. The epigenetic functions of TGS and PTGS, as well as the feedback-loop of PTGS to TGS by RNA-directed DNA methylation (RdDM), are involved in at least two major tasks: suppression of mobile genetic elements, like transposons and viruses, and differential gene control in eukaryotes. For the first task, DNA methylation can be arbitrarily distributed throughout the elements' genome, whereas for the second, methylation marks should appear at more precise positions. In the current study, we will show evidence for both and argue that a differentiation between the two roles is necessary to understand the different geminiviral DNA forms.

Geminiviral circular single-stranded (ss) DNA is encapsidated in twinned icosahedral particles (Böttcher et al., 2004; Zhang et al., 2001). Currently, seven genera have been described (*Begomovirus, Mastrevirus, Curtovirus, Becurtovirus, Eragrovirus, Topocuvirus and Turncurtovirus*), differing in genome organization, host range and insect vector (Varsani et al., 2014). Members of the largest genus, *Begomovirus,* are transmitted by whiteflies and their genomes may consist of one (monopartite) or two (bipartite, DNA A and B) DNA components encoding five to seven proteins that are involved in viral replication, movement, transmission and pathogenesis (Jeske, 2009). DNA A and DNA B are different, except for a common region (CR) which harbors the origin of replication (ori) and promoters for bidirectional transcription. After plant inoculation by the insect vector, viral ssDNA is released into the nuclei of phloem cells, where it is converted to double-stranded (ds) covalently closed circular (ccc) DNA by complementary strand replication (CSR) (Saunders et al., 1992). The cccDNA is wrapped around host histones forming extrachromosomal minichromosomes with defined nucleosome positions and phasing (Abouzid et al., 1988; Pilartz and Jeske, 1992, 2003). For begomoviruses, viral DNA was found to be covered by 11–13 nucleosomes (Abouzid et al., 1988; Pilartz and Jeske, 2003). A recent quantitative survey revealed a most prominent fraction with 12 nucleosomes and a minor fraction with 13 nucleosomes (Paprotka et al., 2015). Upon equal distribution of

* Corresponding author.
E-mail address: holger.jeske@bio.uni-stuttgart.de (H. Jeske).

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nucleosomal DNAs, 13 nucleosomes are necessary for complete coverage of the components' DNA, whereas 12 nucleosomes leave an open gap suitable to bind host transcription factors (Pilartz and Jeske, 2003). Minichromosomes with 12 nucleosomes represent, therefore, transcriptionally active chromatin. Although two different open gaps occurring simultaneously during bidirectional transcription (Frischmuth et al., 1991) and leading to minichromosomes with 11 nucleosomes are not excluded, the recent survey did not support an accumulation of such a stage (Paprotka et al., 2015).

A multitude of viral circular and linear forms of both ss and dsDNA are generated during replication (Paprotka et al., 2015). The viral replication-initiator protein (Rep) initiates rolling circle replication (RCR), while recombination-dependent replication (RDR) produces substantial fractions of heterogeneous linear dsDNA, initially characterized for Abutilon mosaic virus (AbMV) by electron microscopy and two-dimensional (2D) gel electrophoresis (Jeske et al., 2001), and confirmed later for several geminiviruses from different genera, associated satellite DNAs as well as an animal circovirus (Alberter et al., 2005; Cheung, 2012; Erdmann et al., 2010; Jovel et al., 2007; Paprotka et al., 2015; Preiss and Jeske, 2003; Ruschhaupt et al., 2013). An involvement of host repair enzymes for homologous recombination, non-homologous end joining, and translesion DNA synthesis has recently been discovered (Richter et al., 2016a; Richter and Jeske, 2015, 2016b). However, the intriguing role of Rad54 in chromatin-remodeling proposed previously (Kaliappan et al., 2012) has not been confirmed for other geminiviruses (Richter et al., 2015).

Plants can retard virus infection by means of TGS and PTGS, but these defense mechanisms may be counter-balanced by viral suppressors of silencing and replicative evasion of DNA methylation (Csorba et al., 2009; Pooggin, 2013; Raja et al., 2010; Rodriguez-Negrete et al., 2009; Voinnet, 2005). Plants employ symmetric DNA methylation at CG or CNG sites like other eukaryotic cells, but produce higher levels of asymmetric DNA methylation at CHH sites (H: no guanine). Different methyltransferases are involved in these DNA modifications: *de novo* methylation at CHH sites is caused by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2, or its homolog DRM1), whereas maintenance methylation is achieved by CHROMOMETHYLASE 3 (CMT3; maintains the methylation at CHG), METHYLTRANSFERASE 1 (MET1; for the maintenance of CG methylation) and KRYPTONITE (KYP2; for DNA methylation maintenance at CHH and H3K9 methylation) (Raja et al., 2010).

For geminiviruses, chromatin methylation of the viral minichromosomes is one possible host defense mechanism, which is counter-balanced by different suppressor proteins, depending on the virus genus or species. Certain geminiviral gene products (AL2 or L2; syn. AC2, C2) influence, amongst other functions, the methyl cycle of the host (Raja et al., 2010). These proteins target enzymes in the S-adenosylmethionine (SAM) pathway (Buchmann et al., 2009; Zhang et al., 2011). Similarly, the ßC1 protein of a satellite DNA which is associated with the tomato yellow leaf curl China virus (TYLCCNV) was found to interact with S-adenosyl homocysteine hydrolase (SAHH) (Yang et al., 2011). Additionally, a different strategy to suppress silencing was discovered for geminiviral Rep and C4 (Rodriguez-Negrete et al., 2013) which was able to downregulate MET1 and CMT3 and prevent maintenance of *de novo* methylation at CG and CHG sites.

It has been proposed that geminiviruses can evade repressive cytosine methylation and transcriptional silencing by the aid of RCR and RDR (Paprotka et al., 2011; Pooggin, 2013). Previous results have shown that heterogeneous linear (lin) and multimeric (mult) dsDNAs were the main targets of methylation, whereas circular monomeric dsDNA was hardly affected (Paprotka et al., 2011). Bisulfite sequencing-based analyses were compatible with a stochastic distribution of methylated sites in the viral DNAs, although their quantification power was regarded critically in the previous report (Paprotka et al., 2011). Recently determined high levels of C > T mutations in the viral gene pool, which is the same diagnostic exchange in the bisulfite sequencing, add a further argument towards a cautious interpretation of this data (Richter et al., 2016a, 2016b). In spite of these limitations, the available results hint at a role of methylation in host defense to suppress geminiviral gene expression, most prominent during recovery from infection (Buchmann et al., 2009; Ceniceros-Ojeda et al., 2016; Chung and Sunter, 2014; Chung et al., 2014; Liu et al., 2014; Raja et al., 2014, 2008; Rodriguez-Negrete et al., 2013, 2009; Yang et al., 2011).

In order to circumvent the limitations of bisulfite-sequencing and the use of restriction enzymes that are impaired by DNA methylation, we used DNA methylation-dependent restriction enzymes (MdRs) as a complementary diagnostic tool (Paprotka et al., 2011). MdRs can cleave a DNA only if a strand is methylated and introduce a double strand cut (Horton et al., 2012; Loenen and Raleigh, 2014). Intriguingly, one major product of MdR digestion of geminiviral DNA was a lin dsDNA of genomic length indicating that either circular monomeric cccDNA was cut stochastically only once, or that the heterogeneous lin dsDNA - a prominent replication intermediate consisting of concatemeric viral genome units - was cut at a precise position (Paprotka et al., 2011). We can now provide the first evidence that a specific DNA form of monomeric cccDNA with 12 superhelical turns (indicative of minichromosomes with 12 nucleosomes) was preferentially methylated. Furthermore, two specific sites, interestingly flanking the CR sequence, were identified by conventional cloning and sequencing. Circomics (Wyant et al., 2012), however, using a newly developed method of ligation-mediated rolling circle amplification (RCA) combined with Illumina sequencing, revealed many more MdR sites with a preference for an inter-site distance of nucleosomal DNA length scattered throughout the whole genome.

2. Results

2.1. Topoisomer distributions in purified viral minichromosomes

In order to direct the focus on the viral circular dsDNA, monomeric cccDNA-containing minichromosomes were purified and analyzed for the topoisomer distribution of their DNAs using gel electrophoresis in the presence of chloroquine as an intercalator. Three experiments were performed for AbMV as well as for tomato yellow leaf curl Sardinia virus (TYLCSV) in *Nicotiana benthamiana* plants, because these viruses yielded promising amounts of minichromosomes during a survey of several geminiviruses (Paprotka et al., 2015). For each experiment, infected plants were harvested at 14, 28 and 42 days post inoculation (dpi). Young leaf samples were pooled from several plants, and minichromosomes were purified by differential centrifugation, PEG precipitation and rate zonal sucrose-gradient centrifugation in duplicate or triplicate. To compensate for variation between gradients, the DNAs of every fraction of a gradient were first analyzed in gels with ethidium bromide (Figs. 1, 3, 5a), followed by a second separation in gels with chloroquine (Figs. 1, 3, 5b) for the selected minichromosome fractions. To infer the methylation status of the respective DNAs, fractions were treated without or with the MdR enzyme *Mcr*BC in parallel (Figs. 1, 3, 5; -/+). To compare the topoisomer distributions between both treatments, the lanes of Figs. 1, 3, 5b were scanned and evaluated graphically (Figs. 2, 4, 6).

The complete minichromosomes (*m*-chr) were well separated (dark boxes) from geminivirions (gp) in the lower and from incompletely assembled or disassembled minichromosomes in the