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The recombination mediator RAD51D promotes geminiviral infection

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ABSTRACT

To study a possible role for homologous recombination in geminivirus replication, we challenged *Arabidopsis* recombination gene knockouts by Euphorbia yellow mosaic virus infection. Our results show that the RAD51 paralog RAD51D, rather than RAD51 itself, promotes viral replication at early stages of infection. Blot hybridization analyses of replicative intermediates using one- and two-dimensional gels and deep sequencing point to an unexpected facet of recombination-dependent replication, the repair by single-strand annealing (SSA) during complementary strand replication. A significant decrease of both intramolecular, yielding defective DNAs and intermolecular recombinant molecules between the two geminiviral DNA components (A, B) were observed in the absence of RAD51D. By contrast, DNA A and B reacted differentially with the generation of inversions. A model to implicate single-strand annealing recombination in geminiviral recombination-dependent replication is proposed.

1. Introduction

The term “triple R connection” (RRR) (reviewed in Haber, 1999; Stauffer and Chazin, 2004; Hanawalt, 2007) has been coined to emphasize how interwoven replication, repair and recombination are during DNA synthesis. Geminiviruses (reviewed in Jeske, 2009), with their small genomes of 2.5–3 kb per DNA component, are ideal sensors to investigate this interplay in plants. The multitude of DNA intermediates during these processes can be resolved in two-dimensional (2D) agarose gels (Jeske et al., 2001; Preiss and Jeske, 2003) and with circomics, the combination of rolling circle amplification (RCA) selective for their circular DNA with deep sequencing (Wyant et al., 2012). The circomics approach yields a broad view of nucleotides to be repaired and rearrangements within the viral genomic components. Moreover, such investigations probe the little addressed question of specificities of replicating DNA in differentiated cells in the phloem, yielding a novel perspective which complements the broad literature on RRR during mitosis and meiosis (reviewed in Haber, 1999; Schuermann et al., 2005; Jasin and Rothstein, 2013; Knoll et al., 2014). The concerted action of RRR processes during the cell cycle may have important implications for understanding of the epidemiology of geminiviruses, which in past decades have damaged crops globally (Moffat, 1999) through their high capacity to recombine and adapt to new plant cultivars (Martin et al., 2011; Monjane et al., 2014). In particular, recombination and error-prone repair pathways may elevate the reservoir of geminiviral molecules that are potentially able to overcome plant defense and resistance genes.

Geminivirus genomes consist of one or two circular single-stranded (ss) DNA components packed into geminate particles formed of two incomplete icosahedra (Zhang et al., 2001; Böttcher et al., 2004). In nature, viral ssDNA is transmitted by whiteflies or leafhoppers during feeding on the phloem (Czosnek et al., 2001), a tissue to which most geminiviruses remain restricted (Morra and Petty, 2000; Wege et al., 2001).

Only few DNA polymerases, such as translesion DNA synthesis (TLS) polymerases, are available to perform complementary strand replication (CSR) in this tissue (Richter et al., 2016 and references therein). The resulting circular dsDNA is wrapped around nucleosomes and forms minichromosomes with covalently closed circular (ccc) DNA (Abouzid et al., 1988; Pilartz and Jeske, 1992, 2003; Paprotka et al., 2015), which is further amplified by rolling circle replication (RCR) (Saunders et al., 1991). Repair is needed if replication is incomplete due to the low processivity of the polymerases or stalled due to damage on the DNA and recombination-dependent replication (RDR) becomes an important pathway to continue high fidelity replication (Jeske et al., 2001; Preiss and Jeske, 2003; Alberter et al., 2005; Jovel et al., 2007; Ruschhaupt et al., 2013).

Geminiviruses are known to exploit host plant pathways intensively for their multiplication (Hanley-Bowdoin et al., 2013).

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The replication-initiator protein (Rep) is the only viral protein indispensable for replication. Besides its constitutive nicking closing activity at the conserved nonanucleotide of the origin of replication (Laufs et al., 1995; Stanley, 1995) and its helicase activity (Choudhury et al., 2006; Clerot and Bernardi, 2006), its binding to the retinoblastoma protein homolog (pRBR) releases the G1/S block and activates DNA synthesis in differentiated cells (Xie et al., 1996; Ach et al., 1997; Durfee et al., 2000; Settlage et al., 2001; Gutierrez et al., 2004; Ruschhaupt et al., 2013). At this stage, DNA polymerases (α, δ, ε) with high fidelity and processivity as well as a clamp protein (proliferating cell nuclear antigen, PCNA) are available for geminiviral replication (Nagar et al., 2002). Furthermore, Rep can induce re-replication of host DNA without binding to RB in fission yeast, which lacks an RB homolog (Kittelmann et al., 2009), as well as in the phloem (Kong et al., 2000; McGivern et al., 2005; Shepherd et al., 2005; Ruschhaupt et al., 2013), perhaps via an interaction with cyclins (Hipp et al., 2014).

In addition to its influence on host DNA replication, geminivirus infection enhanced homologous recombination of transgenes selectively in vein-associated tissue, which correlated to the phloem-specificity of the virus (Richter et al., 2014). Furthermore, intergenomic recombination within or between viral species occur commonly (reviewed in Lefeuvre and Moriones, 2015). Key players of homologous recombination, such as the RAD51 recombinase or the multifunctional chromatin remodeller RAD54, interacted with the Rep of Mungbean yellow mosaic India virus (MYMIV) and were implicated in replication of geminivirus-derived constructs in *Saccharomyces cerevisiae* and *ex vivo* systems (Kaliappan et al., 2012; Suyal et al., 2013). However, a definitive impact of RAD54 on geminivirus replication *in planta* could not be confirmed (Richter et al., 2015).

Previous work has shown that transcripts for some RRR factors, such as KU80, RAD17 and RAD50, were elevated after infection with Cabbage leaf curl virus (CaLCuV) in *Arabidopsis thaliana* (Ascencio-Ibanez et al., 2008). KU80 is particularly interesting in this context, implicating non-homologous end-joining (NHEJ) as an additional player in the geminivirus RRR concert. The presence of KU80 retards the multiplication of geminiviruses (Richter and Jeske, 2015), possibly by generating deletions, insertions or rearrangements within and between the viral genomic components or by acting as a cytoplasmic viral DNA sensor and ensuing plant defense processes.

With the current study, we have focused on the potential impact of homologous recombination factors on geminiviral replication, in particular RAD51 and its paralogs. Established *A. thaliana* T-DNA insertion lines with the respective gene knockouts were monitored for virus replication in the time course of infection with Euphorbia yellow mosaic virus (EuYMV), a bipartite New World virus that has the advantage to infect *Arabidopsis* with only mild to moderate symptoms (Paprotka et al., 2010b; Richter et al., 2014). Our results implicate the RAD51 paralog RAD51D, pointing to the involvement of RDR and a possible function of single-strand annealing (SSA) during CSR which has not been previously described.

Table 1
A. thaliana T-DNA insertion lines analysed after EuYMV infection.

Gene	Line no.	Gene no.	Expts. no.	Plant no.[a]	Effect on EuYMV[b]
RAD51	GK_134A01	At5g20850	1	7	–
RAD51B	SALK_024755C	At2g28560	1	8	–
RAD51C	SALK_021960	At2g45280	1	7	–
RAD51D	SAIL_564_A06	At1g07745	3	27	+++
XRCC2	SALK_029106	At5g64520	2	18	–
XRCC3	SALK_045564	At5g57450	1	5	–
RAD17	SALK_009384C	At5g66130	1	10	–
MUS81	GK_113F11	At4g30870	1	10	–
RAD52-1	SAIL_25_H08	At1g71310	2	14	–
RAD50	Flag_019F04	At2g31970	2	14	?

[a] Number of mutant plants per line tested in comparison to the same number of wt plants.
[b] Significant effect on early post-infection viral multiplication.

2. Results

Arabidopsis lines with knockouts of genes involved in homologous recombination were examined for their ability to multiply geminiviruses (Table 1). The success of infection was monitored by Southern hybridisation of one-dimensional (1D) gels for quantitative changes (titres), as well as with 2D gels for qualitative alterations of viral intermediates of replication. 1D gels permit quantification of relative amounts of the replication products, such as open circular (oc) and covalently closed circular (ccc) double-stranded (ds) DNA as well as linear and circular single-stranded (ss) in single bands. 2D gels extend this analysis to the intermediates of replication for CSR, RCR and RDR, which are seen as lines, arcs or fields of hybridisation signals as previously described (Jeske, 2007). This approach is superior to other techniques, such as quantitative PCR, because it allows a discrimination between inoculated and replicated DNA as well as between different viral DNA forms which may be especially important in the context of this study as discussed in detail before (Paprotka et al., 2011; Richter et al., 2015).

Experiment-to-experiment variations under greenhouse conditions were compensated by inoculating mutant (mu) and wild-type (wt) plants always in parallel, with sufficient numbers of individual plants to determine the variability of the results. When possible, comparisons involved segregating wt and mutant sibling lines. As reported previously (Paprotka et al., 2015), greenhouse conditions can lead to more dynamic infections in comparison to climate chambers, which may elevate the necessity for repair processes during geminiviral infection.

Notwithstanding these potential sources of variability, it is remarkable how reproducible the infection results were for most

Fig. 1. EuYMV DNA emergence in *rad51* and wt plants. (A) 1D blot hybridization for samples at 7, 14 and 21 dpi, (B) 2D blot hybridization at 14 dpi. Seven plants per genotype were inoculated and total nucleic acids were separated individually in 1D agarose gels (500 ng/lane) or pooled (after RNase A digestion; 300 ng DNA/gel) in 2D agarose gels. Mock controls (m) were inoculated with EuYMV DNA B alone. Viral DNA was hybridized with DIG-labeled probes of full-length EuYMV DNA A. Genomic plant DNA (pDNA) from stained agarose gels served as loading control. HS: hybridization standards 1, 10 and 100 pg of linear EuYMV dsDNA A in different combinations to tag the gels. Indicated viral DNA forms are multimeric (> 1x; mult), open circular (oc), double-stranded linear (lin), covalently closed circular (ccc), linear and circular single-stranded (ss), defective (< 1x; def) for 1D gels, and in addition for 2D gels intermediates of complementary strand replication on circular templates (CSR), rolling circle replication (RCR), recombination-dependent replication (RDR), and heterogeneous double-stranded linear (hdsl). 1x or 2x indicate monomeric or dimeric genomic length. The field of ssDNA-derived intermediates of unknown origin is highlighted by stippled boxes. The box plots in (a) depict integrated pixel densities of viral DNA bands of ocDNA, cccDNA and ssDNA from each sample for 7 and 14 dpi: Boxes represent 50% of the data between upper and lower quartiles; lines the medians; bars the whiskers; diamonds upper or lower outliers. The whiskers are set at 1.5 x interquartile range above the upper quartile and 1.5 x interquartile range below the lower quartile. If minimum or maximum values were outside of this range, they are depicted as outliers. The significance of differences in signal strengths of distinct DNA forms between genotypes was tested statistically (two-way ANOVA with a post-hoc Tukey; with genotypes and DNA forms as selected factors; * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$). (b) 2D analysis of the viral DNA intermediates for the plant lines indicated.