Geminiviral Infection in Interaction with DNA Repair and Damage Tolerance Pathways

Von der Fakultät Energie-, Verfahrens-, und Biotechnik der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.)

Genehmigte Abhandlung

Vorgelegt von Kathrin Siglinde Richter
aus Göppingen

Hauptberichter: Prof. Dr. Holger Jeske
Mitberichter: Prof. Dr. Arnd G. Heyer
Vorsitzender: Prof. Dr. Ralf Mattes

Tag der mündlichen Prüfung: 09. Oktober 2015

Institut für Biomaterialien und biomolekulare Systeme der Universität Stuttgart

2015
<table>
<thead>
<tr>
<th>Inhalt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction ........................................</td>
</tr>
<tr>
<td>Results ................................................</td>
</tr>
<tr>
<td>Discussion ..........................................</td>
</tr>
<tr>
<td>Materials and methods ................................</td>
</tr>
<tr>
<td>Acknowledgements ..................................</td>
</tr>
<tr>
<td>References ..........................................</td>
</tr>
<tr>
<td>Supplementary data ..................................</td>
</tr>
<tr>
<td>Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication</td>
</tr>
<tr>
<td>Abstract ..............................................</td>
</tr>
<tr>
<td>Introduction ........................................</td>
</tr>
<tr>
<td>Experimental section, results and discussion</td>
</tr>
<tr>
<td>Acknowledgements ..................................</td>
</tr>
<tr>
<td>References ..........................................</td>
</tr>
<tr>
<td>Supplementary data ..................................</td>
</tr>
<tr>
<td>The role of homologous recombination factors for geminiviral infection in planta</td>
</tr>
<tr>
<td>Abstract ..............................................</td>
</tr>
<tr>
<td>Introduction ........................................</td>
</tr>
<tr>
<td>Results ................................................</td>
</tr>
<tr>
<td>Discussion ..........................................</td>
</tr>
<tr>
<td>Materials and methods ................................</td>
</tr>
<tr>
<td>Acknowledgements ..................................</td>
</tr>
<tr>
<td>References ..........................................</td>
</tr>
<tr>
<td>Supplementary data ..................................</td>
</tr>
<tr>
<td>The contributions of translesion synthesis polymerases to geminivirus replication</td>
</tr>
<tr>
<td>Abstract ..............................................</td>
</tr>
<tr>
<td>Introduction ........................................</td>
</tr>
<tr>
<td>Results ................................................</td>
</tr>
<tr>
<td>Abkürzung</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>AGE</td>
</tr>
<tr>
<td>AS</td>
</tr>
<tr>
<td>At</td>
</tr>
<tr>
<td>bp</td>
</tr>
<tr>
<td>ccc</td>
</tr>
<tr>
<td>CILCrV</td>
</tr>
<tr>
<td>CR</td>
</tr>
<tr>
<td>CSR</td>
</tr>
<tr>
<td>ds</td>
</tr>
<tr>
<td>DSB</td>
</tr>
<tr>
<td>EuYMV</td>
</tr>
<tr>
<td>hdsl</td>
</tr>
<tr>
<td>HR</td>
</tr>
<tr>
<td>IR</td>
</tr>
<tr>
<td>kb</td>
</tr>
<tr>
<td>kD</td>
</tr>
</tbody>
</table>
Zusammenfassung


Summary

Phytopathogenic geminiviruses are prone to point mutations and recombination events. Thereby, they adapt quickly to new hosts and environments, threatening the yield of crop plants worldwide. Their circular single-stranded DNA genomes are propagated by three modes: complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR). Geminiviruses exclusively infect differentiated cells and many species remain limited to nuclei-containing cells of the phloem. Since they encode only few proteins, their replication largely depends on DNA-amplifying and -modifying enzymes of the host. This study addresses the interaction of geminiviral amplification processes with DNA damage tolerance and repair pathways of the host plants. For this purpose, *Euphorbia yellow mosaic virus* (EuYMV) and *Cleome leaf crumple virus* (CILCrV) infections were monitored in knock-out and reporter lines of the model plant *Arabidopsis thaliana*.

EuYMV infection enhanced homologous recombination (HR) rates of a transgenic reporter gene particularly in vein-associated cells. Since EuYMV was detected exclusively in phloem cells, it is likely that geminiviral infections promote the HR pathway specifically in this tissue. The role of distinct HR factors for geminiviral replication, especially for the RDR mode, was examined by biolistic inoculation of wildtype and knock-out lines. Promising key players of the homologous strand invasion reaction such as Rad51 and Rad54 had no impact. The Rad51 paralog Rad51D, however, promoted geminiviral amplification, probably due to its function in the HR mode of single-strand annealing. Ku80, a key factor of the error-prone non-homologous end-joining repair pathway retarded the progression of systemic EuYMV infection. The inhibitory effect might be caused by the accumulation of erroneous viral DNA molecules. Moreover, Ku80 might act as a viral DNA sensor and induce a defense response of the host plant due to its interaction with the exonuclease Wex. Furthermore, the contribution of translesion synthesis (TLS) polymerases to geminiviral replication, especially to the CSR mode, was analyzed by biolistic inoculation as well as insect transmission. None of the TLS polymerases was necessary for geminiviral amplification. However, comparative mutation analyses of EuYMV sequences imply their involvement and redundant function in geminiviral replication for the first time. Overall, these results indicate a high genetic flexibility of geminiviruses and may explain their high degree of adaptability and epidemic potential.
Einleitung

Reparatur von DNA-Doppelstrangbrüchen


Bei der Detektion von DSBs in Eukaryoten spielt der Proteinkomplex Mre11/Rad50/Nbs1 (MRN), der an die DNA-Enden bindet und sie auch zur Vorbereitung auf Reparaturvorgänge prozessieren kann, eine entscheidende Rolle (Lamarche et al., 2010; Williams et al., 2010). Zur Signalweiterleitung und Induktion der DNA-Reparatur und nachfolgender zellulärer Antworten wie Zellzyklusarrest und Replikationsinhibition wird die Protein kinase Atm (ataxia telangiectasia mutated) aktiviert (Maréchal & Zou, 2013). Dagegen wird die Protein kinase Atr (ataxia telangiectasia and Rad3 related) als Reaktion auf replikativen Stress wie etwa durch blockierte Replikationsgabeln aktiviert. Die meisten Erkenntnisse hierzu beruhen auf Studien mit Hefe- oder Säugerzellen, allerdings sind für die pflanzlichen Homologe ähnliche Funktionen wahrscheinlich (Amiard et al., 2010; Culligan et al., 2006; Waterworth et al., 2007). Atm und Atr aktivieren unter anderem den Transkriptionsfaktor SOG1 (suppressor of gamma response 1), der
die Expression zahlreicher Gene in Reaktion auf DNA-Schäden steuert (Yoshiyama et al., 2014; Yoshiyama et al., 2013). Sog1 ist spezifisch für Pflanzen und stellt möglicherweise ein Analog des tierischen Tumorsuppressors p53 dar.

Es gibt zwei Hauptwege der Reparatur von DSBs, die in ihrer prinzipiellen Form in allen Organismen konserviert vorliegen, zum einen das *non-homologous end-joining* (NHEJ) und zum anderen die homologe Rekombination (HR). Beim NHEJ, auch bekannt als illegitime Rekombination, werden die doppelsträngigen (ds) Enden der beiden Bruchstücke direkt miteinander verbunden (Waterworth et al., 2011). Es ist generell die bevorzugte Art der DSB-Reparatur in somatischen Pflanzenzellen und ist durchgehend über den gesamten Zellzyklus aktiv. NHEJ verursacht allerdings mitunter kleinere oder auch größere Deletionen oder Insertionen in der ursprünglichen Sequenz, da die Enden zur Vorbereitung auf die Ligation prozessiert werden können. Das NHEJ kann abhängig von den beteiligten Reparatur-Faktoren weiter unterteilt werden. Beim klassischen (*canonical*) cNHEJ bindet ein Heterodimer bestehend aus den Proteinen Ku70 und Ku80 an die freien DNA-Enden und bringt diese in räumliche Nähe. Schließlich werden sie unabhängig von Sequenz-Homologien durch Ligase IV und Xrcc4 ligiert (Abbildung 1) (Friesner & Britt, 2003; Tamura et al., 2002; West et al., 2002). Zusätzlich zum cNHEJ gibt es noch alternative Wege des NHEJ (aNHEJ), welche Mikrohomologien an den DNA-Enden nutzen (Frit et al., 2014). Häufig sind diese Reparaturwege fehleranfälliger als das cNHEJ, da die Enden nukleolytisch zu einzelsträngiger DNA (*single-stranded, ss*) prozessiert werden und die Überhänge nach Aneinanderlagerung der wenigen komplementären Nukleotide getrimmt werden. Es gibt mindestens zwei Routen des aNHEJ in Pflanzen mit den jeweiligen Schlüssel-Faktoren Xrcc1, welches auch bei der Reparatur von ssDNA-Brüchen eine Rolle spielt, und Xpf (Charbonnel et al., 2011; Charbonnel et al., 2010). Allerdings sind die genauen Abläufe und beteiligten Faktoren der aNHEJ-Prozesse in Pflanzen bisher wenig charakterisiert.


Beim Modell des synthesis-dependent strand annealing (SDSA) werden die Enden eines DSB zu 3’-ssDNA-Überhängen prozessiert und ein ssDNA-Strang dringt in homologe Bereiche eines intakten dsDNA-Strangs ein. An diesem displacement loop (D-Loop) wird die DNA-Synthese durch Paarung mit der intakten Matrise initiert und der eingedrungene Strang verlängert. Nach Beendigung der Reparatur-Synthese hybridisiert der verlängerte Einzelstrang mit den komplementären Sequenzen des anderen ssDNA-Endes am ursprünglichen DSB, die verbleibenden Lücken werden geschlossen und somit eine Genkonversion als Reparaturprodukt erzielt. SDSA ist vermutlich der bevorzugte Weg der DSB-Reparatur in der S- und G2-Phase des Zellzyklus, da hier das Schwesterchromatid als Matrise zur Verfügung steht (Knoll et al., 2014; Shrivastav et al., 2008; Watanabe et al., 2009). Eine Vielzahl von verschiedenen Faktoren ist an diesen Prozessen beteiligt, das Schlüsselenzym der Strang austausch-Reaktion in somatischen eukaryotischen Zellen ist allerdings die Rekombinase Rad51 (Doutriaux et al., 1998; Shinohara et al., 1992). Durch Bindung von Rad51 an den ssDNA-Strang werden helikale Nukleoprotein-Filamente ausgebildet und das Eindringen und die Homologie-Suche am intakten dsDNA-Strang induziert. Zusätzlich besitzen Pflanzen und Wirbeltiere fünf Rad51-Paralogue (Rad51B, Rad51C,
Einleitung

Rad51D, Xrcc2 und Xrcc3), die sich zu zwei Hauptkomplexen und mehreren kleineren Komplexen zusammenlagern können (Bleuyard et al., 2005; Masson et al., 2001a; Masson et al., 2001b; Miller et al., 2004; Osakabe et al., 2005; Osakabe et al., 2002). Diese spielen eine Rolle bei der Assemblierung und Stabilisierung des Rad51-ssDNA-Filaments und ebenso bei der Migration und Auflösung der Rekombinationsintermediate (Suwaki et al., 2011). Auch die multifunktionelle dsDNA-abhängige ATPase Rad54 trägt durch ihre Bindung an Rad51 und ssDNA wesentlich zur Stabilisierung der Nukleoprotein-Filamente und dem Ablauf der D-Loop Ausbildung bei (Ceballos & Heyer, 2011; Mazin et al., 2010; Osakabe et al., 2006; Wright & Heyer, 2014). Bei der meiotischen Rekombination sind ebenfalls einige der SDSA-assoziierten Faktoren beteiligt, allerdings herrscht hierbei der Mechanismus der DSB-Reparatur (DSBR) vor. Bei diesem Modell werden nach Induktion der DSBs während der Prophase I doppelte Holliday junctions ausgebildet, wobei die jeweiligen homologen Chromosomen als Matrise dienen (Osman et al., 2011; Pradillo et al., 2014). Abhängig von der Auflösung der Intermediate entsteht entweder ebenfalls eine Genkonversion oder ein crossover des elterlichen Erbguts. Das Schlüsselenzym der meiotischen Rekombination ist die Rekombinase Dmc1 (Kagawa & Kurumizaka, 2010; Klimyuk & Jones, 1997).


Translasi onssynthese-DNA-Polymerasen

Neben den verschiedenen DNA-Reparatur-Systemen besitzen Zellen auch Mechanismen, um zeitweise DNA-Schäden tolerieren zu können, bis die jeweilige Reparatur stattfinden kann. Diese werden unter der Bezeichnung der DNA-Schaden toleranz (DNA damage tolerance, DDT) zusammengefasst. DDT trägt wesentlich zum Überleben der Zelle nach DNA-Schädigung bei, kann aber gleichzeitig auch die Entstehung von Mutationen fördern. Aus diesem Grund wird sie auch häufig mit der Entstehung von Tumoren in Verbindung gebracht (Sale, 2013). Ein wesentlicher Bestandteil dabei sind Translasi onssynthese (TLS)-DNA-Polymerasen, die die
Einleitung

Replikation über DNA-Läsionen bewerkstelligen (Goodman & Woodgate, 2013; Waters et al., 2009). Dies wird durch ihre strukturellen Eigenheiten ermöglicht, die einen lockeren Halt an die DNA-Matrize bedingen. TLS-Polymerasen weisen daher eine relativ geringe Prozessivität und eine hohe Fehleranfälligkeit auf, was durch das Fehlen einer 3’-5’-Korrekturlese-Aktivität noch verstärkt wird. Ihre Fehlerraten liegen bei etwa einer Fehlinserion pro $10^{-4} - 10^{-5}$ replizierten Nukleotiden mit unbeschädigter Matrizen-DNA, während high fidelity DNA-Polymerasen wie Polε oder Polδ nur alle $10^{-6} - 10^{-8}$ Nukleotide einen Fehler eingängen (Arana & Kunkel, 2010; McCulloch & Kunkel, 2008). Allerdings sind einige TLS-Polymerasen spezialisiert für die Replikation über spezifische DNA-Läsionen, wobei sie selektiv eine sehr hohe Genauigkeit aufweisen können. In Pflanzen sind momentan die TLS-Polymerasen Polη, Polζ, Polκ und Rev1 charakterisiert und alle vier werden in ausdifferenzierten Pflanzenzellen exprimiert (García-Ortíz et al., 2004; Nakagawa et al., 2011; Sakamoto et al., 2003; Santiago et al., 2008; Takahashi et al., 2005). Polζ besteht aus der katalytischen Untereinheit Rev3 und der akzessorischen Untereinheit Rev7 und ist Teil der B-Familie der high fidelity Polymerasen Polo, Polδ und Polε. Dagegen gehören Polη, Polκ und Rev1 zur Y-Familie, die speziell TLS-Polymerasen umfasst. Die verschiedenen TLS-Polymerasen sind spezialisiert für die Replikation über bestimmte DNA-Läsionen oder die Verlängerung eines fehlgepaarten Primers. Dabei hat jede TLS-Polymerase ein spezifisches Repertoire an Transläsionsaktivitäten.

Einleitung

Geminiviren


In den vergangenen Jahrzehnten kam es durch internationalen Handel mit infiziertem Pflanzenmaterial und die Zunahme der Populationsdichte und Biotypen der übertragenden Insekten zu einer verstärkten globalen Verbreitung von Geminiviren (Morales, 2007; Navas-Castillo et al., 2011). Außerdem weisen geminivirale Genome eine sehr hohe Evolutionsrate von etwa 1,3 × 10⁻⁴ bis zu 1,6 × 10⁻³ Austauschen pro Nukleotid und Jahr auf (Duffy & Holmes, 2008, 2009; Ge et al., 2007; van der Walt et al., 2008). Häufige Rekombinationsereignisse tragen zudem in erheblichem Maße zu ihrer Fähigkeit bei, sich schnell an neue Wirte und Umweltbedingungen anpassen zu können (Lefeuvre & Moriones, 2015).

Die Familie der Geminiviridae besteht aktuell aus den sieben Genera Becurtovirus, Begomovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus, und Turncurtovirus (Varsani et al., 2014). Die Zuordnung einer Art zu einer dieser Gattungen erfolgt aufgrund von Sequenzähnlichkeiten, Genomstruktur, Wirtsspektrum und der übertragenden Insektenvektoren. Die Gattung der Begomoviren enthält die derzeit meisten bekannten Vertreter, die alle durch B. tabaci übertragen werden. Ihr Genom kann sowohl aus einer (monopartit) als auch aus zwei (bipartit) Komponenten von etwa 2,5-3 kb aufgebaut sein. Im Falle eines bipartiten Genoms werden die jeweiligen Komponenten als DNA A und DNA B bezeichnet. In Abbildung 2 sind die bipartiten Genome der in dieser Arbeit verwendeten Neuwelt-Begomoviren Euphorbia yellow mosaic virus (EuYMV) und Cleome leaf crumple virus (CILCrV) dargestellt (Paprotka et al., 2010).
Einleitung

Geminivirale offene Leserahmen (open reading frames; ORF) sind sowohl in viraler als auch in komplementärer Richtung orientiert. Eine Intergene Region (IR) enthält Promotoren für beide Orientierungen. Die dazugehörige Terminationsregion mit Polyadenlyierungssignalen befindet sich auf der gegenüberliegenden Seite der genomischen DNA-Zirkel. Im Falle von bipartiten Begomoviren beinhaltet die IR eine etwa 200 nts umfassende Sequenz, die zwischen DNA A und DNA B eine sehr hohe Homologie aufweist. Diese common region (CR) besitzt allerdings zwischen verschiedenen Virusarten wenig Ähnlichkeit. Innerhalb der CR bzw. IR befindet sich der origin of replication (ori) für die geminivirale rolling circle replication (RCR) (Saunders et al., 1991; Stenger et al., 1991). Dieser enthält eine Haarnadelstruktur mit einer exponierten, in allen Geminiviren hochkonservierten Nonanukleotid-Sequenz. Der erste Schritt des geminiviralen

Abbildung 2: Genomorganisation der bipartiten Neuwelt-Geminiviren Euphorbia yellow mosaic virus (EuYMV, Isolat MGS1) und Cleome leaf crumple virus (CILCrV) mit DNA A und DNA B. Die offenen Leserahmen sind jeweils in viraler (v) und komplementärer (c) Orientierung dargestellt: AV1 (coat protein; CP), AC1 (replication-initiator protein; Rep), AC2 (transcriptional activator protein; TrAP), AC3 (replication enhancer protein; Ren), AC4 (silencing suppressor?), BV1 (nuclear shuttle protein; NSP), BC1 (movement protein; MP). In der common region (CR) befindet sich der origin of replication (ori).
Einleitung


Einleitung

Einleitung

entstehen lineare dsDNA-Produkte von heterogener Länge \((\text{heterogeneous double-stranded linear}, \text{ hdsI})\). Ob hdsI dsDNA-Moleküle zu Monomeren geschnitten oder zirkularisiert werden, ist ebenso ungeklärt wie die generelle Beteiligung viraler Proteine an der RDR. Die für Rep-Oligomere beschriebene Helikase-Aktivität wäre beim Vorgang der loop migration sicher förderlich. Zudem befördert Rep grundsätzlich die geminivirale Replikation. Durch die Bindung von pRBR \((\text{plant retinoblastoma-related protein})\) und vermutlich anderen Zellzyklus-Regulatoren wie Cyclinen hebt es die G1/S-Zellzyklusblockade auf und induziert in der infizierten Zelle einen S-Phase-ähnlichen Zustand mit Re-Replikation \((\text{Arguello-Astorga et al., 2004; Gutierrez et al., 2004; Hipp et al., 2014; Kong et al., 2000; Ruschhaupt et al., 2013})\). Dadurch werden in der anfangs ausdifferenzierten Zelle DNA-Polymerasen und andere Replikationsfaktoren exprimiert und für die virale Replikation zugänglich. Da Geminiviren nicht in meristematischen Geweben mit aktivem Zellzyklus und DNA-Synthese vorkommen, ist dies ein entscheidender Vorgang im Infektionsverlauf. Weiterhin interagiert Rep mit PCNA und RFC \((\text{replication factor C})\), was vermutlich der Vorbereitung der Virusreplikation dient \((\text{Castillo et al., 2003; Luque et al., 2002})\).

Neben Rep kodieren Geminiviren, abhängig von ihrer Genomorganisation, für bis zu sieben weitere Proteine. Das Hüllprotein \((\text{coat protein; CP})\) des AV1 Gens bipartiter Begomoviren bildet das Kapsid zur Verpackung des ssDNA-Genoms aus und ist für die Insektenübertragung von essentieller Bedeutung \((\text{Briddon et al., 1990; Höhnle et al., 2001})\). Das AC2 oder \textit{transcriptional activator protein} (TrAP) erfüllt ebenso wie Rep verschiedene Funktionen. Zum einen kann es die Transkription der Gene AV1 und BV1 transaktivieren \((\text{Sunter & Bisaro, 1991, 1992, 1997})\). Zum anderen wurde für einige Virusisolate eine silencing suppressor Funktion beschrieben \((\text{Chellappan et al., 2005; Wang et al., 2005})\). Zugleich hat TrAP eine erhebliche Wirkung auf die Genexpression des Wirts, beeinflusst den Zucker-, Methyl-, sowie Hormon-Haushalt \((\text{Liu et al., 2014; Saitamo et al., 2012; Trinks et al., 2005; Wang et al., 2003})\) und wirkt hemmend auf die frühe virale Replikation \((\text{Krenz et al., 2015})\). Das AC3 oder \textit{replication enhancer protein} (REn) hingegen verstärkt die virale Replikation. Dies geschieht vermutlich durch Homo-Oligomerisierung und die Interaktion mit Rep, PCNA oder NAC-Transkriptionsfaktoren \((\text{Selth et al., 2005; Settlage et al., 2005})\). Das AC4 Protein ist maßgeblich an der Symtomausprägung beteiligt und wirkt bei einigen Virusisolaten als silencing suppressor, allerdings ist seine genaue Funktionsweise noch nicht vollständig geklärt \((\text{Sunitha et al., 2013; van Wezel et al., 2002; Vanitharani et al., 2004})\).

Für den Transport viraler DNA innerhalb der Wirtspflanze sind bei bipartiten Begomoviren die auf der DNA B kodierten Proteine verantwortlich \((\text{Fondong, 2013; Jeske, 2009; Rojas et al., 2005})\). Hierbei dient das \textit{nuclear shuttle protein} (NSP) des BV1 Gens dem Transport zwischen Nucleus und Cytoplasma durch die Kernporen. Das \textit{movement protein} (MP) des BC1 Gens ist in
Einleitung

Rep ist das einzige virale Protein, welches für die geminivirale Amplifikation essentiell ist. Alle anderen für die zahlreichen DNA-Vermehrungs- und Modifikationsvorgänge notwendigen Faktoren müssen demnach von der Wirtspflanze bereitgestellt werden. Eine geminivirale Infektion ist daher dadurch gekennzeichnet, dass Wirtsprozesse umgestaltet und an die viralen Erfordernisse angepasst werden, wie das Beispiel der Zellzyklus-Kontrolle durch Rep verdeutlicht (Hanley-Bowdoin et al., 2013). Trotz vieler damit zusammenhängender Erkenntnisse, ist letztendlich wenig bekannt über den spezifischen Einfluss der Virusinfektion auf die HR-Maschinerie der Wirtspflanze oder den Beitrag einzelner Wirtswege und -faktoren auf die virale Replikation. Zahlreiche Studien zeigen, dass Rekombinationsereignisse viraler Genome innerhalb einer oder zwischen verschiedenen Arten während Co-Infektionen in beträchtlichem Umfang stattfinden (Garcia-Andres et al., 2006; Kraberger et al., 2013; Kraberger et al., 2015; Martin et al., 2011; Padidam et al., 1999; Rocha et al., 2013; Tiendrébéogo et al., 2012; van der Walt et al., 2009; Varsani et al., 2008; Xie et al., 2013). Diese tragen in hohem Maße zur Erweiterung des viralen Wirtsspektrums und der Entstehung neuer Virusarten bei.
Einleitung


Einleitung

Ergebnisse und Diskussion

Gewebespezifische Verstärkung der somatischen homologen
Rekombination von Transgenen der Wirtspflanze durch geminivirale
Infektion (Manuskript 1)

’Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner’; veröffentlicht in Virology (2014), 452-453: 287-296


In einer ersten Versuchsreihe wurden Pflanzen der vier Linien im 12- bis 14-Blatt Stadium mit rolling circle amplification (RCA)-Produkten der DNA A und DNA B von EuYMV (Isolate MGS1 und MGS2) oder CILCrV biolistisch inokuliert. Als Vergleichsgruppen für den nachfolgenden

Zusammenfassend deuten diese Ergebnisse darauf hin, dass die Infektion mit EuYMV-MGS1 einen spezifischen Einfluss auf den HR-Mechanismus in infizierten Zellen ausübt. Dies wurde deutlich durch die Linie 1445 gezeigt. Die Linie 651 wies eine ähnliche Tendenz auf, allerdings waren die Unterschiede nicht signifikant. Da beide Linien eine invertierte GUS-Sequenzwiederholung von ähnlicher Länge beinhalten, ist es wahrscheinlich, dass entweder der Ökotyp-Hintergrund und/oder der Chromatin-Status der Integrationsstelle des Transgens im Genom der Pflanze für die unterschiedliche Reaktion verantwortlich waren. Auch die Infektion mit RNA-haltigen Tobamoviren induzierte eine etwa zwei- bis dreifache Zunahme der HR-

**Rad54 ist nicht essentiell für die geminivirale Replikation in Pflanzen**

*(Manuskript 2)*

'Rad54 is not essential for any geminiviral replication mode in planta'; veröffentlicht in *Plant Molecular Biology* (2015), 87: 193-202

Das multifunktionelle HR-Enzym Rad54 könnte wesentlich zur Stranginvasion und Chromatin-Umformung während der geminiviralen RDR beitragen. Es besitzt eine dsDNA-abhängige ATPase-Aktivität, bindet an ssDNA sowie an Rad51 und ist dadurch bedeutend an der D-Loop-Ausbildung, der Homologie-Suche, der Migration und der Auflösung der Rekombinationsintermediate beteiligt (Ceballos & Heyer, 2011; Wright & Heyer, 2014). Weiterhin wurde eine Interaktion zwischen Rad54 aus *S. cerevisiae* (ScRad54) und dem Rep von *Mungbean yellow mosaic India virus* (MYMIV) nachgewiesen, was zu einer Verstärkung der nicking-, ATPase- und Helikase-Aktivität von Rep *in vitro* führte (Kaliappan *et al.*, 2012). Genauso war ScRad54 scheinbar für die RCR von MYMIV-basierten Konstrukten *in vitro* und im Hefesystem notwendig. In dieser Studie wurde weiterhin durch transiente Agroinfiltration von Rad54-defizienten (rad54) und Wildtyp (wt-) *A. thaliana*-Linien mit *Tomato leaf curl New Delhi virus* (ToLCNDV)-basierten Konstrukten eine essentielle Rolle von Rad54 für die geminivirale RCR in Pflanzen angedeutet. Allerdings war der hierfür verwendete Nachweis über PCR
unzureichend, um diese Rückschlüsse zu ziehen. Daher wurde in diesem Teil der Arbeit die Rolle von Rad54 für die verschiedenen geminiviralen Replikationsmodi in planta durch biolistische Inokulation der rad54- und wt-Linien mit EuYMV (Isolat MGS1) und CILCrV eingehend überprüft.


Faktoren der homologen Rekombination und des non-homologous end-joining bei geminiviraler Vermehrung in Pflanzen (Manuskript 3 und Manuskript 4)

‘Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication’; veröffentlicht als short communication in Journal of General Virology (2015), 96: 2913-2918

‘The role of homologous recombination factors for geminiviral infection in planta’

Neben Rad54 kommen weitere HR-Faktoren in Frage, die zur geminiviralen Replikation beitragen könnten. Speziell die Rekombinase Rad51 und die Komplexe der fünf Rad51-Paraloge als Schlüsselenzyme der Stranginvasion und Homologie-Suche wären prädestiniert für die Ausführung der RDR. Für Rad51 von A. thaliana wurde, ähnlich wie für ScRad54, eine Interaktion mit dem Rep des MYMIV nachgewiesen und der Ausfall von ScRad51 wurde durch AtRad51 in einem Hefe-basierten ex vivo Replikationsassay von geminiviralen Konstrukten komplementiert (Suyal et al., 2013). Daneben sind Reparaturfaktoren, die durch geminivirale Infektion transkriptionell hochreguliert wurden (Rad17, Rad50, Ku80) (Ascencio-Ibanez et al., 2008), besonders interessante Kandidaten zur weiteren Untersuchung. Durch die transkriptionelle Verstärkung dieser Faktoren könnte sowohl eine unterstützende Rolle bei der viralen Replikation als auch ein Abwehrmechanismus der Pflanze angedeutet werden. Insertionsmutantenlinien verschiedener Reparaturfaktoren und deren wt-Schwesterlinien wurden daher mit EuYMV (Isolat MGS1) inokuliert und die systemische Infektion durch 1D und 2D AGE analysiert.

Rad51 und die Rad51-Paraloge Rad51B, Rad51C, Xrcc2 und Xrcc3 hatten keinen essenziellen Einfluss auf den Infektionsverlauf oder die Akkumulation der verschiedenen viralen DNA-Formen oder Replikationsintermediaten. Der Ausfall des Paralogs Rad51D (rad51d) hingegen hatte zu frühen Zeitpunkten (7 bzw. 14 dpi) eine signifikante Reduktion der viralen DNA-Titer bzw. eine Verzögerung der systemischen Infektion zur Folge. Allerdings wurden im Vergleich zum wt keine Unterschiede von spezifischen Replikationsintermediaten oder -produkten detektiert, die Rückschlüsse über die Funktion von Rad51D bei der geminiviralen Vermehrung ermöglichen würden. Neben der Stranginvasion, die für die RDR notwendig sein könnte, spielt Rad51D, ebenso wie Rad51B und Xrcc2, eine individuelle Rolle während der SSA-Reparatur in Arabidopsis (Serra et al., 2013). Die dreifach knock-out-Linie rad51b rad51d xrcc2 wurde daher gleichermaßen getestet. Auch bei dieser Linie verzögerte sich die systemische Infektion signifikant im Vergleich zum wt, allerdings intensivierte sich dieser Effekt im Vergleich zu rad51d nicht. Wie zuvor für rad51d wurden keine Unterschiede der viralen Replikationsintermediate oder -produkte im Vergleich zu wt-Pflanzen detektiert.
Zusätzlich wurden die Linien rad52-1, mus81, rad17 und rad50 getestet. Rad52-1 ist eines der zwei Arabidopsis-Homologe des HR-Proteins Rad52 (Samach et al., 2011), welches in S. cerevisiae einerseits die Rekrutierung von Rad51 an die ssDNA bewerkstelligt, und ebenso in den SSA-Reparaturweg involviert ist (San Filippo et al., 2008). Mus81 bildet im Komplex mit Eme1 eine strukturspezifische Endonuklease, die Rekombinationsintermediate prozessieren und auflösen kann (Geuting et al., 2009; Hartung et al., 2006). Rad17 ist als Kontroll- und Sensorprotein am Zellzyklusarrest und der Signalweiterleitung nach DNA-Schäden und blockierter Replikation beteiligt (Heitzeberg et al., 2004; Wang et al., 2014). Rad50 spielt als Teil des MRN-Komplexes bei der Detektion und Prozessierung von DSBs eine entscheidende Rolle (Lamarche et al., 2010; Williams et al., 2010). Keiner dieser Faktoren hatte einen nachweislichen Einfluss auf den geminiviralen Infektionsverlauf oder die Replikation. Im Fall der rad50-Linie war allerdings ein verlässlicher Abgleich mit der wt-Linie nicht möglich, da der homozygote knock-out von Rad50 zu erheblichen Wachstumsdefekten der Pflanzen führte.

Rad51D leistete als einziger der untersuchten HR-Faktoren einen wesentlichen Beitrag zur geminiviralen Vermehrung in Pflanzen. Obwohl das Rep von MYMIV und AtRad51 interagieren (Suyal et al., 2013), wurde hier für die EuYMV-Replikation keine Rolle von Rad51 nachgewiesen. Rad51, die Rad51-Paraloge Rad51B, Rad51C, Xrcc2 und Xrcc3 oder Rad52-1, Rad17 und Mus81 könnten dennoch an der geminiviralen Replikation beteiligt sein. Ihre Funktion könnte aber verzichtbar sein oder durch andere Proteine kompensiert werden. Da trotz der förderlichen Wirkung von Rad51D keine Veränderungen von spezifischen DNA-Formen oder Replikationsintermediaten detektiert wurden, kann Rad51D keine genaue Funktion bei der viralen Vermehrung zugeordnet werden. Möglicherweise ist Rad51D an der Stranginvasion bei der RDR beteiligt und quantitative Unterschiede der RDR-Intermediate waren lediglich zu subtil für den Nachweis über 2D AGE. Rad51D ist im humanen Rad51 Paralog-Komplex BCDX2 ein obligatorischer Bestandteil und das Bindeglied zwischen Rad51C und Xrcc2 (Miller et al., 2004).

Falls der pflanzliche Komplex ähnlich aufgebaut sein sollte, würde Rad51D vermutlich nicht als Teil des BCDX2-Komplexes an der RDR beteiligt sein, sondern als Teil eines Subkomplexes oder als individuelles Protein, da die Ausfälle der anderen Rad51-Paraloge keine Folgen hatten. Neben den beschriebenen RDR-Prozessen könnten andere geminivirale rekombinationsabhängige Replikationsformen existieren, bei denen beispielsweise ssDNA als Matrise verwendet wird, und für die Rad51D notwendig ist. Die dazugehörigen Intermediate könnten dem Nachweis über 2D AGE durch die Überlagerung mit anderen DNA-Formen oder Intermediaten bisher entgangen sein. Rad51D wurde außerdem als individueller Faktor der SSA-Reparatur in A. thaliana identifiziert (Serra et al., 2013). Dieser Reparatur-Modus könnte für geminivirale DNAs nützlich sein: Lineare Kopien der geminiviralen Genome oder auch verkürzte virale DNA könnten durch SSA-Aktivität verbunden oder zirkularisiert werden. SSA führt
normalerweise zum Verlust der nicht-komplementären Bereiche zwischen den homologen Sequenzen (Knoll et al., 2014; Puchta, 2005). Im Fall von vollständig komplementären Sequenzen viraler linearer dsDNA-Kopien würden jedoch funktionale und völlig intakte multimere oder zirkuläre Produkte entstehen. Auch die nachgewiesenen Hybridsequenzen zwischen geminiviraler DNA A und DNA B (Gregorio-Jorge et al., 2010; Patil et al., 2007) oder zwischen verschiedenen Genomkomponenten der nah verwandten Nanoviren (Stainton et al., 2012) könnten über SSA der homologen CR-Bereiche entstehen.


In Säugetieren wurde der DNA-PK-Komplex bestehend aus Ku70, Ku80 und der katalytischen Untereinheit der DNA-abhängigen Proteinkinase (DNA-PKcs) als sequenzunspezifischer, cytoplasmatischer Sensor der linearen dsDNA bei Infektionen mit Vaccinia-Viren und humanem
Ergebnisse und Diskussion


Die Beteiligung von Translásionssynthese-Polymerasen an der geminiviralen Replikation (Manuskript 5)

'The contributions of translesion synthesis polymerases to geminivirus replication'

TLS-Polymerasen könnten bei der initialen geminiviralen CSR von Bedeutung sein, da sie in ausdifferenzierten Pflanzenzellen exprimiert werden (Garcia-Ortiz et al., 2004; Nakagawa et al., 2011; Sakamoto et al., 2003; Santiago et al., 2008; Takahashi et al., 2005). Ihre relativ hohe Replikationsungenaugigkeit könnte dabei die hohen Mutationsraten geminiviralen Genome hervorrufen. Um diese Hypothese zu prüfen, wurde zunächst der Verlauf der systemischen geminiviralen Infektion in A. thaliana-Linien mit Ausfällen der TLS-Polymerasen Polη (kodierts durch das Gen POLH), Rev1 (REV1) und Polζ (REV3) kontrolliert. Dafür wurden die etablierten knock-out-Linien polh-1, rev1-2 und rev3-2 und deren jeweilige wt-Schwesterlinie biolistisch mit EuYMV (Isolat MGS1) inokuliert. Keine der drei Linien zeigte eine Veränderung des Infektionsverlaufs oder der viralen DNA-Akkumulation im Vergleich zu wt-Pflanzen bei der 1D AGE-Analyse. Um die Auswirkungen auf die Intermediate der frühen EuYMV Replikation


Ergebnisse und Diskussion


Sowohl die Linien rev3-2, polh-1 und rev1-2 als auch Linien mit kombiniertem knock-out von Polζ/Polη (rev3-1 polh-1) und Pol ζ/Rev1 (rev3-1 rev1-1) (Takahashi et al., 2005) wurden im Vergleich zur wt-Schwesternlinie von rev3-2 der Übertragung von EuYMV durch B. tabaci ausgesetzt. Infektionsverlauf und die viralen DNA-Gehalte waren nicht unterscheidbar zwischen


Zusammenfassend veranschaulichten die verschiedenen Inokulationsexperimente, dass Polη, Polζ und Rev1 individuell und die Kombination aus Polζ/Polη und Polζ/Rev1 für die geminivirale Replikation nicht notwendig sind. Da polk-2- und wt-Schwesterpflanzen zu geringe Infektionsraten bei der Insektenübertragung aufwiesen und beide polk-Linien möglicherweise noch Polk-Aktivität aufweisen bzw. verkürzte Polk-Varianten exprimieren könnten, kann keine endgültige Aussage über die Rolle von Polk getroffen werden. Das Rad6-Homolog Ubc2, das

Interessanterweise sind die TLS-Polymerasen Polη, Polκ, und Rev1 in die DNA-Synthese während der Re-Replikation in humanen Zellen eingebunden (Sekimoto et al., 2015). Da durch die Aktivität des Rep-Proteins Re-Replikation in infizierten Hefe- und Pflanzenzellen induziert wird (Hipp et al., 2014; Kittelmann et al., 2009; Nagar et al., 2002), könnten TLS-Polymerasen nicht nur an der initialen CSR, sondern auch an der späteren Replikation beteiligt sein.


Durch die signifikant veränderten Nukleotidaustauschraten der EuYMV-DNA aus wt- und rev3-1 polh-1-Pflanzen wurde ein Einfluss von Polξ und/oder Polη auf die geminivirale Replikation ersichtlich. Gleichzeitig wurden durch die differentiellen Effekte der DNA A und DNA B ein unterschiedlich wirkender Selektionsdruck für DNA A und DNA B angedeutet. Geminiviren generieren während ihrer Replikation oftmals defekte DNAs mit deletierten Sequenzen und DNA B-abgeleitete Moleküle sind hierbei häufiger vertreten als DNA A-abgeleitete (Bach & Jeske, 2014; Frischmuth et al., 1997; Horn et al., 2011; Patil & Dasgupta, 2006). Diese defekten DNAs enthalten für gewöhnlich eine hohe Anzahl an Sequenzveränderungen, was auf den geringen Selektionsdruck, der auf diesen Molekülen lastet, zurückzuführen ist. Eine weiterführende Sequenzanalyse defekter DNAs könnte daher ein vielversprechender Ansatzpunkt für den Nachweis TLS-Polymerase-spezifischer Fehlersignaturen darstellen, da sich die eingefügten Fehler in diesen Molekülen ungehindert anhäufen könnten.

Hefe-, Maus- oder humane TLS-Polymerasen verursachen einzelne oder komplexere Insertionen und Deletionen, der Großteil der verursachten Fehler sind jedoch Einzel-Nukleotidaustausche (Arana & Kunkel, 2010; Kozmin et al., 2003). Bei der Replikation von unbeschädigter Matrizen-DNA wurden alle zwölf möglichen Austausche nachgewiesen, allerdings tendierten verschiedene
TLS-Polymerasen zu spezifischen Austauschen. Beispielsweise führte die Aktivität von Polη bevorzugt zu T\(\rightarrow\)C Mutationen (Matsuda et al., 2000; Matsuda et al., 2001). Dagegen verursachte Polζ tendenziell A\(\rightarrow\)G und C\(\rightarrow\)G (Zhong et al., 2006) und Polκ T\(\rightarrow\)G Mutationen (Ohashi et al., 2000). Ein Fehlertypus, der fast ausschließlich bei TLS-Polymerasen beobachtet wurde und damit als deren spezifische Fehlersignatur gilt, ist der Doppel-Nukleotidaustausch (Arana & Kunkel, 2010). Sogar Dreifach-Nukleotidaustausche traten nach der Replikation von unbeschädigter Matrizen-DNA durch Polη und Polζ auf (Matsuda et al., 2001; Zhong et al., 2006). Auch bei der Replikation nach komplexer DNA-Schädigung durch künstliches Sonnenlicht wurden Doppelnukleotidaustausche in wt- und verschiedenen TLS-Polymerase-defizienten Hefestämmen verursacht (Kozmin et al., 2003). In wt- und rev3-Stämmen wurden dabei ausschließlich CC\(\rightarrow\)TT Mutationen nachgewiesen, im Rad30-defizienten (Homolog zu Polη) Stamm wurde dagegen ein breiteres Spektrum an Tandem-Mutationen dokumentiert (CC\(\rightarrow\)TT; CC\(\rightarrow\)TA; CC\(\rightarrow\)AT; GC\(\rightarrow\)TT; TC\(\rightarrow\)AT; TC\(\rightarrow\)AA). Der kombinierte knock-out-Stamm rev3 rad30 umfasste ausschließlich CC\(\rightarrow\)AA Mutationen.

Bisherige Sequenzanalysen geminiviraler Genome ließen eine Präferenz für C\(\rightarrow\)T, G\(\rightarrow\)A oder G\(\rightarrow\)T Mutationen erkennen (Duffy & Holmes, 2008, 2009; Ge et al., 2007; van der Walt et al., 2008). Da diese Austausche eine bevorzugt strangspezifische Verteilung aufweisen, entstehen sie vermutlich durch Desaminierung (C\(\rightarrow\)T) oder oxidativ-induzierten Stress (G\(\rightarrow\)T) an der einzelsträngigen viralen DNA-Form (Monjane et al., 2012; van der Walt et al., 2008). Genome von East African cassava mosaic virus neigen außerdem zu T\(\rightarrow\)G Austauschen (Duffy & Holmes, 2009), die mit der Fehlersignatur von Polκ übereinstimmen würden. Für EuYMV sind weiterführende Analysen der Sequenzdaten, idealerweise von defekten DNA-Molekülen, notwendig, um mögliche Signaturen von TLS-Polymerasen aufzeigen zu können.
Publikationsmanuskripte in englischer Sprache

Manuskript 1:
‘Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner’
Kathrin S. Richter, Tatjana Kleinow, Holger Jeske

Manuskript 2:
‘Rad54 is not essential for any geminiviral replication mode in planta’
Kathrin S. Richter, Lukas Ende, Holger Jeske
Veröffentlicht in Plant Molecular Biology (2015), 87: 193-202

Manuskript 3:
‘Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication’
Kathrin S. Richter, Holger Jeske

Manuskript 4:
‘The role of homologous recombination factors for geminiviral infection in planta’
Kathrin S. Richter, Heidi Serra, Charles I. White, Holger Jeske

Manuskript 5:
‘The contributions of translesion synthesis polymerases to geminivirus replication’
Kathrin S. Richter, Monika Götz, Stephan Winter, Holger Jeske
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Kathrin S. Richter, Tatjana Kleinow, and Holger Jeske*

Institute of Biomaterials and Biomolecular Systems, Department of Molecular Biology and Plant Virology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany


#Author for correspondence:

Email: holger.jeske@bio.uni-stuttgart.de
Phone: +49-711-685-65070

Figures: 6
Tables: 1
Supplementary Figures: 4
Supplementary Tables: 2

Abstract

Four transgenic *Arabidopsis thaliana* lines carrying different reporter gene constructs based on split glucuronidase genes were used to monitor the frequency of somatic homologous recombination after geminivirus infections. Euphorbia mosaic virus and Cleome leaf crumple virus were chosen as examples, because they induce only mild symptoms and are expected to induce less general stress responses than other geminiviruses. After comparing the different plant lines and viruses as well as optimizing the infection procedure, Euphorbia mosaic virus
t Enhanced recombination rates significantly in the transgenic reporter line 1445. The effect was tissue-specific in cells of the leaf veins as expected for this phloem-limited virus. The advantage for geminiviruses to activate a general recombination pathway is discussed with reference to an increased fitness by generating virus recombinants which have been observed frequently as an epidemiologic driving force.

---

1 Now renamed to Euphorbia yellow mosaic virus (EuYMV). Renaming of EuMV to EuYMV was implemented according to Fernandes et al., (2011).
Introduction

DNA damage in plants can be repaired by various eukaryotic protein systems (Bray & West, 2005; Kimura & Sakaguchi, 2006), including somatic homologous recombination (SHR) with high fidelity. Several studies have demonstrated that abiotic and biotic stresses increase the SHR frequency in plants (Boyko et al., 2005; Kovalchuk et al., 2003; Lucht et al., 2002; Molinier et al., 2005; Ries et al., 2000). These stressors trigger either a boost of reactive oxygen species or cause DNA damage directly. For some stress types (e.g. UV-C, elicitor flagellin) the SHR frequency was found to be elevated even within the subsequent, non-stressed plant generation (Molinier et al., 2006). This phenomenon was called "transgenerational stress memory" and is likely an epigenetic effect, because it depends on the functional Dicer-like proteins Dcl2 and Dcl3 (Boyko et al., 2010; Boyko & Kovalchuk, 2010). However, the transgenerational effect does not occur generally for all stressors (Pecinka et al., 2009).

In the cited studies, transgenic SHR reporter constructs were used to monitor changes of homologous recombination frequencies (HRF). They consist of two non-functional split parts of a reporter gene with partially overlapping sequences of several hundred base pairs of the β-glucuronidase gene (GUS; Fig. 1a). Homologous recombination of the overlapping sequences restores GUS activity which can be detected by histochemical staining. The recombination events monitored as blue spots or sectors in plant tissues allow the quantitative evaluation of SHR. The reporter constructs may be arranged in direct or indirect orientation enabling further insights into the type of the recombination events (Gherbi et al., 2001; Puchta et al., 1995a). They had been integrated into the Arabidopsis thaliana genome of two ecotypes at different loci with the help of Agrobacterium tumefaciens (Tinland et al., 1994) (Fig. 1a). Consequently, distinct plant lines exhibited different baselines of HRF as well as different responsiveness to stress types which was attributed either to the kind of SHR reporter construction (length or orientation of homologous overlaps), the genomic position or chromatin status of the transgene, the ecotype background, or to a combination of these properties (Pecinka et al., 2009).

Geminiviruses (Jeske, 2009) replicate their circular single-stranded (ss) DNA by three modes of action: complementary strand replication (CSR), rolling-circle replication (RCR) and recombination-dependent replication (RDR) (Alberter et al., 2005; Erdmann et al., 2010; Jeske et al., 2001; Jovel et al., 2007; Preiss & Jeske, 2003). They rely completely on host proteins for replication because they do not encode a DNA polymerase. This is true in particular for the plant homolog of the retinoblastoma protein (pRBR), a cell cycle regulator that blocks replication in differentiated cells (reviewed by Gutierrez et al., 2004; Hanley-Bowdoin et al., 2004). As a consequence, host DNA may be re-replicated, as has been shown for plants (Nagar et al., 2002) and yeasts (Kittelmann et al., 2009).
Recombination is an important factor for the evolution and epidemics of geminiviruses (van der Walt et al., 2009, and references therein). At the same time the RDR mode provides an efficient mechanism for early recombination during infection. This motivates our current study on whether host recombination can be influenced by geminiviruses. A transcriptome analysis of *A. thaliana* after geminivirus (cabbage leaf curl virus) infection revealed several changes in the expression of SHR pathway factors (Ascencio-Ibanez et al., 2008). It is therefore plausible that geminivirus infection may influence this host pathway.

Most of the geminiviruses are confined to the phloem tissue (Horns & Jeske, 1991; Wege et al., 2001) allowing us to differentiate between direct effects in the infected tissue and general, stress-induced effects in the whole plant for the first time. The results show that geminiviruses are indeed promoting SHR in phloem tissue under defined experimental conditions.

**Results**

Monitoring SHR by the help of the reporter constructs as presented in Fig. 1a has been shown to be dependent on the physiological condition of the plants. Most reports have used young plants in axenic cultures for optimal differentiation of baseline and stress-induced SHR. Moreover, the kind of the stressor is important for the outcome of the assay. On the other hand, infection of non-transgenic *Arabidopsis* with the geminiviruses used in this study was found to be optimal at later stages of development in potted plants with vigorous vegetative growth (Paprotka et al., 2010). It was therefore necessary to find a compromise between the optimal experimental conditions for monitoring SHR efficiency and for viral infection. A second difference between this study and previous ones is the phloem-limitation of many geminiviruses. If this tissue tropism is true for the investigated geminiviruses, it would allow discrimination of changes in SHR originated by general stresses from those specifically induced by virus infection in phloem cells.

In a first series of experiments, Euphorbia mosaic virus (EuMV, isolates EuMV-MGS1 or EuMV-MGS2) or Cleome leaf crumple virus (CILCrV) without their alpha satellite DNAs (Paprotka et al., 2010) were delivered biolistically to the four transgenic lines (Fig. 1) at a later stage of plant development (12-14 leaf stage, see 'Material and methods' for details).
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

### a

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Line</th>
<th>Homologous overlap</th>
<th>Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>C24</td>
<td>651</td>
<td>566 bp</td>
<td>At5_10455621</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1213 bp</td>
<td>At2_11758119</td>
</tr>
</tbody>
</table>

| Columbia | IC9C | 1213 bp | At5_8633787 |
|          | 1445*| 618 bp   | At2_14418017 |

### b

- **M**
- EuMV-MGS1
- EuMV-MGS2
- CiLCrV

- Eu1 A
- Eu1 B

- Eu2 A
- Eu2 B

- Ci A
- Ci B

- 1200
- 1138
- 1135
- 902
- 1232
- 893
- 587
- 527
- 503
- 397
- 377
- 376
- 331
- 323
- 283
- 285
- 270
- 245
- 204
- 144
- 140
- 151
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Figure 1: Prerequisites of the analysis. (a) Schematic representation of SHR reporter constructs present in *A. thaliana* transgenic lines 651 (Puchta et al., 1995b), 11 (Swoboda et al., 1994), IC9C (Molinier et al., 2004) and 1445 (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009). Ecotype background, length (bp) and orientation (direct or inverted) of the GUS overlap construct are indicated for each line. The positions of the genomic integration locus are given according to 'The Arabidopsis Information Resource' (TAIR, Apr 02, 2013). LB and RB: left and right border of T-DNA; P35S: cauliflower mosaic virus promoter; T35S: cauliflower mosaic virus terminator; GUS: β-glucuronidase reporter gene; TNos: nopaline synthase terminator; HPT: hygromycin phosphotransferase gene. (b) RFLP analyses to confirm the exclusiveness of full-length DNA A and DNA B from Euphorbia mosaic virus-MGS1 (EuMV-MGS1), Euphorbia mosaic virus-MGS2 (EuMV-MGS2) or Cleome leaf crumple virus (CILCrV) in the applied inocula. RCA products of viral DNAs from systemically infected wild-type *A. thaliana* plants are shown, which were treated with *Hpa*I (five technical replicates) for each virus. Restriction fragments were separated in 2% agarose gels, with 600 ng of *Pst*I-digested λ DNA as molecular weight marker (M) and staining with ethidium bromide afterwards. Black and gray numbers indicate the expected fragment sizes (in bp) for DNA As and DNA Bs, respectively. The corresponding undigested RCA products were used for biolistic inoculation. (c) Characterization of the mock-inoculum by RCA/RFLP as in (b). In order to generate RCA products containing only DNA B, restriction enzymes were chosen to linearize only DNA B, and the resulting fragment was gel-purified, recircularized and amplified by RCA. This product was digested with the diagnostic restriction enzyme showing the absence of DNA A or satellite DNA.

Symptoms, presence of viral DNA and SHR rates of three leaves per tested plant were compared to those of mock-inoculated plants of the four lines treated in parallel. Symptoms were generally mild or not visible macroscopically, with some curling in newly emerging leaves in the combination of EuMV-MGS1 and Col line IC9C (data not shown). Using RCA, restriction fragment length polymorphism (RFLP) combined with Southern blot hybridization to detect viral DNA (Fig. 4), EuMV-MGS1 showed the highest infection rate of more than 90% in all transgenic lines (Table 1). However, none of the combinations of viruses and plant lines revealed a statistically significant difference in the SHR frequency compared to mock-inoculated plants in three tested leaves and at this late stage of plant development (data not shown, ten plants per line and inoculation type, thus 40 plants per transgenic line were tested in total).
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Table 1: Infection rates of EuMV-MGS1, EuMV-MGS2 and CIllCrV on transgenic lines 651, 1445, IC9C and 11.

<table>
<thead>
<tr>
<th>Late inoculation</th>
<th>Line</th>
<th>651</th>
<th>1445</th>
<th>IC9C</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>EuMV-MGS1</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>EuMV-MGS2</td>
<td>6/10</td>
<td>5/10</td>
<td>1/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>CIllCrV</td>
<td>10/10</td>
<td>10/10</td>
<td>8/10</td>
<td>3/10</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Early inoculation</th>
<th>Experiment #</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td>651</td>
<td>1445</td>
<td>651</td>
<td>1445</td>
</tr>
<tr>
<td>EuMV-MGS1</td>
<td>5/10</td>
<td>5/10</td>
<td>11/20</td>
<td>15/20</td>
</tr>
<tr>
<td>EuMV-MGS2</td>
<td>0/10</td>
<td>0/10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CIllCrV</td>
<td>0/10</td>
<td>2/10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In a second series of experiments, plants were inoculated at an earlier stage of development (4-6 leaf stage, see Material and methods for details) where they were at a young rosette leaf stage when harvested at 21 dpi and thus facilitated using the whole plantlets for GUS staining. This procedure reduced the infection rates but provided sufficient infected plants in certain combinations (Table 1). EuMV-MGS1 showed the highest reproducible infection rates in two transgenic lines (651, 1445) and was therefore chosen for the further study.

The punctate signals upon tissue blot hybridization indicated phloem-limitation of EuMV-MGS1 (Fig. 2a), a conclusion confirmed further by microscopic NBT/BCIP-based in situ hybridization (Fig. 2b, c; Suppl. Fig. S1 for mock-control) and fluorescence in situ hybridization (FISH; Fig. 2d, e; Suppl Fig. S2). Very few cells, exclusively associated with vascular tissue, showed virus-specific staining or fluorescence. Infected plants showed mild symptoms (Fig. 3) with some leaf curling indicating that only a low level of general stress was elicited by the treatments. Viral DNA was detected with high sensitivity by a combination of RCA/RFLP and Southern blot hybridization as exemplified for the comparison of EuMV-MGS1 and CIllCrV in two plant lines (Fig. 4; for the results of further analyses see Suppl. Fig. S4). This sensitive detection allowed us to unequivocally distinguish infected from uninfected plants for the following comparison.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.

Figure 2: Phloem-limitation of EuMV-MGS1 in A. thaliana plants. (a) Tissue blots of infected or mock-inoculated (m) lines 651 and 1445 were probed at 33 dpi. Cross-sections of inflorescence shoots or furled rosette leaves from one plant per line and infection type were dappled onto nylon membranes and hybridized with a full-length DNA A probe (3 h exposition time). Punctuate hybridization signals indicate a restriction to vein cells. (b, c) Microscopic images of infected rosette leaves of lines 1445 (b) and 651 (c) harvested at 33 dpi after in situ hybridization with full-length DNA A probes using NBT/BCIP for staining. Mock-controls are provided in Suppl. Fig. S1. (d, e) FISH detection of EuMV-MGS1 in infected (21 dpi) plants of line 1445 with a 5'-Cy3-labeled primer hybridizing within the AV1 ORF. Merged DIC and fluorescent image from Suppl. Fig. S2c, d with two magnifications to show the localization of the hybridization signal within the phloem of the petiole. Corresponding mock controls in Suppl. Fig. S2a, b. Virus-specific signals are indicated by arrows. X: xylem, Ph: phloem; bar = 50 µm.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Figure 3: Symptoms after early inoculation at the 4-6 leaf stage. EuMV-MGS1 infected and mock-inoculated *A. thaliana* plants of the C24 line 651 and the Col line 1445 at 16 dpi. Infected and mock-inoculated plants at higher magnification show mild but distinct leaf rolling in line 1445, which was barely visible in line 651.

EuMV-MGS1 infected and mock-inoculated plants of lines 651 and 1445 showed two types of GUS signal in the SHR assay, either in the mesophyll and epidermis (Fig. 5a, b) or associated with veins (Fig. 5c, d), if investigated under the stereomicroscope. The vast majority of these signals represented single cells rather than patches of tissue. In order to enable at least a gross comparison between our environmental condition with those of other authors, a heat stress experiment according to (Pecinka et al., 2009) was performed and SHR frequencies determined (Fig. 6a). Numbers of blue spots seen under the stereomicroscope were counted and referred to the fresh weight of the respective plants (see material and methods for details). Line 1445 showed a significant increase of total SHR signals under long day conditions after the stress (Fig. 6a, total grey box). Although the vein-associated and the non-vein-associated subset of data revealed the same trend when counted individually, the number of data was too small to pass the statistical test.

Experiment to experiment variation has to be considered for geminivirus infections. Three independent experiments, each with parallel treatments of two plant lines 651 and 1445, revealed different infection rates (Table 1) and varying absolute counts for SHR signals (Fig. 6).
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

The numbers of counts were generally lower for line 651 than for line 1445. In experiment #1 the overall response was similar to that of heat stress but significantly more vein-associated signals were observed. Both lines showed the same trend for more vein-associated signals after infection, but sampling sizes were only large enough to pass the significance test in all three experiments for line 1445 (Fig. 6 asterisks; p<0.001; t-test or Mann-Whitney rank sum test if data was not distributed normally). Although absolute numbers varied between experiments,
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

the relative increase after infection was five times higher for vein-associated SHR signals throughout all experiments with line 1445.

In summary, the phloem-limited EuMV-MGS1 is able to increase significantly SHR in individual vein cells in at least one reporter plant line. This is probably due to a specific impact on the SHR machinery of infected cells by the virus.

**Figure 5**: Recombination events detected by GUS activity. Plants of line 1445 after mock-treatment (a, c) or EuMV-MGS1 (b, d) infection at 21 dpi show GUS signals (blue spots) in single cells. GUS activity (encircled) was observed after histochemical staining with X-Gluc by stereomicroscopy in the mesophyll and epidermal tissues (a, b) or associated to vascular tissues (c, d). Bar=100 μm.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.

**Figure 6**: Statistical analysis of recombination events in SHR reporter plants. Box plot graphs of total, vein- and non-vein-associated GUS signal frequencies per gram plant material for heat-stressed, mock-inoculated, or EuMV-MGS1 infected plants of lines 651 and 1445 at 21 dpi are compared. (a-b) Independent replicate experiments (Expt. 1 - 3) are shown for the infection study. The following numbers of plants were tested: 10 (Expt. 1), 35 (Expt. 2) and 28 (exp. 3) for line 651; 11 (exp. 1), 37 (exp. 2) and 29 (exp. 3) for line 1445. T-tests or Mann-Whitney rank-sum tests, if data were not distributed normally, were used to confirm significant differences (p<0.05 for heat stress, p<0.001 for infection; indicated by asterisks). Boxes comprise 50% of data, bars imply minimum and maximum, *, median.
Discussion

In accordance with the recombination-dependent replication of geminiviruses (Jeske, 2007), the activation of cellular DNA synthesis (Kittelmann et al., 2009; Nagar et al., 2002), and the up-regulation of SHR pathway genes (Ascencio-Ibanez et al., 2008), the results show for the first time that transgenic reporter genes can recombine more frequently after a geminivirus infection. Moreover, this study has distinguished between different tissues in contrast to previous reports which is particularly important for the identification of a specific effect induced by a phloem-limited geminivirus.

The test assay has proven to be sensitive to the experimental conditions as discussed by other authors before (Pecinka et al., 2009), and the proper parameters for geminivirus infection had to be determined here. At least for one combination of virus and transgenic reporter line (EuMV-MGS1 and line 1445), the enhancement of recombination frequencies was significant in three independent experimental sets. The same trend is visible for the other combination of EuMV-MGS1 with line 651 but the difference was too low to pass the statistic tests. Therefore, increasing sample sizes may lead to a similar conclusion. Since both lines (1445 and 651) contain an inverted GUS with approximately the same length of overlap (618 bp and 566 bp), it is likely that the ecotype background and/or the chromatin status at the respective integration sites were the cause of the different SHR baseline level and responsiveness to EuMV-MGS1 infection.

RNA viruses of the genus *Tobamovirus* increased recombination frequencies of their hosts as well. However, the results of the experiments are difficult to compare directly to our data due to differences in the experimental design. Oilseed rape mosaic virus (ORMV) and tobacco mosaic virus infection (TMV) enhanced SHR two- to threefold in *Nicotiana tabacum* (Kovalchuk et al., 2003). The luciferase-based system in this study was nine- to twelve-fold more sensitive compared to the GUS-based system (Ilnytskyy et al., 2004). Moreover, the SHR increase was not limited to infected tissues, but was transmittable to healthy plants via a “systemic recombination signal” as shown by grafting experiments. A further study revealed that ORMV infection promoted HRF (1.6-fold) and genome rearrangements in general (mutation frequency, microsatellite instability) in *A. thaliana* using GUS-based detection systems (Yao et al., 2011). HRF increase was variable depending on the concentration of the inoculated virus, plant age and the time or speed of virus replication (Yao et al., 2013). A boost of reactive oxygen species, salicylic acid-dependent systemic acquired resistance signalling or transport of viral small RNAs were discussed to be responsible for the activation of SHR in uninfected tissue.

Recently, the unambiguity of the results with the SHR reporter lines used in the current study was questioned (Ülker et al., 2012), and the authors postulated alternative explanations (post-
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.

transcriptional or posttranslational event; read-through transcription, alternative splicing, trans-splicing or split protein complementation). In particular, line 1445 raised skepticism as the gene locus was not characterized sufficiently. Puchta and Hohn (2012) have already responded to these claims in general and in some details which we follow. Especially the inverted orientation of the GUS overlap in the tested lines 651 and 1445 rules out that read-through transcription or alternative splicing restore the enzyme activity. Although the fusion of two separately transcribed mRNAs by trans-splicing or transcriptional slippage may occur (reviewed by Dubrovina et al., 2013), we consider this mechanism to be highly unlikely for the lines 651 and 1445, because no promoter is present in these constructs for transcription of the second half (US, Fig. 1). For the same reason, protein complementation seems to be implausible.

The line 1445 has been genotyped in the course of our experiments. The transgene integration of reporter line 1445 is located on chromosome 2 at position 14424870 (corresponding to pos. 14418017 in the latest release of the *A. thaliana* genome; The Arabidopsis Information Resource (TAIR 10), ftp://ftp.arabidopsis.org/home/tair/Sequences/ on www.arabidopsis.org, Apr 02, 2013) according to several reports (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009). One publication (Sun et al., 2008) deviated from this statement mentioning a position on chromosome 5 (Pos. 8633790, corresponding to pos. 8633787 in TAIR10), which was possibly an erroneous assignment only, since this is the integration locus of line IC9C (Molinier et al., 2004) (see Fig.1). After having tested all plants of each transgenic line by PCR-based genotyping (Supplementary data for line 651 and 1445, 11 and IC9C not shown), no doubt remains about the real integration locus.

Although the comparative heat stress test showed similar trend values under our conditions for enhanced vein-associated signal enhancement, the geminiviral infection should exert a more specific effect on phloem cells. All geminiviruses replicate in nuclei-containing cells of the phloem (companion cells, phloem parenchyma cells) as reviewed by Wege (2007) and most of them are confined to this tissue, as shown for EuMV-MGS1 in this study. This limitation can be overcome partially by co-infection with other viruses upon systemic infection (Pohl & Wege, 2007; Sardo et al., 2011; Wege, 2009) or during agroinfiltration in locally infected cells (Zhang et al., 2001). Therefore it is believed that the restriction is caused by host defense mechanisms rather than the inability of geminiviruses to multiply in other tissues.

All geminiviruses need to activate DNA metabolism in differentiated cells, in the first instance in the phloem and some of them in palisade, spongy parenchyma and epidermal cells thereafter (reviewed by Gutierrez et al., 2004; Hanley-Bowdoin et al., 1999; Hanley-Bowdoin et al., 2004). This task is mainly realized by the interaction of the geminiviral replication-initiator protein Rep and the plant retinoblastoma related cell cycle regulator pRBR which promotes DNA synthesis.
but is not absolutely necessary for the replication in the phloem as discussed in detail recently (Ruschhaupt et al., 2013). Interestingly, Rep was also found to interact with key enzymes (Rad54 and Rad51) of the recombination pathway for mungbean yellow mosaic India virus (Kaliappan et al., 2012). The combination of host cell re-replication and recruitment of recombination enzymes would explain convincingly the enhanced recombination-rate observed in the phloem in this study. Alternatively, viral ssDNA could be perceived as DNA damage or a genotoxic stressor by its host (Weitzman et al., 2004). This would trigger an increase in SHR similar to other stressors mentioned before and could in turn be exploited for virus replication. However, geminivirus infection does probably not provoke a general genotoxic stress response as shown by comparing transcriptome profiles of infected and genotoxically stressed A. thaliana plants (Ascencio-Ibanez et al., 2008). Together with our data, this lends support to the conclusion that SHR increase is a specific effect of the geminivirus infection rather than a general stress consequence differing thus from the tobamovirus infections mentioned above.

A general enhancement of the recombination frequency upon geminivirus infection could explain the frequent appearance of recombinant gemini- and related viruses in the field (Stainton et al., 2012 and references therein; van der Walt et al., 2009) and thus increase the fitness of these viruses.

**Materials and methods**

**Plants and viruses**

Transgenic Arabidopsis thaliana plants of two ecotypes (Columbia and C24) were kindly provided by Drs. O. Mittelsten Scheid (Gregor Mendel Institute, Vienna) and H. Puchta (Karlsruhe Institute of Technology, Karlsruhe): Ecotype C24 transgenic lines 651 and 11 according to (Puchta et al., 1995b; Swoboda et al., 1994) and Columbia lines 1445 (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009) and IC9C (Molinier et al., 2004). They contained the GUS reporter constructs described in Fig. 1a.

Rolling circle amplification (RCA) products of Euphorbia mosaic virus (EuMV; isolates -MGS1 and -MGS2) and Cleome leaf crumple virus (CILCrV) DNA without satellite DNA were used to inoculate the plants (Paprotka et al., 2010). For mock inoculation DNAs B alone of EuMV-MGS1 or CILCrV were engineered in the following manner: RCA products of viral DNAs were generated from total nucleic acid extracts from infected A. thaliana plants and linearised with singly cutting restriction enzymes (for EuMV-MGS1 XmaI; for CILCrV NsiI). The resulting fragments were gel-purified and re-circularised by T4 DNA ligase, and used as templates for a second RCA to provide the inoculum. The resulting DNAs B alone were unable to infect the plants.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

**Biolistic inoculation**

RCA products of the respective viral DNAs were amplified from total nucleic acids of previously infected *A. thaliana* (Col) plants (Paprotka *et al.*, 2010) and inoculated biolistically (Biolistic Particle Delivery System, PDS-1000/He; rupture discs for 450 psi or 900 psi, macrocarriers, 1.1 µm tungsten microcarriers or 1 µm gold microcarriers; all from Bio-Rad, Munich, Germany) as described (Zhang *et al.*, 2001). Construct integrity was checked by *Hpa*II digestion and gel electrophoresis beforehand (Fig. 1b and c). Per plant, 1.5 µl of the viral RCA product was diluted with 2 µl of H₂O and mixed with 10 µl of gold or tungsten suspension. 2 µl of 0.1 M spermidine and 5 µl of 2.5 M CaCl₂ were added, briefly mixed and centrifuged at 14,000 rpm for 10 s. The precipitate was washed twice with 20 µl of 70% ethanol and re-suspended in 10 µl of ≥ 99.8 % ethanol.

Inoculation was carried out with a pressure of 450 psi for younger plants (4-6 leaf stage) or 900 psi for older plants (12-14 leaf stage). Infection was determined by symptom evaluation and RFLP analysis of RCA products after 14 dpi. Plants which have been assigned as non-infected by this way were rated like mock-inoculated for the statistical analysis of GUS assay results.

**Plant treatments**

**Late inoculation:** Seeds of lines 651, 11, 1445 and IC9C were kept in standard soil for 7 d under long day conditions (16 h light, 24° C/ 8 h dark, 18° C) and 70 % relative humidity for germination. From day 8 on, conditions were changed to a short day cycle (8 h light, 22° C/ 16 h dark, 18° C) and 65 % relative humidity. On day 15, plantlets were picked and separately planted into clay pots and kept under short day conditions until day 35. On day 36, plants were transferred to long day conditions (16 h light, 22° C/ 8 h dark, 16° C) and 70 % relative humidity. Plants were inoculated from days 40 to 45 (12-14 leaf stage) and were kept further under long day conditions.

**Early inoculation:** Plants of lines 651 and 1445 were kept until day 21 as described before, but inoculation was performed on day 22 (4-6 leaf stage) under short day conditions. After day 29 (7 dpi), conditions were changed to the long day regime.

**Heat stress assay:** In a growth chamber (KBW 400, Binder, Tuttlingen, Germany), ten 18 day old plantlets of *A. thaliana* line 1445 were challenged for 24 h at 37° C, and ten non-stressed plants were kept for control. Both treatments were performed under long day conditions (16 h light/ 8h dark). Plants were initially grown as described before, but were acclimatised to long day conditions since day 16. After the heat stress treatment, plants were grown for four days under non-stress conditions for recovery. The GUS assay was performed at the fifth day after stress treatment (day 23).
**Total nucleic acid extraction**

Leaf samples were ground in liquid nitrogen and nucleic acids were extracted using CTAB or phenol-based techniques as described (Haible et al., 2006; Jeske et al., 2001).

**Genotyping of A. thaliana plants by PCR**

Genotypes of the plants were determined by PCR using Taq polymerase (Qiagen, Hilden, Germany) and two primer pairs (Metabion, Martinsried, Germany) specific for either the transgene or the insertion locus in wild-type (Col or C24) A. thaliana plants (Fig. 1; Suppl. Table S1, S2). Undiluted, 1:10 or 1:100 diluted plant nucleic acids in 1 µl template were mixed with 1 µl Taq polymerase buffer (10×), 0.4 µl of dNTPs (5 mM each), 0.5 µl primer 1 (2 pmol/µl), 0.5 µl primer 2 (2 pmol/µl), 0.5 µl Taq polymerase (0.5 U/µl in 1x buffer) and 6.1 µl ddH₂O and left to react as described in Suppl. Table S1. PCR products were separated by agarose gel electrophoresis and stained afterwards with an 0.5 µg/ml ethidium bromide solution.

**Rolling circle amplification and restriction fragment length polymorphism**

RCA and RFLP were performed as described (Haible et al., 2006) using Illustra TempliPhi Amplification kit (GE Healthcare, Munich, Germany) and restriction enzymes (5-20 U/µl; New England Biolabs, Frankfurt/Main, Germany).

**Fixation, paraffin embedding and sectioning of A. thaliana leaf segments**

Leaf segments of EuMV-MGS1 and mock infected A. thaliana plants (lines 1445 and 651) at 33 dpi were embedded in paraffin, cut with a microtome 1208 (Leica Microsystems, Wetzlar, Germany) into 7-8 µm sections and processed for in situ hybridization as described (Zhang et al., 2001).

**Blotting and hybridization of nucleic acids**

Southern blotting with alkaline (Chomczynski & Qasba, 1984) or neutral transfer (Sambrook & Russell, 2001) was followed by hybridization with DIG-labelled probes as described (Kleinow et al., 2009). For tissue blotting, plant tissue sections were dapped onto N+ membranes (GE Healthcare, Munich, Germany) and DNA was UV-crosslinked to the membrane using UV light of 70,000 mJ/cm² for 2 min. Viral probe DNA was derived from circular DNA of infected A. thaliana (Col) plants which was amplified by RCA, linearized and gel-purified for EuMV-MGS1, EuMV-MGS2, CILCrV DNA A or B (20-40 ng/µl). For Southern and tissue blot hybridization, viral DNA was DIG-labelled with the DIG High Prime DNA Labelling Kit (Roche Diagnostics, Mannheim, Germany), for in situ hybridization it was biotin-labelled (Biotin-Nick Translation Kit, Roche Diagnostics) and purified with the Nucleotide Removal Kit (Qiagen, Hilden, Germany).
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Chemiluminescence was detected with anti-DIG AP conjugate and CSPD (Roche Diagnostics) for blot hybridization or with streptavidin-AP conjugate and NBT/BCIP (Boehringer Ingelheim, Ingelheim Germany) for in situ hybridization of leaf sections. Specimens were examined using an Axioskop microscope (Zeiss, Oberkochen, Germany) with differential interference contrast (DIC) equipment.

**Fluorescence in situ hybridization (FISH) of virus DNA**

Leaves and petioles of EuMV-MGS1- or mock infected *A. thaliana* plants (21 dpi) were cut into 75 µm sections using a hand microtome and were treated for FISH as described by Ghanim et al., (2009) but without RNase treatment. For detection of viral DNA, a 5'-Cy3-labeled primer (Cy3-5' CTGTTATTTCCCTGCTTCTTG '3; Biomers, Ulm, Germany) hybridizing within the AV1 ORF of EuMV-MGS1 DNA A was used. Fluorescent images were merged with DIC images to show the localization of the hybridization signal using Corel Paintshop Pro X4 software (Corel, Munich, Germany).

**GUS assay**

Three leaves classified as older, medium and young were harvested for the first experimental set-up, whereas whole plants were harvested at 21 dpi and weighed immediately for the second experimental set-up. Samples were placed into small Petri dishes, submerged with 4-5 ml of sterile GUS staining solution (Baubec et al., 2009) containing 0.5 mg/ml X-Gluc (Duchefa, Haarlem, Netherlands) and vacuum-infiltrated 3-4 times for 2-3 min, incubated at 37° C overnight, de-stained with 70 % ethanol several times overnight at 37° C, and stored in 70 % ethanol at 4° C. The frequencies of blue spots were evaluated under a MZ16FA stereomicroscope (Leica Microsystems, Wetzlar, Germany) with 40-50 fold magnification and it was determined whether they were close or at a distance to the veins. Assessed signals of each specimen were referred to its fresh weight and statistical analysis was performed with a t-test or a Mann-Whitney rank sum test, if data was not distributed normally, using the SigmaStat program.

**Acknowledgements**

The authors would like to thank Barbara Hohn, Ortrun Mittelsten Scheid and Holger Puchta for providing the transgenic plant lines and valuable advice concerning their handling, Gabi Kepp and Conny Kocher for excellent technical assistance, Sabine Eiben, Christina Wege and Michael Saliba for critical reading of the manuscript and helpful discussions, and the gardeners Diether Gotthardt and Annika Allinger for taking care of the plants. This research was supported by grants from DFG Je116/15-1 and the ERA-PG programme (BMBF 0313986).
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

References


Bray, CM, West, CE (2005) DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. New Phytol 168: 511-528


Somatic homologous recombination in plants is promoted by a geminvirus in a tissue-selective manner


Weitzman, MD, Carson, CT, Schwartz, RA, Lilley, CE (2004) Interactions of viruses with the cellular DNA repair machinery. DNA Repair 3: 1165-1173

Yao, Y, Kathiri, P, Kovalchuk, I (2013) A systemic increase in the recombination frequency upon local infection of Arabidopsis thaliana plants with oilseed rape mosaic virus depends on
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Front Plant Sci* 4: 61


Supplementary data

Figure S1: Mock-control of in situ hybridization for lines 1445 (a) and 651 (b) after development with NBT/BCIP as in Figs. 2b, c.

Figure S2: FISH detection of viral DNA in line 1445 using a 5’Cy3-labeled primer hybridizing within the AV1 ORF of EuMV-MGS1 for mock-inoculated (a, b) or infected (c, d) plants (21 dpi). DIC images (a, c) and corresponding fluorescent images (b, d) of petioles. Virus-specific signal (arrow) were detected only after infection. X: xylem, Ph: phloem; bar = 50 µm. The images B and C were merged for Fig. 2d.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.

**Figure S3:** Genotyping of *A. thaliana* plant lines used. Lines 651 (a), 1445 (b), and two wild type (WT) control plants (C24 for line 651 or Col for line 1445) were tested separately by PCR with either the uidA (GUS) primer pair (respective upper depiction) or primer pairs with binding sites overlapping or flanking the particular integration locus of the transgenic reporter construct (respective lower depiction). In the latter case, no PCR product can be generated in homozygous transgenic plants. PCR products were separated in 1.5% agarose gels, with 600 ng of *PstI* digested λ DNA as a molecular weight marker (M) and staining with ethidium bromide afterwards. Specimens used for the three biological replicates (1, 2, 3) of the GUS assays and two wild type control plants are shown.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner.

**Figure S4**: Detection of EuMV-MGS1 DNA as described in Fig. 4 for the second (a) and third (b) biological replicate.
Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

Table S1: PCR conditions for genotyping with various primer pairs.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
<th>Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>96°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>95°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>56°C</td>
<td>30 s</td>
<td>50</td>
</tr>
<tr>
<td>4.</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>72°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>4°C</td>
<td>pause</td>
<td></td>
</tr>
</tbody>
</table>

Table S2: Primer sequences and expected PCR products for genotyping.

<table>
<thead>
<tr>
<th>Names</th>
<th>Sequences</th>
<th>Genomic locus*</th>
<th>Expected fragment length [bp]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Genomic locus</td>
<td>wt</td>
</tr>
<tr>
<td>uidA-F</td>
<td>GCAATTGCTGTGCCAGGCAGTTT</td>
<td>At2_14417446-14417469</td>
<td>~1000</td>
</tr>
<tr>
<td>uidA-R</td>
<td>CCTGTAAGTGCGCTTGAGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1445wt-F</td>
<td>CAACTCTTTAAATCGTCTATCGTA</td>
<td>At2_14418191-14418172</td>
<td>745</td>
</tr>
<tr>
<td>1445-R</td>
<td>GATATTGT9ACGGGATGAT</td>
<td>At2_14418191-14418172</td>
<td>745</td>
</tr>
<tr>
<td>651wt-F</td>
<td>GCAGCAATGGAAGGAAGAAAAG</td>
<td>At5_10455304-10455322</td>
<td>936</td>
</tr>
<tr>
<td>651wt-R</td>
<td>ACACGATGTCTACATGCTG</td>
<td>At5_10456240-10456222</td>
<td>936</td>
</tr>
<tr>
<td>IC9Cwt-F</td>
<td>ACTGAGTTTGGTGACCTTG</td>
<td>At5_8633613-8633632</td>
<td>295</td>
</tr>
<tr>
<td>IC9Cwt-R</td>
<td>CCAAGATTTAAAATGGAGG</td>
<td>At5_8633908-8633888</td>
<td>295</td>
</tr>
<tr>
<td>11wt-F</td>
<td>TGCTGGTGACACGTTAAGC</td>
<td>At2_11757844-11757863</td>
<td></td>
</tr>
<tr>
<td>11wt-R</td>
<td>CAGTCGCGATGGTGTTTCT</td>
<td>At2_11758400-11758381</td>
<td>556</td>
</tr>
</tbody>
</table>

* Genomic locus according to TAIR10 release.
Rad54 is not essential for any geminiviral replication mode in planta

Kathrin S. Richter, Lukas Ende, and Holger Jeske*

Institute of Biomaterials and Biomolecular Systems, Department of Molecular Biology and Plant Virology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

Reprinted from Plant Molecular Biology (2015), 87: 193-202; doi: 10.1007/s11103-014-0270-1

#Author for correspondence:
Email: holger.jeske@bio.uni-stuttgart.de
Phone: +49-711-685-65070

Figures: 5
Supplementary Figures: 3

Abstract

The circular single-stranded DNA of phytopathogenic geminiviruses is propagated by three modes: complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR), which need host plant factors to be carried out. In addition to necessary host polymerases, proteins of the homologous recombination repair pathway may be considered essential, since geminiviruses are particularly prone to recombination. Among several others, Rad54 was suggested to be necessary for the RCR of mungbean yellow mosaic India virus. This enzyme is a double-stranded DNA-dependent ATPase and chromatin remodeler and was found to bind and modulate the viral replication-initiator protein (Rep) in vitro and in Saccharomyces cerevisiae. In contrast to the previous report, we scrutinized the requirement of Rad54 in planta for two distinct fully infectious geminiviruses with respect to the three replication modes. Euphorbia yellow mosaic virus and Cleome leaf crumple virus were inoculated into Rad54-deficient and wildtype Arabidopsis thaliana plant lines to compare the occurrence of viral DNA forms. Replication intermediates were displayed in the time course of infection by one and two-dimensional agarose gel electrophoresis and Southern hybridization. The experiments showed that Rad54 was neither essential for CSR, RCR nor RDR, and it had no significant influence on virus titers during systemic infection.
Introduction

Geminiviruses cause severe harvest losses in a wide variety of crop plants worldwide, especially in the tropics and subtropics (reviewed in Jeske, 2009). Their genome consists of circular single-stranded DNA (ssDNA) of about 2.6 to 3.0 kb for monopartite geminiviruses, while bipartite begomoviruses have an additional DNA component of again the same size. They replicate within the nuclei of host plants via three modes: complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR) (Jeske et al., 2001; Preiss & Jeske, 2003). The viral replication-initiator protein (Rep) mediates RCR through its nicking/closing (Laufs et al., 1995; Stanley, 1995) and helicase activity (Choudhury et al., 2006; Clerot & Bernardi, 2006). Moreover, it induces an S phase-like environment in infected cells by binding to the plant retinoblastoma-related protein (pRBR) and possibly other cell cycle regulators, thereby removing the G1/S cell cycle block which provides replication factors for virus amplification (Ascencio-Ibanez et al., 2008; Gutierrez et al., 2004; reviewed in Hanley-Bowdoin et al., 2013; Hipp et al., 2014; Kong et al., 2000; Nagar et al., 2002; Ruschhaupt et al., 2013).

Rep is the only virus-encoded factor essential for replication and host factors of DNA repair pathways, including those for homologous recombination, are presumably of prime importance. This is supported by the frequent observation of intra- and interspecies recombinations which are thought to enhance the fitness of the geminiviral populations and thus contributing to their evolution and epidemic spread (Lefeuvre et al., 2007; Padidam et al., 1999; Rocha et al., 2013; van der Walt et al., 2009; Varsani et al., 2008). Recently, we have shown that the frequency of somatic homologous recombination events can also be elevated for Arabidopsis thaliana transgenes, specifically within vein-associated tissues after infection with the phloem-limited Euphorbia yellow mosaic virus (Richter et al., 2014). The RDR mode suggests here a mechanistic principle, by which host recombination factors are utilized for geminiviral amplification and equally lead to enhanced host DNA recombination.

Homologous recombination (HR) is not only crucial for genetic exchange during meiosis in eukaryotes, but it is also necessary for genome stability in general due to its high fidelity repair function for various DNA damage types (reviewed in Jasin & Rothstein, 2013; Li & Heyer, 2008) such as DNA double-strand (DBS) breaks, ssDNA gaps, interstrand crosslinks and also stalled replication forks. Most indispensable key components for HR in eukaryotes are members of the Rad52 epistasis group (reviewed in Symington, 2002; West, 2003) which includes Rad54. This protein acts as a double-stranded (ds) DNA-dependent ATPase (Swagemakers et al., 1998) and belongs to the SWI2/SNF2 family of chromatin remodelling factors. Rad54 is an efficient motor protein and translocates on dsDNA to remodel nucleosomes, but has no strand displacement
Rad54 is not essential for any geminiviral replication mode \textit{in planta}

activity of regular helicases and is presumably involved in a great variety of HR processes (reviewed in Ceballos & Heyer, 2011; Mazin et al., 2010). An interaction between the N-terminus of Rad54 and the Rad51 recombinase (Golub et al., 1997; Jiang et al., 1996) has been shown to increase the stability of Rad51-ssDNA filaments, which probably plays a role at pre-synapsis during HR, though this effect was independent of the Rad54 ATPase activity (Agarwal et al., 2011; Mazin et al., 2003). During synapsis, the Rad51-ssDNA filament conducts homology search on the target dsDNA and forms displacement-loops (D-loop), which was enhanced by binding of Rad54 and dependent on its ATPase activity (Mazina & Mazin, 2004; Petukhova et al., 1998; Solinger et al., 2001). After strand exchange, Rad54 removes Rad51 from the heteroduplex DNA in an ATPase-dependent manner, which enables access and priming of the invading 3’-end by a DNA polymerase (Li & Heyer, 2009; Li et al., 2007; Solinger et al., 2002). These rather opposing activities were recently reconciled by Wright and Heyer (2014) who proposed a model in which Rad54 acts as a heteroduplex DNA pump that is guided and stimulated by Rad51 as well as by the displaced ssDNA strand. In the course of these experiments it was discovered that the Rad54 N-terminus contains a ssDNA-binding domain. In addition, Rad54 promoted branch migration (Bugreev et al., 2006) and the resolution of recombination intermediates, probably by recruiting the Mus81-Mms4 (-Eme1) structure-specific endonuclease (Matulova et al., 2009; Mazina & Mazin, 2008).

Most of the data concerning Rad54 were obtained from \textit{Saccharomyces cerevisiae} (ScRad54) or its mammalian homologs. It is less clear which properties are also accurate for the plant homologs, but amino acid sequence similarities of 54 % to the \textit{S. cerevisiae} and 55 % to the human homologs suggest a functional conservation of \textit{Arabidopsis thaliana} Rad54 (AtRad54) (Klutstein et al., 2008; Osakabe et al., 2006). Equally, an interaction of AtRad51 and AtRad54 has been indicated by yeast two-hybrid analysis (Osakabe et al., 2006). This makes Rad54 and Rad51 interesting candidates for the promotion of geminiviral replication. In particular, they may be key components for the RDR mode, which needs invasion of ssDNA into dsDNA of viral minichromosomes (Jeske et al., 2001; Pilartz & Jeske, 2003). Correspondingly, the combination of Rad54 functions in D-loop generation and chromatin remodelling could be ideal for this process.

For one geminivirus (mungbean yellow mosaic India virus; MYMIV) Raghavan et al. (2004) reported that a DNA A-based dimer can be replicated in \textit{S. cerevisiae} cells. In that regard ScRad54 has been shown to interact with the MYMIV Rep protein and to enhance its nicking, ATPase and helicase activity \textit{in vitro} (Kaliappan et al., 2012). The authors also have suggested that ScRad54 is required for RCR of geminivirus-based constructs in yeast and \textit{in vitro}. Moreover, a transient agroinfiltration test with an artificial construct derived from Tomato leaf curl New Delhi virus
Rad54 is not essential for any geminiviral replication mode in planta

(ToLCNDV) in Rad54-deficient and wildtype (wt) *A. thaliana* was used to propose a necessary role for Rad54 in geminiviral RCR in plants. However, the authors merely used PCR for the detection of viral progeny DNA, which does not allow a differentiation between distinct DNA forms or the respective replication modes. Southern blot hybridizations would be needed to ensure the influence on the proposed replication modes.

Thus, the potential relevance of Rad54 for geminivirus replication needs further clarification. Therefore, we scrutinized the necessity of *AtRad54* for geminivirus replication in planta by visualizing replicative DNA intermediates of RCR and RDR during the time course of infection. Plants of the same Rad54-deficient *A. thaliana* mutant line used by Kaliappan *et al.* (2012) were inoculated in comparison to wt plants with two wt begomoviruses (Euphorbia yellow mosaic virus (formerly Euphorbia mosaic virus), EuYMV; Cleome leaf crumple virus, CILCrV) (Paprotka *et al.*, 2010) in several repeated experimental sets. For the geminiviruses tested in *A. thaliana*, we show for the first time that Rad54 was not essential, neither for CSR, RCR nor for RDR, which is in contrast to previous reports.

**Results**

The *rad54-1* T-DNA insertion line has been characterized and tested before (Kaliappan *et al.*, 2012; Osakabe *et al.*, 2006). Since no hemizygous line and therefore no corresponding wt sibling line for proper control was available, homozygous wt and *rad54-1* F2 progeny lines were unequivocally identified by genotyping PCR, after crossing wt (Col-0) and *rad54-1* plants and self-pollination of this F1 generation (data not shown). The absence of full-length *AtRad54* transcripts in homozygous *rad54-1* lines was confirmed by RT-PCR (see Fig. S1). Plants of the F3 progeny were used for the inoculation experiments described below.

As reported previously (Paprotka *et al.*, 2010; Richter *et al.*, 2014), systemic infection of EuYMV and CILCrV in *A. thaliana* was fully established until 14 dpi. Per virus and genotype, ten plants each were inoculated in parallel for one inoculation experiment and samples were harvested at 7, 14 and 21 dpi from the same plants. This vast experimental data ensured a reliable overview of early and late infection stages and also allowed to assess overall infection rates. For three consecutive, independent experiments, viral DNAs were examined by 1D gel electrophoresis as exemplified in Fig. 1.
Rad54 is not essential for any geminiviral replication mode in planta
Rad54 is not essential for any geminiviral replication mode in planta

At 7 dpi, viral DNAs with distinct forms like ssDNA, covalently closed circular (ccc) and open circular (oc) DNA have emerged for EuYMV and CLCrV, albeit only in some plant samples with varying intensities. Despite the variation, no significant difference in virus titer or the number of infected plants between wt and rad54-1 plants was observed at this stage and the rise of early systemic infection appeared to be rather stochastic. At 14 dpi, systemic infection of both viruses was established for all inoculated plants, irrespective of the genotype. Furthermore, the amounts of the different viral DNA forms were similar in both plant lines for all experiments with CLCrV and two of three consecutive experiments with EuYMV. Only in one inoculation experiment, ocDNA of EuYMV was found to be slightly reduced in most of the plants from the rad54-1 line compared to wt plants (Fig. S2). At 21 dpi, viral titers remained high for both infections without significant differences between the genotypes for all consecutive experiments.
Rad54 is not essential for any geminiviral replication mode in planta

The infection rates of both viruses reached 100 % for either genotype throughout the series of experiments. A lower proportion of ssDNA in relation to the other DNA forms was observed for CilCrV compared to EuYMV infection at 21 dpi. Like other geminiviruses (Bach & Jeske, 2014; Patil & Dasgupta, 2006), EuYMV and especially CilCrV produced occasionally defective DNAs (D-DNA) of subgenomic size during the experiments for some specimens. However, this occurred irrespective of the genetic background of the plant lines.

As reported previously (Osakabe et al., 2006), the knock-out of AtRad54 did not result in an apparent phenotype during plant growth, development or reproduction. After infection, mild symptoms like leaf rolling (EuYMV, CilCrV) or yellow mottling (EuYMV) appeared first at 9-11 dpi with no difference in severity or timing between plant lines (Fig. 2).

In order to discriminate the replicative intermediates, viral DNAs were separated by 2D gel electrophoresis and detected by Southern blot hybridization (Erdmann et al., 2010; Jeske et al., 2001; Preiss & Jeske, 2003). In the course of this procedure, DNA forms are separated in the first dimension primarily due to their molecular masses. In the second dimension, the addition of the intercalator chloroquine delays the mobility of dsDNA compared to ssDNA and also adds positive superhelical turns to the negatively supercoiled cccDNA, whereby distinct topoisomers
of cccDNA can be depicted (Snapka et al., 1991). As a result, a characteristic pattern of arcs, lines and dots is obtained which were assigned previously to the respective geminiviral DNA forms also by using electron microscopy (Jeske, 2007; Jeske et al., 2001). The replication profiles of EuYMV and CLLCrV shown here (Figs. 3-5) are similar to those of earlier geminivirus replication studies and represent the first of its kind in A. thaliana. They were chosen as examples from similar blots of the repeated experiments as they revealed the highest resolution for the DNA intermediates.

To reduce stochastic effects between individual rad54-1 and wt specimens, all samples from either genotype were pooled, each at 14 dpi or 21 dpi. Due to the low virus DNA levels in most samples at 7 dpi, this approach was not adequate for this infection phase, and hence individual specimens with the highest titers of one inoculation experiment were used instead.

Already at 7 dpi, intermediates of all three replication modes (CSR, RCR and RDR) were clearly detectable for each virus with no significant difference in quantities between rad54-1 and wt plants (Fig. 3). Similarly, the end products of replication, like ocDNA, heterogeneous double-stranded (hdsd) DNA and cccDNA were well resolved. At 14 dpi, considerably more viral DNA was separated and relatively high amounts of end products of replication dominated (hdsd and monomer linear dsDNA; monomer, dimer and heterogeneous ocDNA; cccDNA; ssDNA) (see Fig. 4). CSR, RCR and RDR intermediates and also end products of replication were as prevalent in rad54-1 as in wt specimens during all experiments for CLLCrV (Fig. 4b) as well as during two of three inoculation experiments for EuYMV (Fig. 4a).

At 21 dpi, end products of viral replication and only very faint arcs of RDR intermediates were apparent, but with no difference between genotypes and viruses during all inoculation experiments (Fig. 5).

As was mentioned above, during one of the three inoculation experiments the 1D gel analysis of EuYMV DNA showed a slight reduction of ocDNA in most of the rad54-1 compared to wt plants at 14 dpi (Fig. S2). Corresponding to this observation, ocDNA appeared also slightly reduced in the rad54-1 line compared to the wt during the 2D gel analysis of the samples from this particular inoculation experiment (Fig. S3). However, no CSR and RCR but only RDR intermediates were present. Yet those were detected without distinction between the genetic backgrounds.
Figure 3: Examination of replicative intermediates at 7 dpi. Virus DNA was detected after 2D gel electrophoresis of individual samples of EuYMV and CILCrV infected rad54-1 and wt plants. DIG-labeled full-length DNA A probes of the respective virus were used for hybridization. Separation was performed in 0.5 % agarose gels with TBE buffer containing 0.03 % SDS in the first dimension followed by separation in 1.4 % agarose gels with TBE buffer containing 50 µg/ml chloroquine in the second dimension. 100 ng DNA was loaded per gel. DNA forms indicate: CSRC (complementary strand replication on circular templates); RCR (rolling circle replication); RDR (recombination-dependent replication); ccc (covalently closed circular); hdsl (heterogeneous double-stranded linear); oc (open circular); ss (single-stranded linear and circular); hss (heterogeneous single-stranded linear and circular); 2x refers to DNA forms of dimer genomic length.
Rad54 is not essential for any geminiviral replication mode in planta

Figure 4: Examination of replicative intermediates at 14 dpi. Analysis as described in Fig. 3, but with pooled ten samples of EuYMV and CiLCrV from one inoculation experiment per genotype and virus infection. 400 ng of DNA was loaded per gel after RNase A digestion. Additional DNA forms indicated: hoc (heterogeneous open circular); 1x lin (monomer linear double-stranded).
Rad54 is not essential for any geminiviral replication mode in planta.

Figure 5: Examination of replicative intermediates at 21 dpi. Analysis of pooled samples as described in Fig. 4.
Without evidence of RCR intermediates, it is therefore not feasible to assign the effect of ocDNA reduction to a decrease of virus RCR activity in rad54-1 plants, especially since it was not reproduced during the other two experiments.

**Discussion**

Because of their small genome sizes and thus restricted protein coding capacity, geminiviruses depend on various host factors for their amplification (reviewed in Hanley-Bowdoin et al., 2013). Rad54 is considered not only a promising candidate for a role during geminivirus RDR due to its various functions in HR, especially at D-loop formation and as a chromatin remodeler (reviewed in Ceballos & Heyer, 2011; Mazin et al., 2010). It was also considered important during RCR regarding the interaction of ScRad54 with the MYMIV Rep and the ensuing modulation of Rep's ATPase, nicking and helicase activities (Kaliappan et al., 2012). However, we show here that neither RCR nor RDR modes of two geminiviruses depended on Rad54 in A. thaliana.

EuYMV and ClLCrV infection in rad54-1 plants was fully established similar to wt plants with infection rates of 100 % for both genotypes. During every analyzed infection phase, intermediates of all detected replication types were present to equal amounts in wt and rad54-1 plants. The same was true for end products of replication, like hdsDNA, ssDNA, cccDNA and ocDNA. Only during one of three experiment sets, ocDNA of EuYMV was slightly reduced in rad54-1 compared to wt plants at 14 dpi. This effect is particularly interesting because ocDNA not only represents the end product of CSR but also the starting point and an intermediate form of RCR (Jeske et al., 2001). However, CSR and RCR intermediates were neither detected for rad54-1 nor for wt plants in this case, probably since the infection at that point in time had progressed to such an extent that end products of replication prevailed, similar to the infection phase at 21 dpi. Therefore, no conclusion about the activity of the RCR mode can be drawn from this observation. It is nevertheless not excluded that the anticipated involvement of AtRad54 in geminivirus RCR is indicated through this slight ocDNA reduction in Rad54-deficient plants.

The contrasting results compared to those of Kaliappan et al. (2012) may have several reasons. First, although ToLCNDV, MYMIV as well as EuYMV and ClLCrV belong to the Begomovirus genus, it cannot be ruled out that the distinct virus species differ in their requirements for AtRad54 during replication.

Second, the experimental design was different in the number of quantitative repetitions and qualitative diagnostics. Concerning qualitative aspects, Kaliappan et al. (2012) used transient agroinfiltration of an artificial construct consisting of a pCAMBIA1391Z vector backbone and truncated ToLCNDV DNA A flanked by a common region at each side (Pandey et al., 2009). They
detected the replication of the released episomal virus-derived amplicon in both wt and rad54-1 plants at 4 dpi by PCR, but only in the wt at later stages of infection. However, it is well-known that monomeric DNA forms are already released in Agrobacterium cells containing plasmid constructs with two copies of the viral ori (Rigden et al., 1996; Selth et al., 2002). A similar phenomenon has recently been reported for an animal-infecting circovirus in Escherichia coli (Cheung, 2012) and it was shown that replicational release but no ongoing replication occurs in this organism. Corresponding conclusions have been drawn for tomato leaf curl virus (ToLCV) and agrobacteria from 2D gel examinations (Alberter, unpublished results) during the investigations on the replication of ToLCV and satellite DNAs (Alberter et al., 2005). Since the PCR-based assay could also detect construct monomers which have been released replicationally within the remaining agrobacterium cells of the infiltrated plant tissue, it is no proof for in planta replication. Furthermore, it is just as little suitable to differentiate between any replication modes in question.

Third, the release of a viral amplicon from a tandem construct can be achieved not only by a rolling circle-like mechanism but also through HR of the repeated sequences (Lazarowitz et al., 1989; Stenger et al., 1991). Equally, it has been shown recently that all major DBS repair pathways, including HR, are involved in Agrobacterium-mediated transformation of plant cells (Mestiri et al., 2014). It is therefore conceivable that not virus replication itself, but transformation of the tandem construct or the release of the amplicon per se might be impaired when applying local agroinfiltration in HR mutant plants. Avoiding these complications, the biolistic inoculation and the monitoring during systemic infections are thus regarded more meaningful than transient local agroinfiltration.

The rad54-1 T-DNA insertion line used in our experiments and also by Kaliappan et al. (2012) exhibits a 43 bp deletion at the border of the 19th exon and the 19th intron of the AtRAD54 gene, an insertion of 20 bp unknown filler DNA and showed to express no full-length 2.9 kb transcripts but a shorter 2.5 kb transcript (Osakabe et al., 2006). A translation product of this aberrant mRNA was not detectable by Osakabe et al. (2006) in Western blots with an antibody against amino acids 649-910. Further phenotype analyses concerning increased sensitivity to γ-rays, the crosslinking agent cisplatin and reduced somatic HR events endorsed the absence of a functional Rad54 protein within the rad54-1 line.

Although the results in this study did not support the conclusion, that AtRad54 is necessary for the multiplication of two geminiviruses during systemic infection, it is not excluded that the reported interaction with Rep may play a role in infection. It has to be considered that recent years have displayed a complex network of DNA repair pathways (reviewed in Chapman et al., 2012; Knoll et al., 2014; Maher et al., 2011; Puchta, 2005) and several gene products involved in
Rad54 is not essential for any geminiviral replication mode in planta

HR in plants have been described with potentially redundant functions and also preferences in meiotic and mitotic cell cycles (Bleuyard et al., 2005; Da Ines et al., 2013; Wang et al., 2014). Therefore, the function of Rad54 in geminivirus replication may be masked by complementation through other HR proteins.

Utilizing available information of the interactions of host pathways and virus factors is considered an indispensable approach for developing crop resistance strategies against geminiviruses (reviewed in Sahu et al., 2014). In this respect, Rad54 was assessed as a promising candidate for attenuating virus replication via a knock-out or knock-down. It is reasonably possible that this is true for particular geminiviruses or for yeast systems in general. However, we want to emphasize the need to carefully examine host candidate factors for their concrete impact on geminivirus replication in the more complex plant systems.

Material and methods

Plants and viruses

*Arabidopsis thaliana* wt and homozygous RAD54 knock-out plants of the T-DNA insertion line *rad54-1* (Salk_038057C) (Alonso et al., 2003; Kaliappan et al., 2012; Osakabe et al., 2006) have both a Columbia ecotype background and were acquired from the Nottingham Arabidopsis Stock Centre (NASC, Loughborough, UK). The T-DNA insertion within the RAD54 gene (At3g19210) is located on chromosome 3 position 6653391, is flanked by two left borders and caused a deletion of 43 bp at the border of the 19th exon and the 19th intron and an insertion of 20 bp unknown filler DNA (Osakabe et al., 2006). In order to obtain comparable wt and *rad54-1* progeny lines, wt and *rad54-1* plants were crossed and the resulting hemizygous F1 line was allowed to self-pollinate to obtain homozygous F2 *rad54-1* and wt plants. Homozygous progeny F3 plants were used for further experiments. Seeds were germinated in soil in a phytotron under short day conditions (8 h light, 22°C/16 h dark, 18°C). After two weeks, plantlets were separated into individual pots and four weeks after germination, plants were transferred to long day conditions in a greenhouse with additional light (16 h light/8 h dark). After two to four days of acclimatization (10-12 leaf stage), viral DNA was inoculated biolistically, using rolling circle amplification (RCA) products of Euphorbia yellow mosaic virus (EuYMV; DNA A: FN435995 and DNA B: FN435996; formerly Euphorbia mosaic virus) and Cleome leaf crumple virus (CLCrV; DNA A: FN435999 and DNA B: FN436000) (Paprotka et al., 2010). Renaming of EuMV to EuYMV was implemented according to Fernandes et al. (2011).
Rad54 is not essential for any geminiviral replication mode in planta

Biolistic inoculation

Biolistic inoculation was carried out with the Biolistic Particle Delivery System PDS-1000/He, 900 psi rupture discs, 1.1 µm tungsten microcarriers (all Bio-Rad, Munich, Germany) and with RCA products of viral DNA A and DNA B or with DNA B alone as a mock control as described in Richter et al. (2014).

Diagnostics

Rolling circle amplification (RCA) was performed as described in Haible et al. (2006) using the Illustra TempliPhi Amplification kit (GE Healthcare, Munich, Germany). The restriction fragment length polymorphism (RFLP) patterns of RCA products were analyzed by digestion with restriction enzymes according to the supplier’s recommendations (New England Biolabs, Frankfurt/Main, Germany) as described in Richter et al. (2014).

Total nucleic acid extraction

Single rosette leaves having emerged after inoculation were harvested at 7, 14 and 21 days post inoculation (dpi) and frozen in liquid nitrogen. Nucleic acids were extracted with a phenol/chloroform-based method according to Haible et al. (2006) with a minimum of 500 µl homogenization buffer (100 mM Tris-HCl pH 7.0; 10 mM Na-EDTA; 100 mM NaCl; 100 mM dithiothreitol, 10 mM N-ethylmaleimide, 1 % SDS) per 100 mg plant material.

Genotyping and RT-PCR

Plants were genotyped by PCR using the Phire Plant Direct PCR Kit (Fisher Scientific, Schwerte, Germany) with different combinations of primers, which bind either to the RAD54 gene (Rad54-LP: 5’-TTACTCAAGTTTCCTTGGGG-3’; Rad54-RT-LP: 5’-TCCTTCCATGGGATGTAG-3’; Rad54-RP: 5’-CTCGTTGAGAGATAACGGCTG-3’) or to the T-DNA insertion (Salk-LBb1.3: 5’-ATTGTGCGGATTTCGAAC-3’). Insertion mutant and primer design information was obtained from the SIGnAL website (http://signal.salk.edu).

To further verify the absence of full-length RAD54 transcripts in the rad54 line in comparison to wt and hemizygous plants, RNA was extracted from young, uninfected rosette leaves (TRI Reagent RNA Isolation Reagent; Sigma-Aldrich, Hamburg, Germany) according to the manufacturer’s recommendations. RNA (2 µg) was copied by first strand synthesis with M-MuLV Reverse Transcriptase (NEB) primed by dT-oligonucleotides according to the supplier’s protocol. 1 µl of the reverse transcriptase (RT) reaction product served as template in a 32 cycle PCR with Taq DNA polymerase (Qiagen, Hilden, Germany) using Rad54-RT-LP and Rad54-RP primers, which bind within the 19th and 21st exon, respectively. Amplification of APT1 (adenine phosphoribosyl transferase 1) transcripts with primers AtAPT1-F (5’-
Rad54 is not essential for any geminiviral replication mode in planta

CCTCCTATTGGCTGGCTATTG-3') and AtAPT1-R (5'-TCTCCTTTCCCTTAAGCTCTGG-3') was compared as internal control (Santiago et al., 2008). PCR and RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

**Gel electrophoresis**

Standard one-dimensional (1D) agarose gel electrophoresis in TBE buffer containing 5 µg/ml ethidium bromide (Green & Sambrook, 2012) and two-dimensional (2D) gel electrophoresis (0.5 % agarose in TBE with 0.03 % SDS in the first dimension, 1.4 % agarose in TBE with 50 µg/ml chloroquine in the second dimension) was performed according to Jeske et al. (2001). Samples from individual plants were loaded onto the gels with up to 4 µg of total nucleic acids. Alternatively, pooled extracts of total nucleic acids from ten samples per genotype were digested with 10 µg/ml RNase A (Sigma-Aldrich) at 37° C and purified with the PCR purification kit (Qiagen) or using phenol-chloroform extraction followed by ethanol precipitation and dissolving in 30 µl H2O. Up to 500 ng DNA were applied to a gel. DNA concentrations were determined using UV spectrophotometry and semi-quantitative ethidium bromide staining after gel electrophoresis.

**Blotting and detection of nucleic acids**

DNA was transferred under alkaline conditions (Chomczynski & Qasba, 1984) onto nylon N+ membranes (GE Healthcare), hybridized with digoxigenin-labeled full-length viral DNA A probes (DIG High Prime DNA Labelling Kit, Roche Diagnostics, Mannheim, Germany), and chemiluminescence was detected with an anti-DIG AP-conjugated antibody and CSPD (both Roche Diagnostics) as described previously (Richter et al., 2014).

**Acknowledgements**

The authors would like to thank Katharina Hipp and Martin Paul for critical reading of the manuscript and helpful discussions and the gardeners Diether Gotthardt and Marvin Müller for taking care of the plants. This research was supported by the grant Je116/15-1 from the Deutsche Forschungsgemeinschaft (DFG).
Rad54 is not essential for any geminiviral replication mode in planta

References


Rad54 is not essential for any geminiviral replication mode in planta


Rad54 is not essential for any geminiviral replication mode in planta


Li, X, Heyer, WD (2009) RAD54 controls access to the invading 3’-OH end after RAD51-mediated DNA strand invasion in homologous recombination in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **37**: 638-646


Pandey, P, Choudhury, NR, Mukherjee, SK (2009) A geminiviral amplicon (VA) derived from Tomato leaf curl virus (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. *Virol J* **6**: 152


Rad54 is not essential for any geminiviral replication mode in planta


Rad54 is not essential for any geminiviral replication mode in planta

DP (2008) Recombination, decreased host specificity and increased mobility may have driven the emergence of maize streak virus as an agricultural pathogen. J Gen Virol 89: 2063-2074


Wright, WD, Heyer, WD (2014) Rad54 functions as a heteroduplex DNA pump modulated by its DNA substrates and Rad51 during D loop formation. Mol Cell 53: 420-432
Rad54 is not essential for any geminiviral replication mode *in planta*

Supplementary data

**Figure S1:** Detection of RAD54 and APT1 transcripts by RT-PCR. Three individual samples from wt, hemizygous (hz) or rad54-1 plants were tested for presence of RAD54 transcripts. Amplification products of APT1 transcripts were used as internal control. Homozygous rad54-1 plants contained no expected RAD54 amplification products of 480 bp. (M= PstI digested λ phage DNA)

**Figure S2:** Southern Blot hybridization of 1D gel-separated EuMV DNA from each ten inoculated rad54 or wt *A. thaliana* plants at 14 dpi from one inoculation experiment diverging from the other two experiments. OcDNA of most rad54-1 plants appeared slightly reduced compared to wt plants. See Fig. 1 for techniques and abbreviations.
Figure S3: 2D gel analysis of pooled samples of EuMV infected rad54-1 and wt plants at 14 dpi from the one diverging inoculation experiment. OcDNA and also hds1DNA appeared slightly reduced in rad54 samples as already visible in Fig. S2. All ten samples from one inoculation experiment per genotype and virus infection were pooled and 400 ng of DNA was loaded per gel after RNAse A digestion. See Fig. 3 and 4 for techniques and abbreviations.
Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication

Kathrin S. Richter, and Holger Jeske#

Institute of Biomaterials and Biomolecular Systems, Department of Molecular Biology and Plant Virology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany


#Author for correspondence:

Email: holger.jeske@bio.uni-stuttgart.de
Phone: +49-711-685-65070

Figures: 3
Supplementary Figures: 1
Supplementary Table: 1

**Summary**

Ku80 is well-known as a key component of the non-homologous end-joining pathway used to repair DNA double-strand breaks. In addition, the Ku80-containing DNA-dependent protein kinase complex in mammals can act as a cytoplasmic sensor for viral DNA to activate innate immune response. We have now, to our knowledge for the first time, demonstrated that the speed of a systemic infection with a plant DNA geminivirus in *Arabidopsis thaliana* is Ku80-dependent. The early emergence of Euphorbia yellow mosaic virus DNA was significantly increased in *ku80* knock-out mutants compared to the wild type sibling control. The possible impact of Ku80 on geminivirus multiplication by generating non-productive viral DNAs or its role as a pattern recognition receptor against DNA virus infection is discussed.
**Introduction**

Plant pathogenic geminiviruses are comprised of small circular single-stranded (ss) DNA genomes. They are multiplied in the nuclei of host cells by complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR) modes, in the course of which various ssDNA and double-stranded (ds) DNA-containing intermediates arise (reviewed in Jeske, 2007, 2009). The replication-initiator protein (Rep) is the only virus-encoded protein essential for replication. Therefore, the diverse DNA amplification and modification processes are conducted largely by host enzymes, and geminiviruses modulate the respective host machinery strongly (reviewed in Hanley-Bowdoin et al., 2013). In that regard, comprehensive transcriptome profiling of *Arabidopsis thaliana* plants showed increased expression levels of several DNA repair factors after geminivirus infection, which would be well-suited for this purpose (Ascencio-Ibanez et al., 2008). Among those was Ku80, a key component of the non-homologous end-joining (NHEJ) repair pathway in eukaryotes. As part of the Ku70/Ku80 heterodimer, it binds the ends of DNA double-strand breaks (DSBs) and is also involved substantially in telomere function and maintenance (reviewed in Grundy et al., 2014). In addition, mammalian Ku70/Ku80 and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) form a heterotrimer. This complex (DNA-PK) was recently found to be a cytoplasmic, sequence-unspecific sensor for vaccinia virus and human herpesvirus 1 DNA, which are both linear (lin) dsDNA viruses (Ferguson et al., 2012). The DNA-PK complex acts as a pattern-recognition receptor (PRR) in order to activate innate immunity via the interferon regulatory factor 3 (IRF-3) transcription factor pathway. So far, no DNA-PKcs homolog has been identified in plants. The nuclear functions of Ku70/Ku80 in genome maintenance, however, are largely similar in mammals and plants (Gallego et al., 2003b; Tamura et al., 2002; West et al., 2002; Zellinger et al., 2007).

Upregulation of Ku80 transcripts after geminivirus infection (Ascencio-Ibanez et al., 2008) might imply that the Ku80 protein promotes viral amplification or, by contrast, indicates a defense mechanism of the host plant like in the mammalian case. Moreover, it may reflect a side-effect, since geminivirus infection has been shown to induce re-replication and recombination of host DNA in yeasts and plants (Hipp et al., 2014; Kittelmann et al., 2009; Nagar et al., 2002; Richter et al., 2014). So far, Ku80 has not been described in the context of innate immune response for plants. To investigate this issue, the time course of Euphorbia yellow mosaic virus (EuYMV) systemic infection was compared for an *Arabidopsis thaliana* T-DNA insertion mutant knock-out line *ku80* and wild type (wt) sibling plants.
Experimental section, results and discussion

For this purpose, the previously characterized mutant line *ku80* (Flag_049H05; supplied by the Versailles Arabidopsis Stock Centre, Versailles Cedex, France) with abolished KU80 transcription was used (Gallego *et al.*, 2003a; Gallego *et al.*, 2003b). The background Wassilewskija ecotype shows slightly earlier flowering compared to the Columbia ecotype. Homozygous *ku80* or wt progeny plants from the segregating parent line were identified by genotyping PCR with different combinations of primers (KU80-LP: 5’-CTTCAATGTGCTACCTTTCGC-3’; KU80-RP: 5’-GCTCTCGAGCATTGACTCTTG-3’; Tag5: 5’-CTACAAATTGCCTTTTCTTATCGAC-3’). For each experiment, ten plants per genotype were inoculated biolistically with *Euphorbia yellow mosaic virus* (EuYMV) DNA A (FN435995) and/or DNA B (FN435996) EuYMV in parallel, and viral DNA accumulation was monitored at 7, 14 and 21 days post inoculation (dpi) as described by Richter *et al.* (2014). Three independent experiments were performed. Viral infection had established at 7 dpi in most individual plant samples, with slight fluctuations between experiments (exemplarily shown for one experiment, Fig. 1). The main viral DNA forms of ssDNA, covalently closed circular (ccc) and open circular (oc) DNA had emerged with varying intensities among individual samples. Remarkably, the *ku80* plants appeared to contain more viral DNA overall than wt plants, ruling out that Ku80 promotes viral amplification. Measuring and statistical analysis of the signal densities confirmed a significant difference for each of the viral DNA forms (Fig. 1) and for all three experiments (Fig. S1). These results support the hypothesis that Ku80 may be involved in early pathogen response. Among 16 additional T-DNA insertion lines tested (Table S1), with different inactivated genes involved in recombination, DNA damage tolerance and repair like those described in Richter *et al.* (2015, and further unpublished data), none was found to promote geminiviral DNA multiplication. Thus, the behavior of the *ku80* line is so far unique.

These significant differences in virus DNA titers declined with progressing infection, as at 14 dpi all samples contained high levels of EuYMV DNAs in approximately similar quantities regardless of the plant genotype (Fig. 1). Intriguingly, monomeric lin dsDNA cumulated significantly in *ku80* plants at 21 dpi during two out of three experiments. Equally, ssDNA or cccDNA were elevated again in *ku80* plants in those two experiments. During one experiment, however, no differences in any DNA form were detected. In contrast to the viral DNA titers, onset (at 9-10 dpi) and severity of the symptoms of EuYMV infection did not differ significantly between the plant lines (Fig. 2).
Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication

Figure 1: Emergence of EuYMV DNA forms in ku80 and wt A. thaliana plants at 7, 14 and 21 dpi. Total nucleic acids (500 ng each) from ten inoculated plants per genotype were separated in 1.4 % agarose gels in the presence of 5 µg/ml ethidium bromide. Genomic plant DNA (pDNA) is shown as loading control. One plant per genotype was inoculated with EuYMV DNA B alone as mock control (m). Virus DNA was visualized by Southern blot hybridization with DIG-labeled full-length DNA A probes of EuYMV, an anti-DIG alkaline phosphatase-conjugated antibody and CSPD as described (Richter et al., 2015). Hybridization standards with 1, 10 and 100 pg of linear dsDNA are 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),
Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication

defective (<1x; def). Diagrams are depicted for each point in time of infection. Integrated densities of bands for ocDNA, cccDNA and ssDNA from each sample were measured with the ImageJ analysis software (National Institutes of Health; http://rsb.info.nih.gov/ij/) and plotted for the genotype as box plot graphs; linDNAs were analyzed in addition at 21 dpi; Box 50 % of data between upper and lower quartiles; line median; bars 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. Outliers are depicted, if minimum or maximum values were outside of this range. T-tests or, if data was not distributed normally, Mann-Whitney rank-sum tests were performed (SigmaStat program; Systat Software, San Jose, CA, USA) to examine significant differences between genotypes for each DNA form (* p ≤ 0.05; ** p ≤ 0.01).

Figure 2: Symptoms of EuYMV-infected plants at 12 and 18 dpi in comparison to mock-inoculated ku80 and wt A. thaliana. EuYMV infection caused mild leaf rolling, crumpling and mottling without apparent differences between genotypes. Knock-out of Ku80 did not cause an apparent phenotype as described previously (Gallego et al., 2003a; Gallego et al., 2003b).

In order to specify the individual viral DNA forms in closer detail, two-dimensional (2D) agarose gel electrophoresis and Southern blot hybridization was employed as described in Richter et al. (2015). Pooled samples from the first experiment at 7 dpi are shown to assess whether Ku80 has direct influence on the viral replication modes (Fig. 3). In general, intermediates and products of CSR, RCR and RDR were detected in approximately similar amounts for both plant genotypes. This result largely excludes Ku80 having a differential impact on any of the viral replication modes. Only lin dsDNA with a discrete band at monomeric size position as well as the arc
Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication

Figure 3: Intermediates and products of EuYMV replication at 7 dpi in ku80 and wt plants. Nucleic acids of ten samples per genotype (see Fig. 1a) were pooled, digested with RNase A, and DNA (400 ng per gel in total) was separated in 2D gels as described by Richter et al. (2015). Viral DNA was detected as described for Fig. 1. Exposure times were chosen to achieve similar signal strengths for oc, ccc and ssDNA for both samples. In addition to those described in Fig. 1, further DNA forms are indicated as CSRc (complementary strand replication on circular templates), RCR (rolling circle replication), RDR (recombination-dependent replication), hdsl (heterogeneous double-stranded linear); 1x or 2x denote monomeric or dimeric genomic lengths.

of heterogeneous molecules appeared to be increased slightly in ku80 in comparison to wt plants. The lin dsDNA band has been characterized in detail recently (Paprotka et al., 2015), and was identified as a blunt-ended non-productive intermediate lacking the origin of replication (ori). Heterogeneous lin dsDNA is probably the product of RDR (Jeske et al., 2001; Preiss & Jeske, 2003). Taken together with the occasional accumulation of lin dsDNA at 21 dpi observed in 1D gels (Fig. 1), the 2D results may serve as a hint that the NHEJ pathway is utilized to join or circularize geminiviral lin dsDNA. However, in the case of the nearly monomeric lin dsDNA, these repair products do not necessarily promote replication due to the lack of an ori. NHEJ is the prevalent pathway to repair DSB in eukaryotic somatic cells. Nevertheless, it can cause deletions or insertions and thus genetic information might be lost (reviewed in Mannuss et al., 2012; Puchta, 2005). Whereas the luxurious inventory of non-coding intervening DNA sequences in the eukaryote genome allows many NHEJ products without changing coding regions, the condensed geminiviral genome will rather suffer from NHEJ-based repair. The
consecutive accumulation of aberrant coding regions and RNAs in defective geminiviral DNA can lead to secondary effects in the plant defense cascade. Therefore, abolished Ku80-mediated NHEJ in ku80 plants might be advantageous for geminivirus infections by avoiding the accumulation of non-productive, aberrant viral DNAs.

Further on, the observed head start of early systemic infection in ku80 plants might indicate that the Ku70/Ku80 complex in plants acts as a viral DNA sensor similar to the Ku80-containing DNA-PK complex in mammals. Since the Ku70/Ku80 heterodimer binds only to lin dsDNA (Tamura et al., 2002), geminivirus infection would be detected after the first rounds of RDR-producing linear dsDNA forms. This impact might be perceived more effectively during early infection, when viral DNA levels are still low and not yet in signal saturation. However, no homolog of DNA-PKcs has been identified in plants, which is responsible for the downstream, IRF-3 mediated response in mammals. Therefore, further investigations are necessary to elucidate the respective signal transduction pathway or the mechanism behind the observed Ku80-mediated antiviral effect.

A further promising hint comes from the functional interaction between Ku70/Ku80 and the Werner-like exonuclease (Wex, syn. WRNexo) in A. thaliana, which stimulates the exonuclease activity of Wex (Li et al., 2005). Wex is homologous to the 3'-5' exonuclease domain of the human Werner protein (Pilchova et al., 2003), a member of the RecQ helicase family. RecQ helicases are crucial for genome stability and control DSB repair by resolving or disrupting replicative and recombinogenic DNA intermediates in 3’-5’ direction (reviewed in Hartung & Puchta, 2006; Knoll & Puchta, 2011). The plant RecQ2 helicase disrupts displacement loops (D-loop) of homologous recombination intermediates and interacts with Wex. Thus, both plant proteins together probably reconstitute the function of the single human Werner protein in trans (Hartung et al., 2000; Kobbe et al., 2008). Consequently, Ku70/Ku80, Wex and RecQ2 combined might operate in a manner which counteracts homologous recombination and shift repair modes to NHEJ with the detrimental effects for viral genomes mentioned above.

Moreover, Wex has homology to RNase D proteins and was shown to be required for post-transcriptional gene silencing (PTGS) in plants, though the exact underlying mechanism remains uncertain (Glazov et al., 2003). Correspondingly, the Caenorhabditis elegans ortholog of the Werner protein (Mut-7) exhibits RNase D homology and is essentially involved in transposon silencing and RNA interference in general (Ketting et al., 1999; Tops et al., 2005). In this context, Trinks et al. (2005) have identified a putative 3’-5’ exonuclease in A. thaliana with homology to Wex whose transcription was substantially upregulated upon geminiviral AC2 protein expression. This protein was called Wel-1 (Werner exonuclease-like 1), is different from the Werner enzymes discussed above, and showed only limited preservation of the conserved
motifs of other Werner-like exonucleases. It has been suggested to play a role in geminivirus-induced silencing suppression by interfering with Wex's function in PTGS. This might further indicate a certain relevance of Wex-mediated PTGS during geminivirus infections.

The nuclear functions of Ku70/Ku80 in NHEJ are well described. However, both Ku70 and Ku80 localize to the nucleus as well as to the cytoplasm in *A. thaliana* (Tamura *et al.*, 2002). The cytoplasmic localization has not been associated with any functional relevance yet. PTGS in the cytoplasm is a well-known mechanism with antiviral impact in plants (reviewed in Szittya & Burgyan, 2013; Zvereva & Pooggin, 2012), and could be a further indication of a connection between the observed antiviral effect of Ku80, the cytoplasmic localization of Ku70/Ku80 and PTGS via the interaction with Wex. Ku70/Ku80 could be a novel intracellular PRR against viral DNA in plants (for a review on plant PRR, see Zipfel, 2014) and might fulfill a similar role of binding and detecting extranuclear viral linear DNA like the DNA-PK complex in mammals.

**Acknowledgements**

The authors would like to thank Dr. Katharina Hipp for critical reading of the manuscript and helpful discussions and the gardeners Diether Gotthardt and Marvin Müller for taking care of the plants. This research was supported by the grant Je116/15-1 from the Deutsche Forschungsgemeinschaft (DFG).

**References**


Grundy, GJ, Moulding, HA, Caldecott, KW, Rulten, SL (2014) One ring to bring them all—the role of Ku in mammalian non-homologous end joining. *DNA Repair* **17**: 30-38


Kobbe, D, Blanck, S, Demand, K, Focke, M, Puchta, H (2008) AtRECQ2, a RecQ helicase homologue from *Arabidopsis thaliana*, is able to disrupt various recombinogenic DNA structures *in vitro*. *Plant J* **55**: 397-405


Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication


**Supplementary data**

**Table S1:** Further *A. thaliana* T-DNA insertion lines analyzed by EuYMV infection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Line no.</th>
<th>Gene no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51A</td>
<td>GK_134A01</td>
<td>At5g20850</td>
</tr>
<tr>
<td>RAD51B</td>
<td>Salk_024755C</td>
<td>At2g28560</td>
</tr>
<tr>
<td>RAD51C</td>
<td>Salk_021960</td>
<td>At2g45280</td>
</tr>
<tr>
<td>RAD51D</td>
<td>Sail_564_A06</td>
<td>At1g07745</td>
</tr>
<tr>
<td>RAD54</td>
<td>Salk_038057C</td>
<td>At3g19210</td>
</tr>
<tr>
<td>XRCC2</td>
<td>Salk_029106</td>
<td>At5g64520</td>
</tr>
<tr>
<td>XRCC3</td>
<td>Salk_045564</td>
<td>At5g57450</td>
</tr>
<tr>
<td>RAD17</td>
<td>Salk_009384C</td>
<td>At5g66130</td>
</tr>
<tr>
<td>MUS81</td>
<td>GK_113F11</td>
<td>At4g30870</td>
</tr>
<tr>
<td>RAD52-1</td>
<td>Sail_25_H08</td>
<td>At1g71310</td>
</tr>
<tr>
<td>POLH</td>
<td>Salk_129731</td>
<td>At5g44740</td>
</tr>
<tr>
<td>REV3</td>
<td>Salk_029237</td>
<td>At1g67500</td>
</tr>
<tr>
<td>REV1</td>
<td>Salk_005721C</td>
<td>At5g44750</td>
</tr>
<tr>
<td>POLK</td>
<td>Salk_081715*</td>
<td>At1g49980</td>
</tr>
<tr>
<td>&quot;</td>
<td>Flag_566E01*</td>
<td>&quot;</td>
</tr>
<tr>
<td>RAD5A</td>
<td>Salk_047150</td>
<td>At5g22750</td>
</tr>
<tr>
<td>UBC2</td>
<td>Salk_060994</td>
<td>At2g02760</td>
</tr>
</tbody>
</table>

*Knock-out not unequivocally confirmed*
Figure S1: Emergence of EuYMV DNA forms in ku80 and wt A. thaliana plants at 7 dpi for a second (b) and third (c) biological replicate. Viral DNA was separated, detected and analyzed as described for Fig. 1.
The role of homologous recombination factors for geminiviral infection in planta

Kathrin S. Richter1, Heïdi Serra2, Charles I. White2, and Holger Jeske1#

1 Institute of Biomaterials and Biomolecular Systems, Department of Molecular Biology and Plant Virology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

2 Génétique, Reproduction et Développement, UMR CNRS 6293 - Clermont Université- INSERM U1103, Aubière, France

#Author for correspondence:
Email: holger.jeske@bio.uni-stuttgart.de
Phone: +49-711-685-65070

Figures: 8
Tables: 1
Supplementary Figures: 5

Abstract

Phytopathogenic geminiviruses depend largely on host enzyme activities for replication. Their circular single-stranded (ss) DNA genomes are amplified via complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR). Especially for the RDR mode, components of the plant’s homologous recombination (HR) repair pathway might be crucial. Interestingly, the key player of somatic HR, the Rad51 recombinase from Arabidopsis thaliana, was found to interact with the viral Rep protein of mungbean yellow mosaic India virus (MYMIV). This might indicate a vital role for Rad51 or the five Rad51 paralogs during the strand invasion process of geminiviral RDR. We investigated the requirement of several HR proteins, including Rad51 and the Rad51 paralogs, for in planta replication of Euphorbia yellow mosaic virus (EuYMV). Several A. thaliana T-DNA insertion knock-out lines deficient for distinct HR factors were examined in comparison to wildtype sibling lines in the time course of infection using one and two-dimensional agarose gel electrophoresis and Southern hybridization. Interestingly, Rad51D was the only host factor that promoted virus replication, which is rather surprising considering the possible redundancy of Rad51 paralogs in plants.
The role of homologous recombination factors for geminiviral infection in planta

Introduction

Geminiviruses are a serious threat to crop plants worldwide. Their genome consists of one or two circular single-stranded (ss) DNA components and is packed into eponymic twin-shaped particles of two incomplete icosahedra (Böttcher et al., 2004; Zhang et al., 2001). The resulting circular dsDNA is wrapped around nucleosomes and forms minichromosomes with covalently closed circular (ccc) DNA (Paprotka et al., 2015; Pilartz & Jeske, 1992, 2003). Viral DNA is further amplified by recombination-dependent replication (RDR) and rolling circle replication (RCR) (reviewed in Jeske, 2009; Saunders et al., 1991).

The viral replication-initiator protein (Rep) induces a nick within a conserved nonanucleotide sequence of an intergenic region, which represents the origin of RCR (Stanley, 1995). Rep remains covalently joined to the 5’ end of the resulting open circular (oc) DNA, while the 3’ end serves as a primer for a host DNA polymerase. After one or several rounds of replication, the nascent ssDNA is circularized again by Rep (Laufs et al., 1995). In addition, Rep promotes RCR through its helicase activity (Choudhury et al., 2006; Clerot & Bernardi, 2006). No origin of replication is necessary for RDR; instead short ssDNAs or ssDNA overhangs invade into the viral minichromosomes to prime replication with intact homologous cccDNA as template (Jeske et al., 2001; Preiss & Jeske, 2003). This process is similar to homologous recombination in DNA double-strand break (DSB) repair. Yet it is not yet known, whether geminiviral proteins are directly involved in this process. Rep supports virus replication in general by transferring initially differentiated, infected cells to a replication-competent state through binding of cell cycle regulators like the plant retinoblastoma-related protein (pRBR) and presumably cyclins (Arguello-Astorga et al., 2004; Gutierrez et al., 2004; Hipp et al., 2014; Kong et al., 2000). This releases the G1/S cell cycle block and provides host factors for virus replication.

Since Rep is the only viral protein indispensable for replication, the manifold DNA amplification and modification mechanisms are largely accomplished by host enzymes (reviewed in Hanley-Bowdoin et al., 2013). Regarding the RDR mode, DSB repair factors, particularly those involved in somatic homologous recombination (HR), are predestined to conduct the processes connected with ssDNA invasion and elongation. Correspondingly, geminivirus infection enhanced HR events of transgenes selectively in vein-associated tissue, which correlated to the phloem-specificity of the respective virus (Richter et al., 2014). Furthermore, intergenomic recombination within or between viral species occur commonly and contribute to the spread and host adaptation of geminiviruses (reviewed in Lefeuvre & Moriones, 2015). Key players of HR, such as the Rad51 recombinase or the multifunctional chromatin remodelller Rad54, interact with Rep of mungbean yellow mosaic India virus (MYMIV) and were implicated in geminiviral replication in Saccharomyces cerevisiae or ex vivo systems (Kaliappan et al., 2012; Suyal et al., 2001; Preiss & Jeske, 2003).
The role of homologous recombination factors for geminiviral infection in planta

2013). These results could indicate a role either in RCR, RDR or both for these proteins. However, a definitive impact on virus replication in planta could not be confirmed for Rad54 (Richter et al., 2015).

During mitotic HR, Rad51 forms a helical nucleoprotein filament with ssDNA, and conducts the strand exchange reaction and homology search on the intact template DNA. Furthermore, it supports the activity of the meiosis-specific recombinase Dmc1 during meiotic recombination (Bishop, 2012; Cloud et al., 2012; Da Ines et al., 2013b; Pradillo et al., 2014). S. cerevisiae codes additionally for two Rad51 paralogs, Rad55 and Rad57, which are composed in a heterodimer and promote Rad51 activity (Johnson & Symington, 1995; reviewed in Krogh & Symington, 2004; Sung, 1997). Vertebrates and plants encode five paralogs (Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3), which share up to 30% amino acid similarity with Rad51 and are present in two major complexes, namely BCDX2 and CX3 and several subcomplexes (Bleuyard et al., 2005; Bleuyard & White, 2004; Masson et al., 2001a; Masson et al., 2001b; Miller et al., 2004; Osakabe et al., 2005; Osakabe et al., 2002). These complexes are involved in the assembly and the stability of the Rad51-ssDNA nucleoprotein filament during early stages of HR, in the migration and resolution of Holliday junctions during late stages of HR, and exhibit in vitro strand exchange activity (Chun et al., 2013; Kurumizaka et al., 2001; Kurumizaka et al., 2002; Liu et al., 2004; reviewed in Suwaki et al., 2011; Yokoyama et al., 2004). With these properties, Rad51-like proteins are promising candidate factors for geminiviral replication, especially the RDR mode. In A. thaliana, all Rad51 paralogs are involved in somatic HR, since knock-out mutations of these proteins decreased somatic HR frequencies or promoted the hypersensitivity to DNA damage (reviewed in Bleuyard et al., 2006; Da Ines et al., 2013a; Wang et al., 2014). Moreover, Rad51D activated pathogenesis-related (PR) genes transcriptionally during systemic acquired resistance (SAR) against pathogens and consequently, RAD51D-disrupted A. thaliana plants showed increased susceptibility to Pseudomonas syringae infection (Durrant et al., 2007; Song et al., 2011).

Beyond their functions in the canonical strand invasion-dependent HR pathway, Xrcc2, Rad51B and Rad51D are individual key players in the alternative, Rad51-independent single-strand annealing (SSA) HR pathway in somatic cells of A. thaliana (Serra et al., 2013). This pathway is active when DSBs in tandemly repeated sequences occur. After 5'-3' exonucleolytic processing of both adjacent DNA ends, complementary ssDNA sequences can anneal. Non-complementary ssDNA-tails are resected and the remaining gaps are filled in, leading to the loss of sequences between the repeats (reviewed in Heyer et al., 2010; Puchta, 2005).

Another interesting candidate gene for geminivirus amplification might be RAD52. The Rad52 protein from S. cerevisiae is involved in SSA and mediates Rad51 recruitment to DSBs by binding
The role of homologous recombination factors for geminiviral infection in planta

to ssDNA and Rad51 (reviewed in San Filippo et al., 2008; Symington, 2002). In A. thaliana, two homologs of RAD52 (RAD52-1, RAD52-2) with several splice variants have been characterized and the respective proteins probably have a similar role in somatic HR like the yeast homolog (Samach et al., 2011). Both Arabidopsis homologs encode two open reading frames, respectively (RAD52-1A, RAD52-1B, RAD52-2A, RAD52-2B). The Rad52-1A protein was present throughout the nucleus, whereas the Rad52-1B protein localized in mitochondria. The Rad52-2A protein was present in the periphery of the nucleus and throughout the chloroplasts, while the Rad52-B was found in chloroplasts only.

Transcripts of some DNA repair factors, such as Rad17 and Rad50, were elevated after infection with a geminivirus (Cabbage leaf curl virus; CaLCuV) (Ascencio-Ibanez et al., 2008). Rad17 is a checkpoint control factor involved in cell cycle arrest in response to DNA damage or stalled replication (reviewed in Parrilla-Castellar et al., 2004). Knock-out of the RAD17 homolog in Arabidopsis led to impaired and deregulated DSB repair with increased HR and diminished non-homologous end joining (NHEJ) activity (Heitzeberg et al., 2004). Rad50 is part of the MRN complex (Mre11-Rad50-Nbs1) which is a key player for binding and processing of DSBs before repair processes can proceed. Moreover, it is involved in meiosis, telomere maintenance and further DNA damage signalling responses (reviewed in Lamarche et al., 2010; and Williams et al., 2010). Whether transcriptional upregulation of Rad17 and Rad50 indicates their role in geminivirus replication or rather hints at a defense mechanism or an unspecific response of the host plant remains to be shown.

With the current study, we examined the potential impact of several host plant's HR repair factors on geminiviral replication, in particular proteins such as Rad51 and its paralogs which are interesting candidate factors for the RDR mode. To this aim, established A. thaliana T-DNA insertion knock-out lines, which were deficient for various HR factors, were monitored for virus replication in the time course of infection with Euphorbia yellow mosaic virus (EuYMV, formerly Euphorbia mosaic virus) using one- (1D) and two- (2D) dimensional agarose gel electrophoresis and virus-specific hybridization. Given the possible redundancy of Rad51 paralogs in plants it is interesting to note that Rad51D was the only host factor that promoted virus replication.
Results

Rad51

Due to its interaction with Rep of MYMIV (Suyal et al., 2013), we assumed Rad51 might be a first, promising target for investigating its presumed participation in EuYMV RCR and/or RDR modes in planta as described explicitly for Rad54 in Richter et al. (2015). The T-DNA insertion line rad51 harbors an insert within the fourth exon and was verified as loss-of-function allele for AtRad51 before (Li et al., 2004). The original line from the GABI-KAT collection contained a second T-DNA insertion in a non-coding region of chromosome 3, but had been outcrossed by Li and colleagues. Since homozygous rad51 plants were sterile, progeny from hemizygous, segregating parent plants were genotyped by PCR for their zygosity. The absence of the original insertion on chromosome 3 was also verified by PCR. Homozygous rad51 and the corresponding wt siblings were identified and inoculated biolistically in parallel with EuYMV. Leaf samples were harvested at 7, 14 and 21 dpi from the same plants and viral DNAs were analyzed by 1D gel electrophoresis followed by Southern hybridization (Fig. 1). Viral DNA forms of ssDNA, cccDNA and ocDNA emerged at 7 dpi with varying titers between plant samples, but without overall significant differences between wt and rad51 plants. At 14 dpi, EuYMV infection was fully established in all inoculated plants of both genotypes and viral DNA titers remained high until 21 dpi. In accordance with viral DNA titers, symptoms like leaf rolling and yellow mottling did not differ significantly in their onset (at 9-10 dpi) or severity between rad51 and wt plants (not shown).

This first result largely ruled out that the deficiency of Rad51 has a severe effect on the overall geminiviral infection process in planta. In order to examine whether Rad51 is required for a specific geminiviral replication mode, replicational intermediates from samples at 14 dpi were examined by 2D gel electrophoresis and hybridization, a convenient technique to visualizes geminiviral replication processes (Jeske et al., 2001; Preiss & Jeske, 2003; Richter et al., 2015). Intermediates of CSR, RCR and RDR modes were present for wt and rad51 samples to similar amounts (Fig. 2). Correspondingly, end products of replication such as heterogeneous double-stranded (hds1) DNA, which is the output of RDR, as well as ocDNA, ssDNA and cccDNA were equally prevalent.
The role of homologous recombination factors for geminiviral infection in planta

Figure 1: EuYMV DNA emergence in rad51 and wt A. thaliana plants at 7, 14 and 21 dpi. Seven plants per genotype were inoculated and total nucleic acids (500 ng each) were separated in agarose gels (1.4%) with 5 μg/ml ethidium bromide. Mock controls (m) were inoculated with EuYMV DNA B alone. Virus DNA was hybridized with DIG-labeled full-length DNA A probes of EuYMV. Genomic plant DNA (pDNA) from agarose gels served as loading control. 1, 10 and 100 pg of linear EuYMV dsDNA As were used as hybridization standards. 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).
The role of homologous recombination factors for geminiviral infection in planta

Rad51 paralogs and Rad52

In mammals, Rad51 paralog complexes are able to conduct the homologous pairing reaction in vitro (reviewed in Suwaki et al., 2011). Therefore, knock-out mutant lines of the five A. thaliana Rad51 paralogs were tested in the same manner as described above. Neither of the rad51b, rad51c, xrc2 or xrc3 lines showed any significant differences compared to wt plants in viral DNA accumulation or in the occurrence of replicational intermediates (Fig. S1a-f).

By contrast, rad51d plants accumulated less viral DNA at 7 dpi compared to the wt genotype background (Fig. 3). Measuring of signal densities and a two-way Anova combined with a post-hoc Tukey test selecting genotypes and DNA forms as factors confirmed an overall significant decrease of viral DNA accumulation in rad51d plants. However, no specific DNA forms were significantly altered. At 14 dpi, all plants of both genotypes were fully infected. Total DNA levels appeared slightly lower in rad51d plants and a significant difference was verified by a two-way Anova combined with a post-hoc Tukey test for each of the viral DNA forms (Fig. 3).
Figure 3: EuYMV DNA emergence in rad51d and wt A. thaliana plants at 7, 14 and 21 dpi as described in Fig. 1. The box plot graph depicts integrated densities of viral DNA bands of ocDNA, cccDNA and ssDNA from each sample for 7 and 14 dpi. Box ± 50% of data between upper and lower quartiles; line = median; bars = whiskers; diamonds = upper or lower outliers. The whisker 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 evaluated by a two-way ANOVA with a post-hoc Tukey test with genotypes and DNA forms as selected factors was performed. * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001
Symptoms of EuYMV infection emerged at 9-11 dpi for both genotypes with leaf curling and mottling slightly less severe in rad51d plants (Fig. S2). At 21 dpi, DNA levels were detected without any significant differences between genotypes (Fig 3). 2D analysis was conducted to determine whether the different DNA amounts were due to a changed activity of the RDR mode. However, at 14 dpi all intermediates of replication were equally present in rad51d and wt plants (Fig. 4).

Since A. thaliana Rad51D is also involved in Rad51-independent SSA recombination, as are Rad51B and Xrcc2 (Serra et al., 2013), triple knock-out rad51b rad51d xrcc2 plants were inoculated in parallel with rad51d and the respective wt siblings. The reduction of viral DNA in rad51d compared to wt plants was even intensified: Viral DNA was nearly absent at 7 dpi in all rad51d plants in this experiment (Fig. 5). The same trend was observed for the rad51b rad51d xrcc2 triple mutant plants. Only one out of ten plants contained considerable amounts of viral DNA. At 14 dpi, all wt plants, nine out of ten rad51d plants, but only five out of ten of the triple mutant plants were fully infected (Fig. 5).
However, the difference in infection manifestation was not accompanied by reduced levels of viral DNAs in rad51d or triple mutant plants at 14 dpi in this inoculation experiment. At 21 dpi, viral infection was established with similar viral DNA levels in all plants from all three genotypes (Fig. 5). Symptom onset corresponded to the observations from DNA analysis, as mutant specimens with a delayed viral DNA emergence exhibited a delayed symptom onset a reduced symptom severity as well (data not shown). 2D analysis of viral DNA from triple mutant and wt plants (Fig. 6) confirmed the presence of equal amounts of all replicational intermediates and end products at 14 dpi.
The role of homologous recombination factors for geminiviral infection in planta

Figure 6: Replicative intermediates and end products, described in Fig. 2, at 14 dpi in \textit{rad51b rad51d xrcc2} and \textit{wt} plants from the samples shown in Fig. 5 (500 ng DNA per gel).

In a further biological repetition of this inoculation experiment, viral DNA was present at 7 dpi in most plants of all three genotype backgrounds, but with differing quantities (Fig. S3). A two-way Anova with a post-hoc Tukey test revealed significantly lower ssDNA and cccDNA levels in \textit{rad51d} and triple mutant plants compared to \textit{wt} plants, but not between \textit{rad51d} and triple mutant plants. Signals of ocDNA were overall too low for a reliable analysis. At 14 dpi and 21 dpi (Fig. S3), all three genotype backgrounds harbored similar amounts of viral DNA, yet infection of two \textit{rad51d} plants was delayed or not manifested at all.

Since Rad52 of \textit{S. cerevisiae} is involved in SSA in addition to recruiting Rad51 to ssDNA overhangs (San Filippo et al., 2008; Symington, 2002), a \textit{rad52-1} line with nearly abolished transcription of \textit{RAD52-1} mRNA (Samach et al., 2011) was tested. The \textit{RAD52-1} gene codes for the two splice variants Rad52-1A and Rad52-1B, which localize in nuclei (except for the nucleolus) or mitochondria of \textit{A. thaliana}, respectively. However, viral DNA accumulation or intermediate composition in \textit{rad52-1} plants showed no difference compared to \textit{wt} sibling plants (Fig. S4).
Rad17, Rad50 and Mus81

The up-regulation of Rad17 and Rad50 transcripts upon geminivirus infection might indicate a role in viral replication or a defense response of the host plant. The endonuclease Mus81 in complex with Eme1 is important for the procession and resolution of Holliday junctions, stalled replication forks or other recombination intermediates (Geuting et al., 2009; Hartung et al., 2006; reviewed in Matos & West, 2014). A homozygous knock-out line (rad17) was tested in parallel with a mus81 and its respective wt sibling line. No differences in infection rates or viral DNA amounts were detected in the time course of EuYMV infection for both mutants compared to the wt sibling line of mus81 (Fig. 7). All replicative intermediates were present in the mutant plants, as identified in 2D gels (Fig. 8).

None of the Arabidopsis lines tested so far showed any vegetative growth or developmental defects, except for the sterility of homozygous rad51, rad51c and xrcc3 lines. In contrast, homozygous rad50 plants grew with stunted rosettes, a reduced number of rosette leaves and slightly deformed leaf blades (Fig. S5). Nevertheless, EuYMV DNA was detected in rad50 plants (data not shown), but a further evaluation was meaningless under these differential growth conditions.
Figure 7: EuYMV DNA emergence in mus81, wt and rad17 plants at 7, 14 and 21 dpi. Ten plants per genotype were inoculated and analyzed as described in Fig. 1.
The role of homologous recombination factors for geminiviral infection in planta

Figure 8: Replicative intermediates and end products, described in Fig. 2, at 14 dpi in mus81, wt and rad17 plants with the samples shown in 7 (500 ng DNA per gel).
Discussion

Geminiviruses are known to exploit plant host pathways intensively for their multiplication (Hanley-Bowdoin et al., 2013) and their distinct replication modes probably require the activities of host repair factors. Several *A. thaliana* knock-out lines for candidate HR proteins were tested, from which only *rad51d* and *rad51b rad51d xrcc2* triple mutants showed a significant reduction of viral DNA titers or a delayed infection manifestation when compared to wt lines. Considering experiment-to-experiment as well as plant-to-plant variation, no significant difference was observed between the single and triple mutants, implicating a distinct impact of Rad51D. However, since intermediates of viral replication were unaltered between wt, *rad51d* and *rad51b rad51d xrcc2* plants, no specific function can be assigned to Rad51D in geminiviral amplification. Possibly, Rad51D promotes RDR due to a role in the process of strand invasion, but differences in RDR intermediates might have been too subtle for detection. Besides the reported RDR processes, other types of RDR might exist, whereby, for example, ssDNA serves as template for replication and to which Rad51D might contribute. Due to an overlap with other DNA forms or intermediates in 2D gel electrophoresis, respective intermediates might have escaped detection so far.

One of the most interesting HR factors which may participate in viral RDR is Rad51. As the somatic key recombinase (Bleuyard et al., 2006; Jasin & Rothstein, 2013), it seems perfectly fitted to realize ssDNA invasion and homology search during RDR. Furthermore, AtRAD51 was found to interact with the MYMIV Rep by yeast two-hybrid and co-immunoprecipitation assays, and to complement ScRad51 in an *ex vivo* viral replication restoration assay (Suyal et al., 2013). In spite of these promising results, the knock-out of Rad51 had no impact on RCR, RDR or the progress of virus infection in our experiments with *Arabidopsis*. This discrepancy resembles that of Rad54: ScRad54 was shown to interact with the MYMIV Rep protein, to enhance Rep's various enzymatic activities *in vitro* and to be essential for a viral replication restoration assay (Kaliappan et al., 2012), but a knock-out of Rad54 in *A. thaliana* had no influence on any replication mode or the overall infection process *in planta* for two distinct begomoviruses (Richter et al., 2015). It cannot be ruled out, however, that the reported interaction of Rad51 and Rep may have a role for geminivirus infection as discussed previously (Richter et al., 2015). The absence of Rad51 might be complemented by other HR proteins with redundant or overlapping functions, especially since the mammalian Rad51 paralog complexes BCDX2 and CX3 have *in vitro* strand exchange activity as well (Kurumizaka et al., 2001; Kurumizaka et al., 2002; Wang et al., 2010; Yokoyama et al., 2004).

To account for a potential redundancy of Rad51, single Rad51 paralogs or paralog subcomplexes in viral replication, multiple knock-out lines should be used. However, this is difficult to perform
due to the role of Rad51, Rad51C and Xrcc3 in meiosis and corresponding sterility phenotypes in *A. thaliana* (Bleuyard *et al.*, 2005; Bleuyard & White, 2004; Li *et al.*, 2004; Li *et al.*, 2005). Furthermore, the activity of the meiotic recombinase Dmc1 has to be considered: Although Dmc1 was shown to be expressed exclusively in pollen mother cells or megaspore mother cells by RNA *in situ* hybridization in *A. thaliana* (Klimyuk & Jones, 1997), expression was also observed in mitotically active cells from suspension cultures by Northern analysis (Doutriaux *et al.*, 1998). Further, microarray analysis from rosette leaves of *A. thaliana* showed significant expression of Dmc1 after geminivirus infection (Ascencio-Ibanez *et al.*, 2008). This might suggest that Dmc1 is indeed present in infected cells after the induction of the S phase-like state via Rep and could provide a recombinase activity for RDR.

In *Arabidopsis*, all five Rad51 paralogs were tested here for the first time in the context of geminivirus infection. Rad51C and Rad51D serve as integral connectors of the human BCDX2 paralog complex (Miller *et al.*, 2004). However, only Rad51D but neither Rad51B, Rad51C, Xrcc2 nor Xrcc3 had any impact on geminiviral infection. In case the *Arabidopsis* paralog complex has a similar structure like the human one, the impact of Rad51D on geminiviral amplification is probably not due to a function of the BCDX2 complex, but due to a role of Rad51D-containing subcomplexes or the individual Rad51D protein. Interestingly, human Rad51C and a DX2 subcomplex were able to catalyze the homologous pairing reaction on their own *in vitro* (Kurumizaka *et al.*, 2001; Kurumizaka *et al.*, 2002).

Rad51D, Rad51B and Xrcc2 of *A. thaliana* are key players in the SSA pathway of recombination and there are strong indications that they do not participate as part of the BCDX2 complex, but play individual roles in SSA (Serra *et al.*, 2013). This would also be compatible with our observations about the exclusive impact of Rad51D on geminivirus infection. Rad51, Rad51C and Xrcc3 on the other hand are apparently not or only marginally involved in this alternative pathway of recombination (Roth *et al.*, 2012). SSA ensures recombinational repair of DSBs for tandem repeats or homologous sequences in close vicinity and geminiviral DNAs might benefit from this repair mode as well. Linear copies of geminiviral genomes or aberrant viral DNA molecules such as defective DNAs (Bach & Jeske, 2014) could be joined or circularized by SSA activity. SSA processes normally lead to the loss of non-complementary sequences between the homologies (Heyer *et al.*, 2010; Puchta, 2005). By using perfectly complementary sequences of viral linear dsDNA copies, functional and intact multimeric or circular viral DNAs would be obtained however. This error-free approach of processing viral DNAs by SSA would be contrary to the alternative, more error-prone Ku80-mediated NHEJ pathway suggested for linear viral DNA (see manuscript 3). Correspondingly, hybrids of distinct genome components have been observed for geminiviruses (Gregorio-Jorge *et al.*, 2010; Patil *et al.*, 2007) and for the closely
related nanoviruses (Stainton et al., 2012). Since the common region (CR) of bipartite geminiviruses is nearly identical over 200 nts, this region provides a platform for SSA recombination between DNA A and DNA B.

In addition, Rad51D is involved in the transcriptional regulation of PR genes during SAR (Durrant et al., 2007), which is an important resistance response of plant immunity against various pathogens. Presumably, Rad51D interacts with the Ssn2 protein to replace the transcriptional repressor Snf1 at PR promoters, leading to the expression of defense genes (Song et al., 2011). Equally, Rad51 promotes this process (Wang et al., 2010). Geminiviruses probably lead to SAR responses via salicylic acid (SA) signaling and PR genes expression as well (Ascencio-Ibanez et al., 2008). However, it remains to be shown whether this PR gene induction is a crucial defense reaction and has an impact on the progress of infection.

Other promising factors like Mus81, Rad52-1 or Rad17 showed no impact on geminivirus replication or the overall infection process, which does not rule out that they might be involved somehow in geminiviral replication in concert with other proteins.

In conclusion from all tested HR repair factors, Rad51D was the only one with a major impact on geminivirus infection, possibly during SSA, which might be utilized to join or circularize viral linear dsDNAs in an error-free manner.

Material and methods

Plants, viruses and biolistic inoculation

Most A. thaliana T-DNA insertion lines were in the Columbia (Col-0) ecotype background and were acquired from the Nottingham Arabidopsis Stock Centre (Loughborough, UK): rad51b (Salk_024755C) (Bleuyard et al., 2005), rad51c (Salk_021960) (Abe et al., 2005; Bleuyard et al., 2005), rad51d (Sail_564_A06) (Da Ines et al., 2013a), xrcc2 (Salk_029106) (Bleuyard et al., 2005), xrcc3 (Salk_045564) (Bleuyard & White, 2004), rad52-1 (Sail_25_H08) (Samach et al., 2011), and rad17 (Salk_009384C), (Heitzeberg et al., 2004). The rad51 knock-out line GK_134A01 (Li et al., 2004) was kindly provided by Bernd Reiss (Max Planck Institute for Plant Breeding Research, Cologne, Germany) and the mus81 line GK_113F11 (Hartung et al., 2006) by the group of Holger Puchta (Karlsruhe Institute of Technology, Karlsruhe, Germany). The xrcc2 rad51b rad51d triple mutant was obtained by crossing of the single knock-out lines Salk_029106, Salk_024755C and Sail_564_A06 (Serra et al., 2013). Only the rad50 line (Flag_019F04) (Gallego et al., 2001) was in the Wassilewskija (Ws) ecotype background and was supplied by the Versailles Arabidopsis Stock Centre (Versailles Cedex, France).
All lines used have been characterized and checked for their respective HR repair knock-out phenotypes and absence of functional transcripts before. If a segregating seed stock was available for the respective insertion line, wildtype (wt) sibling plants were used as a control. In case of a homozygous seed stock (rad51b; rad17; rad51b rad51d xrc2), wt siblings of another insertion line of the same ecotype background were used.

Plantlets were grown and inoculated with rolling circle amplification (RCA) products of *Euphorbia yellow mosaic virus* (EuYMV) DNA A (FN435995) and/or DNA B (FN435996) at 10-12 leaf stage with the Biolistic Particle Delivery System PDS-1000/He, 650 psi or 900 psi rupture discs and 1.1 µm tungsten microcarriers (all Bio-Rad, Munich, Germany) as described previously (Paprotka et al., 2010; Richter et al., 2015; Richter et al., 2014).

**Isolation of total nucleic acids**

At 7, 14 and 21 days post inoculation (dpi), individual rosette leaves that emerged after inoculation from each plant were harvested, frozen and ground in liquid nitrogen. Total nucleic acids were isolated using a phenol/chloroform-based technique with a minimum of 500 µl homogenization buffer per 100 mg plant material as described (Haible et al., 2006; Richter et al., 2015).

**Genotyping**

Plant lines were tested for their designated insertion and homozygosity by PCR with the aid of the Phire Plant Direct PCR Kit (Fisher Scientific, Schwerte, Germany) and various combinations of primers (Table 1) binding to the T-DNA insertion or the adjacent gene sequences. Size, absence or presence of PCR products were examined by agarose gel electrophoresis and ethidium bromide staining. Sequence, insertion and primer design details were provided by the ‘Salk Institute Genome Analysis Laboratory’ (http://signal.salk.edu) or ‘The Arabidopsis Information Resource’ (http://www.arabidopsis.org/) websites.
The role of homologous recombination factors for geminiviral infection in planta

Table 1: Primer for genotyping PCRs of various T-DNA insertion lines.

<table>
<thead>
<tr>
<th>Knock-out</th>
<th>Line</th>
<th>Gene no.</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad51</td>
<td>GK_134A01</td>
<td>At5g20850</td>
<td>LP: 5’CTCCCCCTTCAGAGAAATCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’ATGCCAAGGGTGACAGATTG</td>
</tr>
<tr>
<td>Rad51B</td>
<td>Salk_024755C</td>
<td>At2g28560</td>
<td>LP: 5’AAAGACCAGTCGGATGTTAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’CTTTATACGCTTTGTTCCAG</td>
</tr>
<tr>
<td>Rad51C</td>
<td>Salk_021960</td>
<td>At2g45280</td>
<td>LP: 5’TACACAGAGGAAGGACATTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’TTTTTGGCAAGCCTTGAAC</td>
</tr>
<tr>
<td>Rad51D</td>
<td>Sail_564_A06</td>
<td>At1g07745</td>
<td>LP: 5’GGCTTTTCTTTGTTGGTTCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’GCAGAAATATATGGCCACCGG</td>
</tr>
<tr>
<td>Xrcc2</td>
<td>Salk_029106</td>
<td>At5g64520</td>
<td>LP: 5’TTTACATCTGGCGAGTTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’ATCATCATGGCGATTGGAC</td>
</tr>
<tr>
<td>Xrcc3</td>
<td>Salk_045564</td>
<td>At5g57450</td>
<td>LP: 5’TGAGAGATAGCAACAAAGTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’AAGACACAGCTTCGCTTCAG</td>
</tr>
<tr>
<td>Rad50</td>
<td>Flag_019F04</td>
<td>At2g31970</td>
<td>LP: 5’CTGTCATCTGTTGAAATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’CAGGAGCAATGTTAGTCAG</td>
</tr>
<tr>
<td>Rad52</td>
<td>Sail_25_H08</td>
<td>At5g47870</td>
<td>LP: 5’AGGCCAGAGGCAAGAACCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’CAAGCCTTGGCTTTCAGATG</td>
</tr>
<tr>
<td>Rad17</td>
<td>Salk_009384C</td>
<td>At5g66130</td>
<td>LP: 5’CAGTCTGGTCAGAAAGTGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’ATGTGGGGTGTCACCCTTG</td>
</tr>
<tr>
<td>Mus81</td>
<td>GK_113F11</td>
<td>At4g30870</td>
<td>LP: 5’GTGGAAAAATCTGGAAGAGGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’TGATTCATACCAAACAGGCCAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insertion lines</th>
<th>Primer name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salk</td>
<td>Lb1.3</td>
<td>5’ATTTTGCAGTCTGGGAAC</td>
</tr>
<tr>
<td>Sail</td>
<td>Lb3</td>
<td>5’TACATCTGGAATTCATAACCTC</td>
</tr>
<tr>
<td>GK</td>
<td>Lbo8409</td>
<td>5’ATTTGACACCATCACCTGAGC</td>
</tr>
<tr>
<td>Flag</td>
<td>Tag5</td>
<td>5’CTACAAATGCTTTTCTATCGAC</td>
</tr>
</tbody>
</table>

Gel electrophoresis, Southern blotting and nucleic acid detection

Standard one-dimensional (1D) and two-dimensional (2D) agarose gel electrophoresis, followed by alkaline Southern blotting, hybridization and detection of viral DNA was conducted as described in Richter et al. (2015). Signal strengths of viral DNA bands were measured with the ImageJ analysis software (National Institutes of Health; http://rsb.info.nih.gov/ij/). To evaluate the significance of differences in signal strengths of distinct DNA forms from different genotypes, two-way Anovas were combined with post-hoc Tukey analysis (SigmaStat program; Systat Software, San Jose, CA, USA).

Acknowledgements

The authors would like to thank Holger Puchta and Bernd Reiss for kindly providing plant lines, Lukas Ende for technical assistance and the gardeners Diether Gotthardt and Marvin Müller for taking care of the plants. This research was supported by the grant Je116/15-1 from the Deutsche Forschungsgemeinschaft (DFG).


Bishop, DK (2012) Rad51, the lead in mitotic recombinational DNA repair, plays a supporting role in budding yeast meiosis. *Cell Cycle* **11**: 4105-4106


Bleuyard, JY, Gallego, ME, White, CI (2006) Recent advances in understanding of the DNA double-strand break repair machinery of plants. *DNA Repair (Amst)* **5**: 1-12


Klimyuk, VI, Jones, JD (1997) AtDMC1, the Arabidopsis homologue of the yeast DMC1 gene: characterization, transposon-induced allelic variation and meiosis-associated expression. Plant J 11: 1-14


Matos, J, West, SC (2014) Holliday junction resolution: regulation in space and time. DNA Repair (Amst) 19: 176-181


components of Indian cassava-infecting geminiviruses generate defective molecules in *Nicotiana benthamiana*. *Virus Res* **124**: 59-67

**Pilartz, M, Jeske, H (1992)** Abutilon mosaic geminivirus double-stranded DNA is packed into minichromosomes. *Virology* **189**: 800-802


**Song, J, Durrant, WE, Wang, S, Yan, S, Tan, EH, Dong, X (2011)** DNA repair proteins are directly involved in regulation of gene expression during plant immune response. *Cell Host Microbe* **9**: 115-124


**Sung, P (1997)** Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev* **11**: 1111-1121


**Suyal, G, Mukherjee, SK, Choudhury, NR (2013)** The host factor RAD51 is involved in mungbean yellow mosaic India virus (MYMIV) DNA replication. *Arch Virol* **158**: 1931-1941
The role of homologous recombination factors for geminiviral infection in planta


Williams, GJ, Lees-Miller, SP, Tainer, JA (2010) Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair (Amst) 9: 1299-1306


The role of homologous recombination factors for geminiviral infection *in planta*

Supplementary data

<table>
<thead>
<tr>
<th></th>
<th>xrc2</th>
<th>m</th>
<th>wt</th>
<th>m</th>
<th>rad51B</th>
<th>m</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 dpi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pDNA</td>
</tr>
<tr>
<td>14 dpi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pDNA</td>
</tr>
<tr>
<td>21 dpi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pDNA</td>
</tr>
</tbody>
</table>

- 7 dpi: 7 days post-infection
- 14 dpi: 14 days post-infection
- 21 dpi: 21 days post-infection

Legend:
- pDNA: parental DNA
- oc: open complex
- lin: linear DNA
- ccc: covalently closed circular DNA
- ss: single-stranded DNA
- mult: multimeric reactions
- def: defined reaction product
The role of homologous recombination factors for geminiviral infection in planta
The role of homologous recombination factors for geminiviral infection in planta
The role of homologous recombination factors for geminiviral infection in planta
The role of homologous recombination factors for geminiviral infection in planta
The role of homologous recombination factors for geminiviral infection in planta

Figure S1: EuYMV DNA emergence at 7, 14 and 21 dpi and replicative intermediates at 14 dpi in rad51b, xrcc2 and wt sibling plants of line xrcc2 (a and b), rad51c and wt sibling plants (c and d) and xrcc3 and wt sibling plants (e and f). Plants were inoculated and DNA was detected as described in Fig. 1.

Figure S2: Symptoms of EuYMV-infected rad51d and wt plants at 16 dpi. Infected plants are shown in comparison to uninfected, mock-inoculated plants of their respective genotypes. EuYMV infection with leaf rolling, crumpling and yellow mottling appeared slightly milder in rad51d plants.
The role of homologous recombination factors for geminiviral infection in planta
The role of homologous recombination factors for geminiviral infection *in planta*

Figure S3: EuYMV DNA emergence in *rad51b rad51d xrcc2*, *wt* and *rad51d* plants at 7, 14 and 21 dpi, a biological replicate of the inoculation experiment shown in Fig. 6. Ten plants per genotype were inoculated and analyzed as described in Fig. 1. Statistical analysis was performed as described in Fig. 3.
The role of homologous recombination factors for geminiviral infection in planta

![Diagram showing the role of homologous recombination factors for geminiviral infection in planta.](Image)
The role of homologous recombination factors for geminiviral infection in planta

Figure S4: EuYMV DNA emergence at 7, 14 and 21 dpi (a) and replicative intermediates at 14 dpi (b) in rad52-1 and wt sibling plants. Plants were inoculated and DNA was detected as described in Fig. 1.

Figure S5: EuYMV-infected homozygous and hemizygous rad50 and wt plants at 21 dpi. Homozygous rad50 plants exhibited stunted rosettes, a reduced number of rosette leaves and slightly deformed leaf blades, which prevents a meaningful evaluation of virus infection on DNA and symptom level.
The contributions of translesion synthesis polymerases to geminivirus replication

Kathrin S. Richter¹, Monika Götz², Stephan Winter², and Holger Jeske¹#

¹ Institute of Biomaterials and Biomolecular Systems, Department of Molecular Biology and Plant Virology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany
² Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Messeweg 11-12, 38104 D-Braunschweig, Germany

#Author for correspondence:
Email: holger.jeske@bio.uni-stuttgart.de
Phone: +49-711-685-65070

Figures: 9
Tables: 4
Supplementary Figures: 1
Supplementary Tables: 1

Abstract

The replication of phytopathogenic geminiviruses depends on host DNA polymerases and is strictly confined to the nuclei of differentiated cells. By binding to the plant retinoblastoma-related protein (pRBR) and probably other cell cycle regulators, the viral Rep protein transfers the infected cell to an S phase-like state whereby replication factors become available. However, initial complementary strand replication (CSR) of their circular single-stranded (ss) DNA genomes depends on DNA polymerases expressed in differentiated cells. This is the case for translesion synthesis (TLS) polymerases, which makes them promising candidates for the first rounds of geminiviral replication. TLS polymerases are specialized enzymes for the replication across DNA lesions. They exhibit high error rates matching the high mutation rates observed for geminiviral genomes. We elucidated whether TLS polymerases are involved in Euphorbia yellow mosaic virus (EuYMV) replication by biolistic inoculation and insect transmission experiments of Arabidopsis thaliana wildtype (wt) and TLS polymerases knock-out lines. None of the tested TLS factors was necessary for geminiviral replication. However, our results do not exclude that they might be involved in the viral amplification processes, since the lack of single TLS polymerases might be complemented by a redundant action of these enzymes. Moreover, their contribution to geminiviral replication was indicated by deep sequencing and comparative mutation analysis of EuYMV genomes.
Introduction

Geminiviruses are severe phytopathogens with circular single-stranded (ss) DNA genomes. They do not code for a DNA polymerase and therefore rely completely on host enzyme activities for DNA synthesis. They do not invade meristems, where active replication occurs, but are restricted to differentiated tissues, predominantly the phloem (reviewed in Wege, 2007). Their genomes are encapsulated into twin-shaped particles of two incomplete icosahedra for transmission by phloem-feeding whiteflies, leafhoppers or treehoppers (Böttcher et al., 2004; reviewed in Gray et al., 2014; Wei et al., 2014; Zhang et al., 2001). After injection into the nuclei-containing companion or phloem parenchyma cells, ssDNA of the disassembled virus particles is converted to a double-stranded (ds) DNA which serves as template for transcription of viral genes and further replication. At this very first event, complementary strand replication (CSR) needs DNA polymerases other than the main S phase dependent cell cycle polymerases Polα, Polδ und Polε. Only after transcription and expression of virus proteins, the viral replication initiator protein Rep can convert the infected cell to an S phase-like state by binding to the plant retinoblastoma-related protein (pRBR) and probably other cell cycle regulators like cyclins (Arguello-Astorga et al., 2004; reviewed in Hanley-Bowdoin et al., 2013; Hipp et al., 2014; Kong et al., 2000). Then, the host DNA synthesis is re-activated due to the removal of the G1/S block and cell cycle DNA polymerases and other replication factors like PCNA (proliferating cell nuclear antigen) or RFC (replication factor C) are made available (Gutierrez et al., 2004; Luque et al., 2002; Nagar et al., 1995). Further virus DNA amplification by rolling circle replication (RCR), recombination-dependent replication (RDR) and ensuing CSR can proceed using these host factors thereafter (reviewed in Jeske, 2009). During RCR, Rep is essential due to its nicking, closing and helicase activity (Choudhury et al., 2006; Clerot & Bernardi, 2006; Laufs et al., 1995; Stanley, 1995). Arising circular dsDNA can be wrapped around host histones to compose covalently closed circular (ccc) DNA in viral minichromosomes as template for transcription and replication (Abouzid et al., 1988; Paprotka et al., 2015; Pilartz & Jeske, 1992).

In case of bipartite begomoviruses, transport of viral DNA throughout its host plant is promoted by the activities of the nuclear shuttle protein (NSP) and the movement protein (MP) (reviewed in Fondong, 2013; Levy & Tzfira, 2010; and Rojas et al., 2005). In this process, NSP mediates trafficking of viral DNA from the nucleus to the cytoplasm and back through the nuclear pores, and plasma membrane-associated MP conveys viral DNA to neighbouring cells through plasmodesmata. So far, it has not been shown conclusively whether transfer of geminiviral DNAs to adjacent cells or during long-distance transport in the phloem occurs with ssDNA or dsDNA molecules. If ssDNA is transported between cells and Rep is not co-transported, the first event of
The contributions of translesion synthesis polymerases to geminivirus replication

viral CSR in newly infected cells would require DNA polymerases in differentiated cells as discussed above.

Four translesion synthesis (TLS) DNA polymerases (Polη, Polζ, Polκ and Rev1) have been identified for *Arabidopsis thaliana*, which are promising candidates for geminiviruses' first round of CSR, since they are expressed in differentiated plant tissues of leaves and stems (Garcia-Ortiz *et al.*, 2004; Nakagawa *et al.*, 2011; Sakamoto *et al.*, 2003; Santiago *et al.*, 2008a; Santiago *et al.*, 2008b; Takahashi *et al.*, 2005). TLS polymerases are specialized for replication across DNA lesions and hence contribute to DNA damage tolerance (DDT) (reviewed in Goodman & Woodgate, 2013). They have a low processivity and a relatively loose hold on the DNA template. Due to the lack of a 3'-5' proofreading activity, they are relatively error-prone when replicating undamaged DNA, although they may insert nucleotides opposite of specific DNA damages with high accuracy. Error frequencies of mammalian and yeast TLS polymerases range from one incorrect for every $10^1$ to $10^4$ nucleotides with undamaged DNA as template compared to $10^6$ to $10^8$ for the main cell cycle polymerases (reviewed in Arana & Kunkel, 2010; McCulloch & Kunkel, 2008; and Waters *et al.*, 2009). Geminiviruses exhibit high mutation frequencies as well (Duffy & Holmes, 2008, 2009), which are not or only partly due to recombination events and the susceptibility of ssDNA to oxidative damage and deamination (Monjane *et al.*, 2012). Therefore, these mutations might indicate a role for TLS polymerases in geminiviral replication as well.

TLS polymerases and DDT in general are regulated by a complex consisting of the E2-ubiquitin ligase Rad6 and the E3-ubiquitin ligase and DNA binding protein Rad18 (reviewed in Lee & Myung, 2008; Ulrich, 2005). In response to stalled replication or DNA damage, the Rad6/Rad18 heterodimer monoubiquitinates the processivity factor PCNA, whereby TLS polymerases' affinity to PCNA is promoted due to their ubiquitin binding motifs. Distinct eukaryotic TLS polymerases are specialized for replicating over certain DNA lesions or extending DNA synthesis from ends of mismatched primers. The Y-family Polη is encoded by the POLH gene (RAD30 in *S. cerevisiae*), is involved in bypassing of UV-induced cyclobutane pyrimidine dimers and contributes thereby to UV resistance (Anderson *et al.*, 2008; Curtis & Hays, 2007; Johnson *et al.*, 1999; McDonald *et al.*, 1999; Washington *et al.*, 2001). It bypasses several other DNA lesions and abasic sites *in vitro* (Choi *et al.*, 2010; Patra *et al.*, 2015; Waters *et al.*, 2009). The B-family Polζ heterodimer has a higher fidelity than the Y-family TLS polymerases, consists of a catalytic (Rev3) and an accessory subunit (Rev7), and extends preferentially DNA from mismatched deoxynucleotides inserted by other TLS polymerases opposite to DNA lesions (Haracska *et al.*, 2003; Johnson *et al.*, 2000). The Y family Polκ (POLK) can extend mispaired terminal primers on damaged or undamaged template DNA (Garcia-Ortiz *et al.*, 2004; Haracska *et al.*, 2002a;
Washington et al., 2002), and bypasses G lesions with bulky N(2)-adducts (Avkin et al., 2004; Jia et al., 2008; Minko et al., 2008). The Y family Rev1 has nucleotidyl transferase activity, inserting only one or two nucleotides with subsequent extension probably accomplished by Polζ (Nelson et al., 1996; Takahashi et al., 2005; Zhang et al., 2002). A. thaliana Rev1 preferentially inserts Cs regardless of the template nucleotide and opposite to certain DNA lesions such as abasic sites (Takahashi et al., 2007). However, Rev1’s main function is attributed to recruiting other TLS proteins to DNA damage sites and to serve as interaction platform due to its various binding partners (Waters et al., 2009).

By inoculation of Arabidopsis thaliana wildtype (wt) and TLS polymerases knock-out lines with Euphorbia yellow mosaic virus (EuYMV), we elucidated whether TLS polymerases are involved in geminiviral CSR. Although none of the tested TLS factors was necessary for geminiviral replication, their contribution was indicated by extensive sequencing and mutation analysis.

Results

Biolistic inoculation of polh-1, rev3-2 and rev1-2 plants

A. thaliana T-DNA insertion lines polh-1, rev3-2 and rev1-2 were biolistically inoculated with RCA products of EuYMV as described previously (Richter et al., 2015). The lines were characterized earlier and are verified loss-of-function alleles with inhibited transcription of the respective genes (Anderson et al., 2008; Sakamoto et al., 2003; Takahashi et al., 2005). Individual, homozygous mutant and wt sibling plants were identified by genotyping PCR, ten plants per genotype were inoculated in parallel and viral DNA accumulation was examined at 7, 14 and 21 days post inoculation (dpi) by Southern hybridization. Since viral titers remained constant from 14 to 21 dpi, data from 7 and 14 dpi are shown exemplarily (Fig. 1a-c). At 7 dpi, discrete EuYMV DNA forms of open circular (oc), ccc and ssDNA arose for some specimens with diverging quantities. Virus infection established until 14 dpi in all samples without significant differences between mutant and wt plants. Thus EuYMV replication can proceed in the absence of one of the three TLS polymerases. The polh-1 mutant plants seemed to exhibit more viral DNA than their wt siblings at 7 dpi in this experiment (Fig. 1a), but this effect was not reproduced during two repetitions with lines polh-1 and rev3-2. Furthermore, the inoculation of these lines with Cleome leaf crumple virus (CILCrV) DNA (Fig. S1a and b) did not reveal any reliable difference to wt plants. Correspondingly, mild symptoms such as leaf rolling and mild crumpling emerged at the same time and with similar severity in mutant and wt plants for EuYMV as well as CILCrV infections (data not shown).
The contributions of translesion synthesis polymerases to geminivirus replication

(a) polh-1

(b) rev3-2

7 dpi

14 dpi
The contributions of translesion synthesis polymerases to geminivirus replication

Figure 1: EuYMV DNA emergence at 7 and 14 dpi in polh-1 (a), rev3-2 (b) and rev1-2 (c) A. thaliana plants in comparison to sibling wt plants after biolistic inoculation. Total nucleic acids (500 ng each) from ten plants per genotype were separated in agarose gels (1.4 %) in the presence of 5 µg/ml ethidium bromide. Mock controls (m) were inoculated with EuYMV DNA B alone. Virus DNA was hybridized with DIG-labeled full-length EuYMV DNA A probes and detected as described (Richter et al., 2015). Genomic plant DNA (pDNA) from agarose gels served as loading control, different quantities of linear EuYMV dsDNA As (1, 10 or 100 pg) as hybridization standards. 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 (def).

In order to investigate the modes of early EuYMV replication, individual samples from 7 dpi of polh-1, rev1-2 and respective wt sibling lines were resolved in two-dimensional (2D) gel analyses (Fig. 2). Intermediates of CSR, RCR and RDR as well as end products of replication (ocDNA, cccDNA, hdsLDNA) were clearly present for all samples with no or only minor quantitative differences between mutant and wt plants. For rev3-2 and respective wt plants, DNA amounts at 7 dpi were insufficient for 2D analysis.
The contributions of translesion synthesis polymerases to geminivirus replication

**Figure 2:** Replicative intermediates of EuYMV from individual polh-1 (a) and rev1-2 (b) plants and their respective wt siblings at 7 dpi. Separation by 2D gel electrophoresis was conducted in 0.5% agarose gels with TBE buffer containing 0.03% SDS in the first dimension followed by 1.4% agarose gels with TBE buffer containing 50 µg/ml chloroquine in the second dimension. 100 ng total DNA was loaded per gel and detection of viral DNA was conducted as described in Fig. 1.
The contributions of translesion synthesis polymerases to geminivirus replication

**Biolistic inoculation of rev3 polh double mutants and ubc2-1**

The so far reported lack of differences may hint at redundant functions of the TLS polymerases. Therefore, double mutant lines and a knock-out line of the *A. thaliana* Rad6 homolog were tested. In complex with Rad18, Rad6 regulates the activity of several TLS polymerases by monoubiquitination of PCNA. A *rev3-2 polh-1* T-DNA insertion double mutant and its wt sibling line were obtained by crossing the homozygous single mutant plants and subsequent genotyping of the F2 generation. At the same time, a homozygous *rev3-1 polh-1* line was kindly provided by A. Sakamoto, which resulted from a crossing of the same *polh-1* T-DNA insertion line and the chromosomal rearrangement line *rev3-1* (Nakagawa et al., 2011; Sakamoto et al., 2003). Similar to the single knock-out lines, *rev3-1 polh-1, rev3-2 polh-1* and wt plants exhibited equal levels of viral DNA or overall infection after biolistic inoculation with EuYMV and CILCrV (Fig. 3a for EuYMV, Fig S1c for CILCrV).

Ubc2 is the *A. thaliana* homolog of the E3 ubiquitin ligase and putative TLS regulator *Rad6* of *S. cerevisiae* (Zwirn et al., 1997). EuYMV inoculation of the previously characterized knock-out line *ubc2-1* (Xu et al., 2009) in comparison to the wt sibling line showed no significant difference in infection rates or virus DNA accumulation (Fig. 3b).
Expression analysis and biolistic inoculation of polk-1

In contrast to the well-characterized mutant lines described so far, no A. thaliana knock-out line for Polκ has been examined to a comparable degree. The database of the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu) was screened and the Salk_081715 line was identified and named polk-1. It harbors a T-DNA insertion within the intron between the 13th and 14th exon of the POLK gene (Fig. 4a), which was confirmed by genotyping PCR. RT-PCR using primers flanking the insertion confirmed that no full-length transcript was present in polk-1 rosette leaves (Fig. 4b). In addition however, we have detected amplification products of about 350 bp in both wt and polk-1 plants, which might represent a new alternative AtPOLK splice variant. Furthermore, RT-PCR analysis with both primers binding upstream of the T-DNA insertion revealed that POLK-derived mRNA is expressed up to the T-DNA insertion in wt and polk-1 plants (Fig. 4c). According to Garcia-Ortiz et al. (2004), at least three splice variants of POLK mRNA are synthesized in A. thaliana. In comparison to the full-length transcript coding for the 671 aa Polκ protein, the two alternative transcripts would encode N-terminal proteins of 345...
Figure 4: Gene organization of POLK (At1g49980) and RT-PCR analysis. (a) A gene model with exon/intron structure of *A. thaliana* POLK, the positions of the T-DNA insertions polk-1 and polk-2 and primer binding sites for RT-PCR are depicted. (b) and (c) POLK transcripts from two individual samples of polk-1 and wt plants were detected by RT-PCR with primers flanking the polk-1 T-DNA insertion (b), or with both primers binding upstream of the T-DNA insertion (c). Amplification products of APT1 (adenine phosphoribosyl transferase 1) transcripts were used as internal control. To control genomic DNA contamination, PCR was conducted without RT (-RT). Molecular weights of RT-PCR products (expected and alternative) and genomic DNA contaminations were depicted. (M: PstI digested λ phage DNA)
The contributions of translesion synthesis polymerases to geminivirus replication

or 184 aa. A recombinant N-terminal AtPolκ variant of 478 aa still retained polymerase activity and exhibited even higher processivity and fidelity than full-length AtPolκ (Garcia-Ortiz et al., 2004; Garcia-Ortiz et al., 2007).

To determine whether truncated Polk variants are expressed in polk-1 or wt plants, a polyclonal antiserum against the human N-terminal portion of Polκ was used for Western blot analysis. A multitude of bands was obtained for both wt and polk-1 samples as well as for a human HEK293 cell lysate used as a positive control (Fig. 5). However, none of the bands unequivocally matched the expected full-length AtPolκ and no difference between wt and polk-1 samples was apparent. The most prominent band at approximately 52 kD probably represented an unspecific signal for the great subunit of RuBisCO. Two conspicuous signals appeared at approximately 36 and 30 kD.

The expected molecular mass of the 345 aa AtPolκ variant would be 38 kD and could thus match the band at 36 kD. The 30 kD band, however, could not be assigned, since the 184 aa AtPolκ variant would exhibit an expected molecular mass of 20 kD. Altogether, these data indicate that Polk variants might be expressed in wt as well as polk-1 plants, and it cannot be completely excluded that those contain residual polymerase activity.

![Figure 5: SDS-PAGE/Western blot to analyze expression of full-length Polk or putative N-terminal Polk fragments in polk-1 and wt plants. Total protein extracts were separated by 10% SDS-PAGE. Polk was detected with a polyclonal rabbit anti-Polk antiserum against the N-terminus of human Polk, alkaline phosphatase-conjugated goat anti-rabbit IgG and NBT/BCIP substrate. A human HEK293 cell lysate was used as detection control. Signals derived from unspecific binding (probably RuBisCO 52 kD), the expected full-length proteins or alternative splicing or degradation products for AtPolk and HsPolk are indicated. A prominent band at approximately 30 kD could not be assigned to any expected protein and is marked with an asterisk. M: Prestained protein ladder ‘Page Ruler’, Fisher Scientific, Schwerte, Germany.](image-url)
Nonetheless, wt sibling and *polk-1* plants were inoculated biolistically with EuYMV and ClLCrV. As shown before for the other TLS polymerase mutant lines, no significant difference in virus DNA levels or overall infection rates was detectable compared to wt plants (Fig. 6 for EuYMV, Fig S1d for ClLCrV).

**Figure 6:** EuYMV DNA emergence at 7 and 14 dpi in *polk-1* and wt *A. thaliana* plants as described in Fig. 1.

**Expression analysis of *polk-2***

On account of these circumstances, databases were once again screened for alternatives and a further plant line (Flag_566E01) was identified in the collection of the National Institute for Agricultural Research Versailles. It was named *polk-2* and its T-DNA insertion is located within the intron between the 1\textsuperscript{st} and 2\textsuperscript{nd} exon of the POLK gene. The T-DNA/POLK junctions were sequenced to determine the insertion in more detail (Fig. 7a and 7 b). Its 5’ border is located at position 181 of the POLK gene followed by a 28 bp right border sequence of the TR-DNA from pRiA4 comprised in the pGKB5 plasmid used for the generation of Flag insertion lines (Bouchez *et al.*, 1993; Jouanin *et al.*, 1989) and an unknown filler sequence. Its 3’ border is located
The contributions of translesion synthesis polymerases to geminivirus replication

Figure 7: Analysis of A. thaliana line polk-2. (a) Sequence of the T-DNA/Polk junctions. The color code indicates AtPOLK, RB or LB T-DNA sequences of pRIA4. (b) Gene organization of POLK (At1g49980) with exon/intron structure, the positions of the T-DNA insertions polk-1 and polk-2 and primer binding sites. (c) POLK transcripts from three individual samples of polk-2 and wt plants detected by RT-PCR with primers flanking the T-DNA insertion of polk-2 as described in Fig 3. Molecular weights (in bp) of RT-PCR products and expected genomic DNA contamination were depicted.
at position 194 of the POLK gene succeeding a 167 bp stretch of a left border sequence of pRiA4 and 3 bp filler DNA. This implies a deletion of 12 bp in the intron of the POLK gene. Whereas strong signals for the expected 235 bp amplification product were detected by RT-PCR with primers flanking the insertion in wt sibling plants, only very faint signals appeared in two of three polk-2 plant samples (Fig. 7c), showing that POLK transcription is considerably reduced, but not completely abolished in polk-2 plants.

Alternative inoculation procedures

Since geminiviral ssDNA is delivered into the plant, it might be possible that inoculation with dsDNA of RCA products circumvents a need for TLS polymerases during initial infection. For this purpose, several inoculation approaches were tested with wt Nicotiana benthamiana or A. thaliana plants. First, biolistic inocula with EuYMV and CILCrV ssDNA were generated from viral particle preparation followed either by Cs2SO4 density gradient centrifugation or by Sau3AI digestion to dispose of viral dsDNA. This approach led to an infection rate of only 10-20 % with CILCrV and to no infection at all with EuYMV with both plant species (Table 1). Hence, this approach was unsuitable for a reliable comparison of infection rates between genotypes for further experiments. Second, infectious Agrobacterium tumefaciens clones for EuYMV and CILCrV DNA A and B were established, which revealed 100 % infection of N. benthamiana for both viruses. For A. thaliana, merely EuYMV infection was tested which, however, generated infection of only 40 % of the inoculated plants. In addition, the systemic infection was less homogeneous in the whole plant, leaving only some rosette leaves with symptoms and viral DNA. Third, insect transmission with B. tabaci was established for the first time for Arabidopsis. Both viruses were transmittable from and to A. thaliana during an initial test, with EuYMV exhibiting a higher infection rate than CILCrV (Table 1), which is why EuYMV was chosen for subsequent experiments with T-DNA insertion mutants. Viral symptoms were not or barely visible (not shown) after this insect transmission. The subsequent experiments were limited to one point in time (14 dpi) and samples of whole plantlets were pooled to compensate for sampling errors.
The contributions of translesion synthesis polymerases to geminivirus replication

### Table 1: Infection rates of alternative inoculation tests.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Inoculation procedure</th>
<th>EuYMV</th>
<th>CILCrV</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. thaliana</em></td>
<td>Biolistics(^a)</td>
<td>0/10</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>Agroinoculation</td>
<td>8/20</td>
<td>n.t.</td>
</tr>
<tr>
<td></td>
<td><em>B. tabaci</em> transmission</td>
<td>7/7</td>
<td>3/7</td>
</tr>
<tr>
<td><em>N. benthamiana</em></td>
<td>Biolistics(^b)</td>
<td>0/10</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>Agroinoculation</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td><em>B. tabaci</em> transmission</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

Numbers represent infected individuals per inoculated individuals.

\(^a\) After virus particle preparation and Cs\(_{2}SO_{4}\) density centrifugation.

\(^b\) After virus particle preparation and *Sau*3AI digestion.

n.t.: not tested

### B. tabaci transmission experiments

The single mutant lines *rev3-2, polh-1* and *rev1-2* as well as the double mutant lines *rev3-1 polh-1* and *rev3-1 rev1-1* were challenged by insect transmission in comparison to the wt sibling line of *rev3-2*. The homozygous *rev3-1 rev1-1* line was kindly donated by A. Sakamoto and is a crossing of the *rev1-1* T-DNA insertion line (Takahashi *et al.*, 2005) and the chromosomal rearrangement line *rev3-1* (Nakagawa *et al.*, 2011; Sakamoto *et al.*, 2003). Infection was established at 14 dpi in all inoculated plants irrespective of their genotypes, except for one specimen of line *rev3-2* (Fig. 8). Some variations in viral DNA titers between single samples were detected, but overall no significant difference between any of the genotypes was verified.

Since *polk-2* and its wt sibling line were in a different ecotype background (Wassilewskija), they were inoculated in a separate transmission experiment. Infection rates for this ecotype were low (Fig. 8) and none of the wt and only two out of seven *polk-2* plants contained considerable amounts of EuYMV DNA at 14 dpi. Although this experiment precludes a reliable comparison between wt and mutant line, it confirms that EuYMV can replicate in plants with Polκ deficiency.

A summary of all inoculation experiments is given in Table S1.
The contributions of translesion synthesis polymerases to geminivirus replication

Figure 8: *B. tabaci* transmission of EuYMV DNA: emergence at 14 dpi after in *A. thaliana* (a) *polh*‐1, *rev3*‐2, *rev1*‐2, *rev3*‐1 *polh*‐1, *rev3*‐1 *rev1*‐1 and wt siblings of line *rev3*‐2 and (b) in *polk*‐2 in comparison to its wt siblings as described in Fig. 1.

Deep sequencing of EuYMV genomes

The inoculation experiments so far allow two conclusions: Either TLS polymerases are not involved in geminiviral replication, or the redundant action of these enzymes masks their effects and the applied tests are not sensitive enough to detect subtle differences.

Since yeast or mammalian TLS polymerases exhibit higher error frequencies and specific error types (reviewed in Arana & Kunkel, 2010), extensive sequence analysis is a promising approach for further investigation. A difference in the frequencies, distribution, or specificity of mutations in viral DNAs from wt or TLS mutant plants could implicitly demonstrate a contribution of TLS polymerases in geminiviral replication. To examine these hypotheses, RCA-amplified EuYMV DNA from wt or *rev3*‐1 *polh*‐1 double mutant plants, which had been infected with the help of *B.
tabaci, was deep-sequenced (Illumina HiSeq2500 technology; GATC, Konstanz, Germany). RCA products of pBluescript SK(+) plasmids which had been propagated in *E. coli* were added to both viral DNA samples to serve as a technical control for error rates of the high fidelity Φ29 polymerase used during RCA amplification, the library construction, and for the sequencing procedure in general. The resulting data were analysed using a Python script for wt and mutated single nucleotides as well as larger deletions and inversions. The sequences were determined for the viral and the complementary orientations for the geminiviral components A and B, as well as for the respective sense and antisense orientation of the plasmid completely for four data batches each containing ~ 6 Mio reads with 100 nts for each batch.

First evaluations of the data (Fig. 9) reveal that the genotype of the host plant has a significant differential influence on the nucleotide substitution rates of DNA A and DNA B. This effect is biologically significant as the technical control plasmid sequence had a significantly lower exchange rate than the viral samples. Subtracting the exchange rates of the technical base line from the viral DNA data, the substitution rates of the viral samples still lies within the observed high rate of mutations ($10^{-4}$) reported for several geminiviruses before (Duffy & Holmes, 2008, 2009). Interestingly, the substitution rate for DNA A was significantly reduced in mutant plants whereas it was unchanged for DNA B indicating a differential role of selection for DNA A and

---

**Figure 9:** Total nucleotide substitution rates (mean values, standard deviations, n = 4 data batches each) for viral DNA A or DNA B replicated in rev3-2 polh-1 (mut) or wt plants compared to the technical control of plasmid DNA (p) added each to the respective plant samples. Student’s t-tests or Mann-Whitney rank-sum tests were performed using SigmaStat program to determine significant differences (p<0.05) indicated by brackets.
Discussion

Geminiviruses do not encode a DNA polymerase and thus completely dependent on host enzymes for replication of their ssDNA genomes. Reprogramming of the host’s cell cycle by the viral Rep protein for inducing expression of replicative DNA polymerases like Polδ and Polε is, hence, a crucial feature of geminivirus infection (Hanley-Bowdoin et al., 2013). However, the first event of CSR after phloem-injection certainly has to involve DNA polymerases, which are expressed in differentiated cells.

We hypothesized that TLS polymerases could be suitable candidate factors to meet this purpose. Therefore, *A. thaliana* T-DNA insertion lines of distinct TLS enzymes were analysed for their ability to replicate EuYMV and/or ClLCrV DNA. Besides biolistic inoculation with viral RCA products, several inoculation procedures using ssDNA as inoculum were tested to approximate natural conditions for viral CSR. Finally, an insect transmission procedure for *A. thaliana* and EuYMV was established for the first time here. However, none of the tested TLS mutants showed any significant change in infection rates or viral DNA titers, neither after biolistic- nor after insect-based inoculation. In conclusion, Polη, Polξ and Rev1 are not necessary individually for geminivirus replication. The necessity of Polκ is not yet ultimately elucidated, since both polk lines may retain residual Polκ activity from full-length or C-terminally truncated Polκ variants. Furthermore, the infection rates during the insect transmission experiment with polk-2 were too low to enable final evidence for or against a necessary role of Polκ in geminivirus replication.

Equally, the Rad6 homolog Ubc2, which probably regulates TLS by monoubiquitinating PCNA and the combination of Polξ/Polη or Polξ/Rev1 were dispensable for geminivirus infection, as the corresponding mutants showed no effect in inoculation assays. These results, however, do not exclude that TLS polymerases might be involved somehow in geminivirus replication. Yeast and human TLS polymerases exhibit affinity to unmodified PCNA (Haracska et al., 2001; Haracska et al., 2002b) or the alternative sliding clamp 9-1-1 as well (Sabbioneda et al., 2005) and would thus be able to conduct highly processive replication without PCNA modification. Since PCNA is expressed only after the induction of the S phase-like state in infected plant cells (Nagar et al., 1995), PCNA binding would be, in any case, of no importance for initial CSR. Furthermore, the replication of small viral genomes could possibly proceed even with low processivity and without PCNA. Remarkably, human TLS polymerases Polη, Polκ, and Rev1 were shown to be involved in DNA synthesis during re-replication (Sekimoto et al., 2015). Since geminivirus infection induces re-replication in yeast and plant cells (Hipp et al., 2014; Kittelmann et al., 2009; Nagar et al., 2002), TLS polymerases might play a role during geminivirus infection, not only in early CSR but during later stages of replication.
Moreover, the activity of single TLS polymerases might be dispensable due to redundancy of and complementation by other TLS polymerases. To further investigate a possible redundancy of TLS polymerases in geminiviral replication, it would be required to generate additional double, or even triple and quadruple mutant combinations. However, a double-mutant for POLH and REV1 is not obtainable, since both genes are directly adjacent to each other on chromosome 5 (Santiago et al., 2006; Takahashi et al., 2005). Besides, plants with multiple TLS polymerase knock-outs may exhibit growth or developmental defects and would, therefore, be unsuitable for infection analysis.

TLS polymerases exhibit high error rates and specific error signatures. Thus, deep-sequencing analysis of rev3-1 polh-1- and wt-replicated viral DNA was conducted to detect possible footprints of Polη and/or Polζ replication in viral genomes. A significant differential influence of the genotype on the substitution rates of DNA A and DNA B was revealed, indicating a role for Polζ and/or Polη during geminiviral infection and a differential role of selection for DNA A and DNA B. Geminiviruses are known to produce defective DNAs of subgenomic size and DNA B tends to generate a higher number of such molecules than DNA A (Bach & Jeske, 2014; Frischmuth et al., 1997; Horn et al., 2011; Patil & Dasgupta, 2006). Defective DNAs often accumulate high amounts of sequence changes, which is likely to be due to a low selection pressure. Explicitly screening those molecules or specific viral DNA regions under low selection pressure for footprints of TLS replication is a promising approach for further sequence analysis, since errors may accumulate more unrestricted here.

The majority of errors originated from yeast or human TLS polymerases’ activity are single nucleotide substitutions (Arana & Kunkel, 2010; Kozmin et al., 2003). If replicating undamaged DNA, TLS polymerases generate all twelve possible single nucleotide substitutions and a multitude of other error types, but there are biases for specific changes for certain polymerases (Arana & Kunkel, 2010). A summary of specific error signature for TLS polymerases on undamaged DNA is given in Table 2. Polη generates most prevalently dGMP misinsertions opposite a template T, which leads to a T→C mutation in the original sequence (Matsuda et al., 2000; Matsuda et al., 2001). It causes single and multiple deletions (1-100 nucleotides) and insertions (1-3 nucleotides). Polζ most commonly produces dCMP mispairs opposite template A or C, thereby inducing A→G or C→G mutations (Zhong et al., 2006). Equally, multiple nucleotide deletions and clusters of multiple single nucleotide errors including substitutions, deletions and insertions have been observed. Polκ most frequently causes T→G substitutions due to misinsertion of dCMP opposite template T (Ohashi et al., 2000). Single nucleotide insertions, single and double nucleotide deletions, substitution-insertion and substitution-deletion errors
Table 2: Error signatures of TLS polymerases.

<table>
<thead>
<tr>
<th>TLS pol</th>
<th>Error phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polη</td>
<td>• Mainly single base substitutions: most prevalently dGMP opposite template T (T→C)</td>
</tr>
<tr>
<td></td>
<td>• Double and triple base substitutions</td>
</tr>
<tr>
<td></td>
<td>• Insertions of 1-3 bases</td>
</tr>
<tr>
<td></td>
<td>• Single and multiple (2-100) base deletions</td>
</tr>
<tr>
<td>Polζ</td>
<td>• Mainly single base substitutions: most prevalently dCMP opposite template A or C (A→G; C→G)</td>
</tr>
<tr>
<td></td>
<td>• Double and triple base substitutions</td>
</tr>
<tr>
<td></td>
<td>• Clusters of multiple single base errors (substitutions, deletions, insertions)</td>
</tr>
<tr>
<td></td>
<td>• Multiple base deletions</td>
</tr>
<tr>
<td>Polκ</td>
<td>• Mainly single base substitutions: most prevalently dCMP opposite template T (T→G)</td>
</tr>
<tr>
<td></td>
<td>• Double base substitutions</td>
</tr>
<tr>
<td></td>
<td>• Single base insertions and deletions</td>
</tr>
<tr>
<td></td>
<td>• Double base deletions mainly at 5’-GCT-3’ template sites</td>
</tr>
<tr>
<td></td>
<td>• Substitution-insertion and substitution-deletion</td>
</tr>
</tbody>
</table>

have been detected as well. Interestingly, double nucleotide deletions were mainly located at 5’-GCT-3’ template sites. An error event almost uniquely observed for TLS polymerases replicating undamaged DNA are double nucleotide substitutions, and those are therefore considered as specific error signatures of Polη, Polζ and Polκ (Arana & Kunkel, 2010). For Polη and Polζ, even triple nucleotide substitutions have been reported (Matsuda et al., 2001; Zhong et al., 2006). After induction of a complex DNA damage spectrum by synthetic sunlight in S. cerevisiae, double substitutions were detected in wt, rad30, rev3 and double mutant rev3 rad30 strains as well (Kozmin et al., 2003). Interestingly, only CC→TT mutations were detected in wt and rev3 strains, whereas a broader variety of those was detected in the rad30 strain (CC→TT; CC→TA; CC→AT; GC→TT; TC→AT; TC→AΔ). The rev3 rad30 strain, however, harboured only CC→AA double mutations.

Geminiviruses are known to exhibit high evolutionary rates ranging from 1.6 × 10⁻³ to 1.3 × 10⁻⁴ substitutions per site and year with variations between distinct genome regions (Duffy & Holmes, 2008, 2009; Ge et al., 2007; van der Walt et al., 2008). Notably, biases for certain substitutions including an overrepresentation of C→T and G→A for East African cassava mosaic virus (EACMV) (Duffy & Holmes, 2009), Tomato yellow leaf curl virus (TYLCV) (Duffy & Holmes, 2008) and Tomato yellow leaf curl China virus (TYLCCNV) (Ge et al., 2007), or G→T for Maize
streak virus (MSV) (van der Walt et al., 2008) and TYLCV have been detected. These substitutions are likely to originate from deamination from C to U (C→T) or are derived from oxidation-induced damage of G during which 8-oxo-guanine is formed and copied to A by Pols α, δ or ε (G→T) and affect especially ssDNA, which is indicated by the strand-specific distribution of mutations (Monjane et al., 2012; van der Walt et al., 2008). However, for EACMV T→G substitutions were overrepresented as well, which fit to the error signature of Polκ. Further investigations concerning specific substitutions in genome regions with low selectivity pressure and a comparison with TLS polymerases’ error signatures are necessary to elucidate this issue for EuYMV in more detail.

In conclusion, single TLS polymerases and the combination of Polζ/Polη and Polζ/Rev1 are not necessary for geminivirus replication, yet further analyses are required for Polκ. Nevertheless, sequence analysis revealed significant differences of error frequencies in viral DNAs replicated in wt and rev3-1 polh-1. These differences indicate a role for TLS polymerases in geminivirus replication, but at the same time imply a complex network of TLS replication, selection pressure and DNA repair. Further TLS polymerase mutants and specific errors need to be analyzed in more detail preferably in genome regions with low selection pressure to elucidate these remaining ambiguities.

Materials and Methods

Plants and viruses

*Nicotiana benthamina* DOMIN plants were kept in a greenhouse with additional illumination and a 16 h photoperiod for biolistic and agroinoculation experiments. Seeds of the segregating, hemizygous *Arabidopsis thaliana* (ecotype Col-0) T-DNA insertion lines polh-1 (Salk_129731) (Anderson et al., 2008), rev3-2 (Salk_029237) (Sakamoto et al., 2003), ubc2-1 (Salk_060994) (Xu et al., 2009), polk-1 (Salk_081715) and the homozygous insertion line rev1-2 (Salk_005721C) (Takahashi et al., 2005) were acquired from the Nottingham Arabidopsis Stock Centre (NASC, Loughborough, UK). The rev3-2 polh-1 double mutant line was obtained via crossing of the single T-DNA insertion lines. Seeds of the segregating *A. thaliana* (ecotype Wassilewskija) line polk-2 (Flag_566E01) were acquired from the Versailles Arabidopsis Stock Centre (Versailles Cedex, France). Homozygous double mutant lines rev3-1 rev1-1 and rev3-1 polh-1 (Nakagawa et al., 2011) were kindly provided by Ayoko Sakamoto (Life Science and Biotechnology Division, Japan Atomic Energy Agency, Takasaki, Gumma, Japan). The absence of transcripts for characterized lines polh-1, rev3-2, rev1-2, ubc2-1, rev3-1 rev1-1 and rev3-1 polh-1 has been verified before. Wildtype (wt) sibling plants from each individual segregating line were used as a control. For the homozygous line rev1-2, wt siblings of line rev3-2 were used as a control. Plantlets were
initially grown under short day conditions in an acclimate chamber as described previously (Richter et al., 2015). After 28 days, plants were transferred to a greenhouse with additional illumination and a 14 h photoperiod for insect transmission experiments or a 16 h photoperiod for agro- or biolistic inoculation. Plants were inoculated with Euphorbia yellow mosaic virus (EuYMV) DNA A (FN435995) and/or DNA B (FN435996) or Cleome leaf crumple virus (CILCrV) DNA A (FN435999) and/or DNA B (FN436000) after one to four days of acclimatization.

**Genotyping**

The Phire Plant Direct PCR Kit (Fisher Scientific, Schwerte, Germany) and different combinations of primer pairs (Table 3) binding to the T-DNA insertion and respective neighboring gene sequences were used to validate the particular insertions of the distinct plant lines and the zygosity of individual plants. PCR products were assessed with agarose gel electrophoresis. The ‘Salk Institute Genome Analysis Laboratory’ (http://signal.salk.edu) or ‘The Arabidopsis Information Resource’ (http://www.arabidopsis.org/) websites supplied necessary sequence information concerning insertion lines and primer design.

**Table 3:** Primers for genotyping PCRs of various T-DNA insertion lines.

<table>
<thead>
<tr>
<th>Knock-out line</th>
<th>Line no.</th>
<th>Gene no.</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>polh-1</td>
<td>Salk_129731</td>
<td>At5G44740</td>
<td>LP: 5’GAGGACTGCTAGTGCAGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’GTCAGTGGTCAAGCTAGGCC</td>
</tr>
<tr>
<td>rev3-2</td>
<td>Salk_029237</td>
<td>At1G67500</td>
<td>LP: 5’GATTGCTAAGTGCTGGACTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’TATACGTGCAAGGCGATTG</td>
</tr>
<tr>
<td>rev1-2</td>
<td>Salk_005721C</td>
<td>At5g44750</td>
<td>LP: 5’CTCTTCAAAAGGATTTTGGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’CGTTACGCTCAGGGCAAC</td>
</tr>
<tr>
<td>polk-1</td>
<td>Salk_081715</td>
<td>At1g49980</td>
<td>LP: 5’GGTAAGCGAGATCGGAAATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’ATAGCCGTTCTGTTGATAGG</td>
</tr>
<tr>
<td>polk-2</td>
<td>Flag_566E01</td>
<td>At2g01760</td>
<td>LP: 5’ATTTTGGCGATTGCAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP: 5’CTACAAATTGCCCTTCTATAG</td>
</tr>
</tbody>
</table>

**Biolistic inoculation**

*A. thaliana* (10-12 leaf stage) and *N. benthamiana* (4-6 leaf stage) plants were biolistically inoculated with undigested rolling circle amplification (RCA) products of EuYMV or CILCrV DNA A and B or with DNA B only as a mock control as described previously (Richter et al., 2015; Richter et al., 2014).
Alternatively, to obtain viral ssDNA as inoculum, EuYMV and CILCrV particles were prepared from infected *N. benthamiana* plants and purified by Cs₂SO₄ density gradient centrifugation (Kittelmann & Jeske, 2008). Virus particle containing fractions were pooled, heated (95°C, 5 min) and ssDNA was purified by phenol/chloroform based extraction (Haible *et al.*, 2006; Richter *et al.*, 2015). ssDNA was dissolved in 30 µl H₂O for the production of microcarriers for biolistics as described (Richter *et al.*, 2014). During a second particle preparation experiment, density gradient centrifugation was omitted to increase the yield of ssDNA. Instead, DNA from particle preparations were digested with *Sau*3AI to dispose of remaining viral dsDNA and prepared for biolistics as described before.

**Preparation of infectious *Agrobacterium tumefaciens* clones and agroinoculation**

Partial tandem repeats of EuYMV and CILCrV DNA A and DNA B were obtained by partial digestion of RCA products with *Sau*3AI according to Wyant *et al.* (2011), ligated into *Bam*HI-digested *pGreen0029* vector plasmids (Hellens *et al.*, 2000) and transformed to *Escherichia coli* DH5α cells. Positive clones were tested by colony RCA followed by *Xba*I and *Eco*RI (DNA A inserts) or *Xba*I and *Pst*I (DNA B inserts) restriction and sequencing of both insert ends. Colony RCA products from constructs containing two viral origins of replication were tested for infectivity by biolistic inoculation of *N. benthamiana* plants. All constructs proved to be infectious and were used for transforming *Agrobacterium tumefaciens* LBA4404 cells by electroporation (Shen & Forde, 1989) in combination with the helper plasmid pSoup (Hellens *et al.*, 2000). For agroinoculation, the stem of 4-leaf stage *N. benthamiana* or of 8-leaf stage *A. thaliana* plants were punctured with a thin pin and bacterial suspensions of DNA A and DNA B (1:1) transformants or DNA B alone as a mock control. Single, newly emerged leaves were harvested in liquid nitrogen after 7, 14 or 21 dpi.

**Bemisia tabaci** transmission

A laboratory population of *B. tabaci* Middle East-Asia Minor 1 (MEAM1) initially collected in Gezira (Sudan) was maintained on cotton plants (*Gossypium hirsutum*) at 26°C and a photoperiod of 14 h in insect-proof cages under quarantine conditions at the DSMZ Braunschweig. The insect population was assigned to the cryptic species MEAM1 on the basis of partial mitochondrial cytochrome oxidase subunit I (mtCOI) sequence (mtCOI Genebank accession number: KP941428) (Boykin & de Barro, 2014; Frohlich *et al.*, 1999). Analysis of symbiont communities was performed according to Chiel *et al.* (2007) showing that the population harbored the primary symbiont *Portiera* and the secondary symbionts *Hamiltonella, Rickettsia* and *Wolbachia* (Kollenberg *et al.*, 2014). For EuYMV inoculation, insects were given an acquisition access period of three days on EuYMV infected *A. thaliana* Col-0 plants (10-16 dpi).
Viruliferous insects (at least 1000) were transferred to *A. thaliana* mutant and wt plants (10-12 leaf stage, n=14-35 in total) for a 7 days inoculation access period and cultivated as described before. Single leaves or whole rosettes were harvested in liquid nitrogen at 7, 14 or 21 dpi.

**Total nucleic acid extraction**

Ground plant material of single rosette leaves from biolistic inoculation or of complete rosettes (100 mg) from insect transmission experiments were used for nucleic acid isolation with a phenol/chloroform-based method as described (Haible et al., 2006; Richter et al., 2015).

**Diagnostics**

For inoculation tests, infection of *N. benthamiana* and *A. thaliana* plants was verified by RCA-RFLP as described (Richter et al., 2014) or by PCR with Taq polymerase and EuYMV- or ClLCrV-specific primers.

**Gel electrophoresis, Southern blotting and nucleic acid detection**

One-dimensional (1D) and two-dimensional (2D) agarose gel electrophoresis followed by alkaline Southern blotting, hybridization with digoxigenin (DIG)-labelled viral DNA A and DIG-based viral DNA detection was performed as previously described (Jeske et al., 2001; Preiss & Jeske, 2003; Richter et al., 2015). Signal strength of viral DNA was determined with the ImageJ analysis software (National Institutes of Health; http://rsb.info.nih.gov/ij/) and statistics were conducted with the SigmaStat program (Systat Software, San Jose, CA, USA).

**RNA extraction and RT PCR**

In order to analyze the occurrence or absence of POLK transcripts in *polk*-1 and *polk*-2 insertion mutant in comparison to wt plants, total RNA was extracted from young, uninfected rosette leaves and total mRNA was reversely transcribed as described in Richter et al. (2015). The reverse transcriptase (RT) reaction product served as template in a 35 cycle PCR with Taq DNA polymerase (Qiagen, Hilden, Germany) with different combinations of primers (see Fig. 4a and Table 4). Amplification of APT1 (adenine phosphoribosyl transferase 1) transcripts according to Richter et al. (2015) served as internal control.

**Table 4:** Primers for RT-PCRs of *polk*-1 and *polk*-2 lines.

<table>
<thead>
<tr>
<th>Knock-out line</th>
<th>Line no.</th>
<th>Primer name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>polk</em>-1</td>
<td>Salk_081715</td>
<td>polk-1 F</td>
<td>5’GTTAGCCTGCAGAGGTACAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polk-1 R</td>
<td>5’AGGATAACATCTCCGCTCTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F5</td>
<td>5’TGTATGAAAAAGAAGAAGGCTGAAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R6</td>
<td>5’ATCTTCATTGAGAAATGATCTGAG</td>
</tr>
<tr>
<td><em>polk</em>-2</td>
<td>Flag_566E01</td>
<td>polk-2 F</td>
<td>5’ TAATGCGCGAATTATATAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polk-2 R</td>
<td>5’ATCAAGGGAAGAAAGCCTTTGC</td>
</tr>
</tbody>
</table>
The contributions of translesion synthesis polymerases to geminivirus replication

Protein extraction and SDS-PAGE/ Western blotting

Fresh rosette leaves from uninfected polk-1 and wt plants were homogenized on ice with 1 ml grinding buffer (Von Arnim et al., 1993) per 200 mg plant material and additional protease inhibitors for plant extracts (Sigma Aldrich, Hamburg, Germany). Crude protein extracts (7.5 µl) were mixed with SDS loading buffer (end concentration: 50 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue, 100 mM dithiothreitol) and heated at 100° C for 3 min. Proteins were separated by 10 % SDS-PAGE according to Laemmli (1970) and blotted semidry onto nitrocellulose membranes. Detection of Polκ was conducted by using a polyclonal rabbit anti-Polκ antisemur (1:1000) against the N-terminus of human Polκ (ABIN502985; antibodies-online, Aachen, Germany), an alkaline phosphatase-conjugated goat antirabbit IgG (1:2000) (Rockland Immunochemicals Inc, Gilbertsville, PA, USA), and nitroblue tetrazolium/chloride-5-bromo-4-choro-3'-indolylphosphate. A positive detection control of human embryonic kidney cell line (HEK-293) lysates in SDS loading buffer was kindly provided by Kornelia Ellwanger (Institute of cell biology and immunology, University of Stuttgart).

Deep sequencing of EuYMV DNA

EuYMV DNA replicated in wt or rev3-1 polh-1 plants from B. tabaci transmission experiments was pooled for each genotype, amplified by RCA and sequenced by Illumina HiSeq2500 technology with approximately reads 6 million (~ 100 nts) per sample (GATC, Konstanz, Germany). As a technical control, RCA products of E. coli replicated pBluescript SK (+) were added to both samples. Sequences were analyzed with a Python-based (Phyton Software Foundation) workflow and t-tests or Mann-Whitney rank-sum tests were carried out with the SigmaStat program (Systat Software, San Jose, CA, USA).

Acknowledgements

The authors would like to thank Dr. Ayoko Sakamoto (Life Science and Biotechnology Division, Japan Atomic Energy Agency, Takasaki, Gumma, Japan) for consigning the rev3-1 rev1-1 and rev3-1 polh-1 double mutant lines, Dr. Kornelia Ellwanger (Institute of cell biology and immunology, University of Stuttgart) for providing HEK293 cell lysates and the gardeners Annika Allinger, Diether Gotthardt and Marvin Müller for taking care of the plants. This research was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG, Je116/15-1).
The contributions of translesion synthesis polymerases to geminivirus replication

References


156


157


Ulrich, HD (2005) The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. Chembiochem 6: 1735-1743


Supplementary data

(a) 7 dpi

(b) 7 dpi

Supplementary data
Figure S1: ClLCrV DNA emergence at 7 and 14 dpi in polh-1 (a), rev3-2 (b), rev3-2 polh-1, rev3-1 polh-1 (c), polk-1 (d) and respective wt sibling plants. Virus DNA was hybridized with DIG-labeled full-length ClLCrV DNA A probes and detected as described in Fig. 1.
Table S1: Summary of individual inoculation experiments.

<table>
<thead>
<tr>
<th>Inoculation procedure</th>
<th>Simultaneously tested plant lines</th>
<th>EuYMV</th>
<th>CILCrV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biolistics</td>
<td><em>polh-1</em> + wt siblings</td>
<td>3 x 10</td>
<td>3 x 10</td>
</tr>
<tr>
<td></td>
<td><em>rev3-2</em> + wt siblings</td>
<td>3 x 10</td>
<td>3 x 10</td>
</tr>
<tr>
<td></td>
<td><em>rev1-2</em> + wt siblings of <em>rev3-2</em></td>
<td>1 x 10</td>
<td>n.t.</td>
</tr>
<tr>
<td></td>
<td><em>polk-1</em> + wt siblings</td>
<td>1 x 10</td>
<td>1 x 10</td>
</tr>
<tr>
<td></td>
<td><em>rev3-2 polh-1</em> + wt siblings + <em>rev3-1 polh-1</em></td>
<td>2 x 10</td>
<td>2 x 10</td>
</tr>
<tr>
<td>Insect transmission</td>
<td><em>polh-1</em> + <em>rev3-2</em> + <em>rev1-2</em> + <em>rev3-1</em> <em>polh-1</em> + <em>rev3-1</em></td>
<td>1 x 5</td>
<td>n.t.</td>
</tr>
<tr>
<td></td>
<td><em>rev1-1</em> + wt siblings of <em>rev3-2</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>polk-2</em> + wt siblings</td>
<td>1 x 7</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

Numbers indicated: Quantity of individual experiments x quantity of inoculated plants per genotype and experiment.

n.t.: not tested
Author contributions

**Manuskript 1: 'Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner':**

Study conception and design: Kathrin S. Richter, Tatjana Kleinow and Holger Jeske
Acquisition, analysis and interpretation of data: Kathrin Richter
Writing of the manuscript: Kathrin Richter and Holger Jeske

**Manuskript 2: ‘Rad54 is not essential for any geminiviral replication mode in planta’**

Study conception and design: Kathrin S. Richter and Holger Jeske
Acquisition of data from two inoculation experiments: Kathrin S. Richter
Acquisition of data from one inoculation experiment: Lukas Ende
Analysis and interpretation of data: Kathrin Richter
Writing of the manuscript: Kathrin Richter

**Manuskript 3: ‘Ku80, a key factor for non-homologous end-joining, retards geminivirus multiplication’**

Study conception and design: Kathrin S. Richter and Holger Jeske
Acquisition, analysis and interpretation of data: Kathrin Richter
Writing of the manuscript: Kathrin Richter

**Manuskript 4: ‘The role of homologous recombination factors for geminiviral infection in planta’**

Study conception and design: Kathrin S. Richter and Holger Jeske
Crossing and genotype analysis of the rad51b rad51d xrcc2 triple mutant: Heïdi Serra and Charles I. White
Acquisition of data from inoculation experiments: Kathrin S. Richter
Analysis and interpretation of data: Kathrin Richter
Writing of the manuscript: Kathrin Richter
Acknowledgements to Lukas Ende for acquisition of data from one replicate inoculation experiment for the line xrcc2

**Manuskript 5: ‘The contributions of translesion synthesis polymerases to geminivirus replication’**

Study conception and design: Kathrin S. Richter and Holger Jeske
Acquisition of data from expression analysis, biolistic and *Agrobacterium-mediated* inoculation procedures: Kathrin S. Richter
Acquisition of data from insect transmission experiments: Monika Götz and Stephan Winter
Acquisition of data from sequence analysis: Holger Jeske
Analysis and interpretation of data: Kathrin Richter
Writing of the manuscript: Kathrin Richter
Literaturverzeichnis


Friesner, Fondong, Donson, both distinct and additive roles in response to ionizing radiation. Ferguson, IRF-3-dependent innate immunity.

Choudhury, Chapman, Duffy, the RAD51 and DMC1 homologs from Arabidopsis thaliana. Diversity in the native plant reservoir species of recombinant nature.}

Frischmuth, Frit, linked to its size. Radiation and defective in T-DNA integration.}

Bernardi, Charbonnel, repair pathway choice. 


Ferguson, BJ, Mansur, DS, Peters, NE, Ren, H, Smith, GL (2012) DNA-PK is a DNA sensor for IRF-3-dependent innate immunity. Elife 1: e00047


Glazov, Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA. EMEM1 complexes from Hartung, 2022

Goodman, encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in Gray, 2021. Biol


Kołb, D, Blanck, S, Demand, K, Focke, M, Puchta, H (2008) AtRECQ2, a RecQ helicase homologue from *Arabidopsis thaliana*, is able to disrupt various recombogenic DNA structures in vitro. *Plant J* 55: 397-405


Mazin, AV, Mazina, OM, Bugreev, DV, Rossi, MJ (2010) Rad54, the motor of homologous recombination. DNA Repair 9: 286-302


Ulrich, HD (2005) The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. Chembiochem 6: 1735-1743


Williams, GJ, Lees-Miller, SP, Tainer, JA (2010) Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair 9: 1299-1306

Wright, WD, Heyer, WD (2014) Rad54 functions as a heteroduplex DNA pump modulated by its DNA substrates and Rad51 during D loop formation. Mol Cell 53: 420-432


Yao, Y, Kathiria, P, Kovalchuk, I (2013) A systemic increase in the recombination frequency upon local infection of Arabidopsis thaliana plants with oilseed rape mosaic virus depends on plant age, the initial inoculum concentration and the time for virus replication. Front Plant Sci 4: 61


Zhang, SC, Wege, C, Jeske, H (2001a) Movement proteins (BC1 and BV1) of Abutilon mosaic geminivirus are cotransported in and between cells of sink but not of source leaves as detected by green fluorescent protein tagging. Virology 290: 249-260


Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ausschließlich mit den angegebenen Hilfsmitteln und Quellen angefertigt habe.

Stuttgart, den

Kathrin S. Richter
**Danksagung**

Eine Promotion dauert einige Jahre und kann nicht ohne Unterstützung bewerkstelligt werden. Ich möchte daher meinen Freunden, Bekannten, Kollegen und allen, die meinen Weg kreuzten, ganz herzlich danken.


Ebenso möchte ich mich bei Prof. Dr. Arnd Heyer für seine Bereitschaft, das Zweitgutachten zu übernehmen, und das Interesse an meiner Arbeit bedanken.

Nach vielen Jahren des Studentenlebens weiß jeder, dass eine WG nur so gut ist, wie die Leute, mit denen man zusammen lebt. Eine schlechte Atmosphäre, auch in einer WG in bester Halbhöhenlage, drückt auf Dauer auf das Gemüt. Eine gute Atmosphäre hingegen hilft einem jeden Tag aufs Neue, auch schwerere Zeiten durchzustehen. Die Molbios sind die beste „Wissenschafts-WG“, die ich mir wünschen konnte und ich möchte mich bei allen für ihre Hilfsbereitschaft, den geistigen Input, die anregenden Diskussionen und die tolle Stimmung (inklusive Verpflegung) bedanken. Ganz speziell gilt dies für Dr. Kathrin Deuschle (auch für die großartige Zeit im gemeinsamen Büro), Dr. Sabine Eiben, Dr. Fabian Eber, Angela Schneider, Nana Wenz, Gabi Kepp, Dr. Tatjana Kleinow, Prof. Dr. Christina Wege und Dr. Katharina Hipp. Annika Allinger, Diether Gotthardt und Marvin Müller danke ich ganz herzlich für die Pflege meiner Pflanzen.

Ein großer Dank geht an Dr. Monika Götz und Dr. Stephan Winter aus dem DSMZ in Braunschweig für die Planung und Durchführung der Insektenübertragungsversuche.

Special thanks go to Prof. Dr. Charles White and Heidi Serra from the Clermont Université in Aubière and Dr. Ayoko Satamoto from the Life Science and Biotechnology Division of the Atomic Energy Agency in Takasaki who provided important mutant lines for my work.

Der Peer Mentoring Gruppe des Instituts und speziell Katrin Brauner möchte ich für die hilfreichen Gespräche, nicht nur über Statistik, danken.

Zuletzt geht ein ganz besonderer Dank an meine Freunde, Familie und speziell an Dr. Michael Saliba für die Unterstützung, aufmunternden Worte und für die Bereitschaft, meine vereinzelten Anfälle von Launenhaftigkeit zu ertragen.

Danke an euch alle, dass ihr mich in einer prägenden Phase meines Lebens begleitet habt.