Geminivirale Infektion in Interaktion mit DNA-Reparatur und -Schadenstoleranz

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
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Abkürzungen

AGE	Agarosegelelektrophorese
AS	Aminosäuren
At	*Arabidopsis thaliana*
bp	Basenpaare
ccc	*covalently closed circular*
ClLCrV	*Cleome leaf crumple virus*
CR	*common region*
CSR	*complementary strand replication*
ds	doppelsträngig
DSB	Doppelstrangbruch
EuYMV	*Euphorbia yellow mosaic virus*
hdsl	*heterogeneous double-stranded linear*
HR	Homologe Rekombination
IR	Intergene Region
kb	Kilobasen
kD	Kilodalton
MYMIV	*Mungbean yellow mosaic India virus*
NHEJ	*non-homologous end-joining*
nts	Nukleotide
oc	*open circular*
RCA	*rolling circle amplification*
RCR	*rolling circle replication*
RDR	*recombination-dependent replication*
RT	Reverse Transkription
Sc	*Saccharomyces cerevisiae*
SDSA	*synthesis-dependent strand annealing*
ss	*single-stranded*
SSA	*single-strand annealing*
TLS	Transläsionssynthese
wt	Wildtyp

Zusammenfassung

Durch ihre Neigung zu Rekombinationen und Punktmutationen können sich Geminiviren schnell an neue Wirte und Umweltbedingungen anpassen und bedrohen so weltweit die Erträge wichtiger Nutzpflanzen. Die Vermehrung ihres zirkulären einzelsträngigen DNA-Genoms erfolgt über drei Replikationsmodi: *complementary strand replication* (CSR), *rolling circle replication* (RCR) und *recombination-dependent replication* (RDR). Geminiviren infizieren ausschließlich ausdifferenzierte Zellen und viele Arten bleiben auf kernhaltige Phloemzellen beschränkt. Da sie nur für wenige Proteine kodieren, ist ihre Replikation in erheblichem Maße von DNA-amplifizierenden und -modifizierenden Enzymen des Wirtes abhängig. Diese Arbeit befasst sich mit dem Zusammenspiel der geminiviralen Vermehrungsprozesse und verschiedenen Wegen der DNA-Schadenstoleranz und –Reparatur der Pflanze. Dafür wurden die Infektionen mit *Euphorbia yellow mosaic virus* (EuYMV) und *Cleome leaf crumple virus* (ClLCrV) in *knock-out*- und Reporterlinien der Modellpflanze *Arabidopsis thaliana* analysiert.

Die EuYMV-Infektion erhöhte die Häufigkeit der homologen Rekombination (HR) eines Reportertransgens speziell in Leitgewebe-assoziierten Zellen. Da EuYMV ausschließlich im Phloem nachgewiesen wurde, fördert die geminivirale Infektion vermutlich den HR-Reparaturweg der Wirtspflanze spezifisch in diesem Gewebe. Die Beiträge verschiedener HR-Faktoren zur geminiviralen Replikation, speziell der RDR, wurden durch biolistische Inokulationen von Wildtyp- und *knock-out*-Linien überprüft. Vielversprechende Schlüsselfaktoren des homologen Strangaustausches wie Rad51 oder Rad54 hatten keinen Einfluss. Das Rad51-Paralog Rad51D trug jedoch wesentlich zur geminiviralen Vermehrung bei, wahrscheinlich durch seine Funktion im HR-Reparaturmodus des *single-strand annealing*. Ku80, ein Schlüsselfaktor der fehleranfälligen *non-homologous end-joining* Reparatur, verzögerte den Verlauf der systemischen EuYMV-Infektion. Dieser hemmende Effekt könnte durch die Anhäufung fehlerhafter viraler DNA-Moleküle verursacht werden. Möglicherweise fungiert Ku80 außerdem als Sensor für virale DNA und löst durch die Interaktion mit der Exonuklease Wex eine pflanzliche Abwehrreaktion aus. Darüber hinaus wurde der Beitrag von Transläsionssynthese (TLS)-Polymerasen zur geminiviralen Replikation, speziell zur CSR, mittels biolistischer Inokulation sowie Insektenübertragung analysiert. Keine der TLS-Polymerasen war für die geminivirale Vermehrung notwendig. Jedoch deuten vergleichende Mutationsanalysen von EuYMV-Sequenzen erstmalig auf ihre Beteiligung und redundante Funktion bei der geminiviralen Replikation hin. Diese Ergebnisse lassen insgesamt die genetische Flexibilität der Geminiviren erkennen und könnten so ihre hohe Anpassungsfähigkeit und ihr epidemiologisches Potential erklären.

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* (ClLCrV) 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

Alle Zellen stehen unter dem ständigen Einfluss von endogenen und exogenen Faktoren, die die Integrität ihres Genoms gefährden. Reaktive Sauerstoffspezies, UV-Strahlung, chemische Substanzen oder auch spontane chemische Veränderungen der Nukleinsäuren wie Desaminierungen sind nur einige von vielen möglichen Auslösern von DNA-Schäden. Insbesondere Pflanzen sind durch ihre Abhängigkeit vom Sonnenlicht und ihre sessile Lebensweise intensiv DNA-schädigenden Umweltbedingungen ausgesetzt und darauf angewiesen, die verschiedensten Formen von DNA-Läsionen effizient beheben zu können. Diese betreffen entweder chemische oder strukturelle Veränderungen des DNA-Einzelstrangs bzw. eines einzelnen Nukleotids und können durch verschiedene Reparatur-Wege, wie beispielsweise der DNA-Einzelstrangbruchreparatur, der Basenexzisionsreparatur, der Nukleotid-exzisionsreparatur, der Photoreaktivierung durch Photolyasen oder der *mismatch* Reparatur beseitigt werden (Caldecott, 2014; Iyama & Wilson, 2013; Mannuss *et al.*, 2012). Eine der schwersten Formen von DNA-Schäden sind allerdings DNA-Doppelstrangbrüche (DSBs), da sie zur Umgestaltung und Aberration kompletter Chromosomen und dadurch letztendlich zum Zelltod führen können. Gleichzeitig werden DSBs und ihre Auflösung über Rekombination der homologen Chromosomen während der meiotischen Teilung intrinsisch induziert, was essentiell zur Neukombination des elterlichen Erbguts und damit zur genetischen Vielfalt und Evolution beiträgt. Da manche Reparatur-Faktoren sowohl in meiotischen als auch in verschiedenen somatischen Reparatur-Prozessen beteiligt sein können, ist eine strikte Regulation ihrer Aktivitäten, abhängig vom zellulären und umweltbedingten Kontext, unumgänglich (Chapman *et al.*, 2012; Schuermann *et al.*, 2005).

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 Proteinkinase Atm (*ataxia telangiectasia mutated*) aktiviert (Maréchal & Zou, 2013). Dagegen wird die Proteinkinase 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.

Im Gegensatz zum NHEJ ist HR ein Reparaturweg mit sehr hoher Genauigkeit, da homologe Sequenzen verwendet werden. Außerdem trägt es als Mechanismus neben seiner Rolle in der somatischen DNA-Reparatur essentiell zum genetischen Austausch während der Meiose bei. Es können darüber hinaus nicht nur DSBs, sondern auch andere DNA-Schäden wie DNA *interstrand crosslinks*, ssDNA-Lücken oder blockierte Replikationsgabeln behoben werden (Jasin & Rothstein, 2013; Li & Heyer, 2008). Auch bei der HR werden verschiedene Mechanismen unterschieden (Abbildung 1).

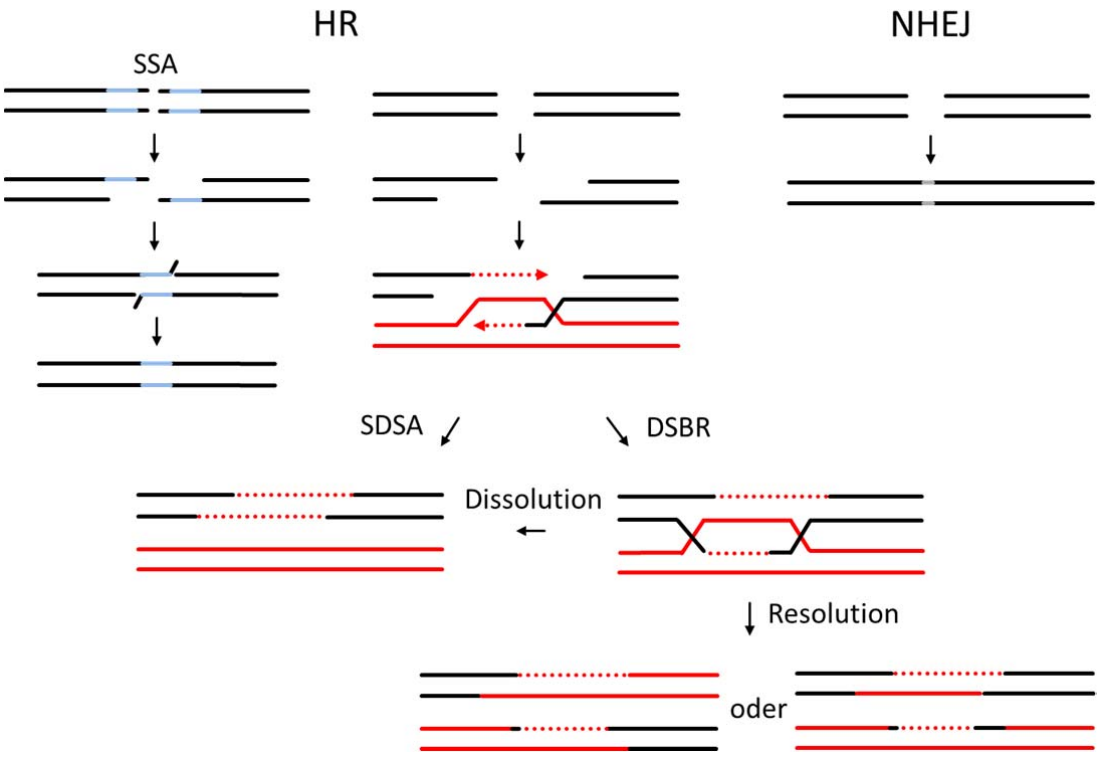

Abbildung 1: Modelle der Reparatur nach DSBs. Homologe Rekombination (HR) über *single-strand annealing* (SSA) findet im Falle eines DSB zwischen Tandem-Sequenzen statt. Beim *synthesis-dependent strand annealing* (SDSA) und der DSB-Reparatur (DSBR) dringen 3'-ssDNA-Überhänge in die homologe dsDNA-Matrize ein und werden verlängert. Beim DSBR wird im weiteren Verlauf eine zweite *Holliday junction* ausgebildet und abhängig von der Auflösung dieser Struktur entstehen verschiedene Reparaturprodukte. Beim *non-homologous end-joining* (NHEJ) werden die gebrochenen Enden direkt ligiert, wobei Insertionen oder Deletionen entstehen können. Beim klassischen NHEJ findet meist nur eine minimale Prozessierung der Enden statt. Der Ablauf des alternativen NHEJ hingegen erfolgt vermutlich ähnlich wie beim SSA-Modell.

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 Matrize initiiert 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 G_2-Phase des Zellzyklus, da hier das Schwesterchromatid als Matrize 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 Strangaustausch-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-Paraloge (Rad51B, Rad51C,

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 Matrize 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).

Im Falle eines DSB zwischen tandemartig wiederholten DNA-Sequenzen wird in somatischen Zellen der *single-strand annealing* (SSA) Reparatur-Mechanismus angewandt (Knoll *et al.*, 2014; Puchta, 2005). Dabei werden die Enden des DSB in 5‘-3‘-Richtung exonukleolytisch prozessiert und die komplementären ssDNA-Bereiche beider Enden hybridisieren. Verbleibende Überhänge werden anschließend zurechtgeschnitten bzw. ssDNA-Lücken wieder aufgefüllt und ligiert. Die zwischen den Tandem-Wiederholungen liegenden Bereiche der ursprünglichen Sequenz gehen somit verloren. Vom prinzipiellen Ablauf her ähnelt das SSA damit entfernt dem Mikrohomologien-nutzenden aNHEJ, mit dem Unterschied, dass die homologen Sequenzbereiche beim SSA wesentlich länger sind. In *Arabidopsis thaliana* wurden die drei Rad51-Paraloge Rad51B, Rad51D und Xrcc2 als individuelle Schlüsselfaktoren des SSA identifiziert (Serra *et al.*, 2013). Ihre genaue Wirkungsweise in diesem Prozess ist allerdings noch unklar.

Transläsionssynthese-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-Schadenstoleranz (*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 Transläsionssynthese (TLS)-DNA-Polymerasen, die die

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 Fehlinsertion pro 10^1-10^4 replizierten Nukleotiden mit unbeschädigter Matrizen-DNA, während *high fidelity* DNA-Polymerasen wie Polε oder Polδ nur alle 10^6-10^8 Nukleotide einen Fehler einfügen (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 Pflanzengeweben exprimiert (Garcia-Ortiz *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 Polα, 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.

DDT wird in Hefe- oder tierischen Zellen durch die Aktivität der E2-Ubiquitin-Ligase Rad6 und die E3-Ubiquitin-Ligase Rad18 reguliert (Andersen *et al.*, 2008; Ulrich, 2005). Diese bilden ein Heterodimer, das in Reaktion auf DNA-Läsionen oder blockierte Replikation PCNA (*proliferating cell nuclear antigen*) monoubiquitiniert. Durch die Ubiquitin-Bindemotive der TLS-Polymerasen wird ihre Affinität zu PCNA verstärkt und der TLS-Weg aktiviert. Neben dem TLS-Zweig existiert noch ein weiterer DDT-Zweig, bei dem PCNA durch einen Komplex bestehend aus Mms2, Ubc13 und der E3-Ubiquitin-Ligase Rad5 weiter polyubiquitiniert wird. Im weiteren Verlauf spielen vermutlich keine speziellen Polymerasen, die über die Läsion replizieren können, eine Rolle. Stattdessen findet ein Matritzentausch statt (Unk *et al.*, 2010). Bei dieser fehlerfreien Variante der DDT dient der neu synthetisierte Strang des unbeschädigten Schwesternduplexes als Matrize. Die pflanzlichen Homologe von Rad5 und Rad6 sind ebenfalls bekannt und haben vermutlich ähnliche Funktionen wie im tierischen oder Hefe-System (Strzalka *et al.*, 2013; Wang *et al.*, 2011; Zwirn *et al.*, 1997).

Geminiviren

Phytopathogene Geminiviren verursachen weltweit, aber vor allem in den Tropen und Subtropen, schwere Ernteausfälle (Moffat, 1999; Shepherd *et al.*, 2010; Varma & Malathi, 2003). Ihr breites Wirtsspektrum beinhaltet wirtschaftlich relevante Nutzpflanzen wie Mais, Weizen, Tomaten, Bohnen, Baumwolle, Maniok, Zuckerrüben und viele mehr. Ihren Namen verdanken Geminiviren ihrem Zwillingskapsid bestehend aus zwei unvollständigen Ikosaedern (Böttcher *et al.*, 2004; Zhang *et al.*, 2001b), in die ihr zirkuläres ssDNA-Genom verpackt wird. Sie werden durch Phloem-saugende Insekten wie Zwergzikaden, Buckelzikaden oder die Weiße Fliege *Bemisia tabaci* übertragen (Gray *et al.*, 2014; Whitfield *et al.*, 2015). Während ihres Infektionsverlaufs bleiben viele Geminiviren auf die kernhaltigen Phloem-Parenchymzellen oder Geleitzellen beschränkt und nur wenige Arten wurden im Schwamm- und Palisadenparenchym oder der Epidermis nachgewiesen (Morra & Petty, 2000; Wege, 2007; Wege *et al.*, 2001).

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 $1{,}3 \times 10^{-4}$ bis zu $1{,}6 \times 10^{-3}$ 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* (ClLCrV) dargestellt (Paprotka *et al.*, 2010).

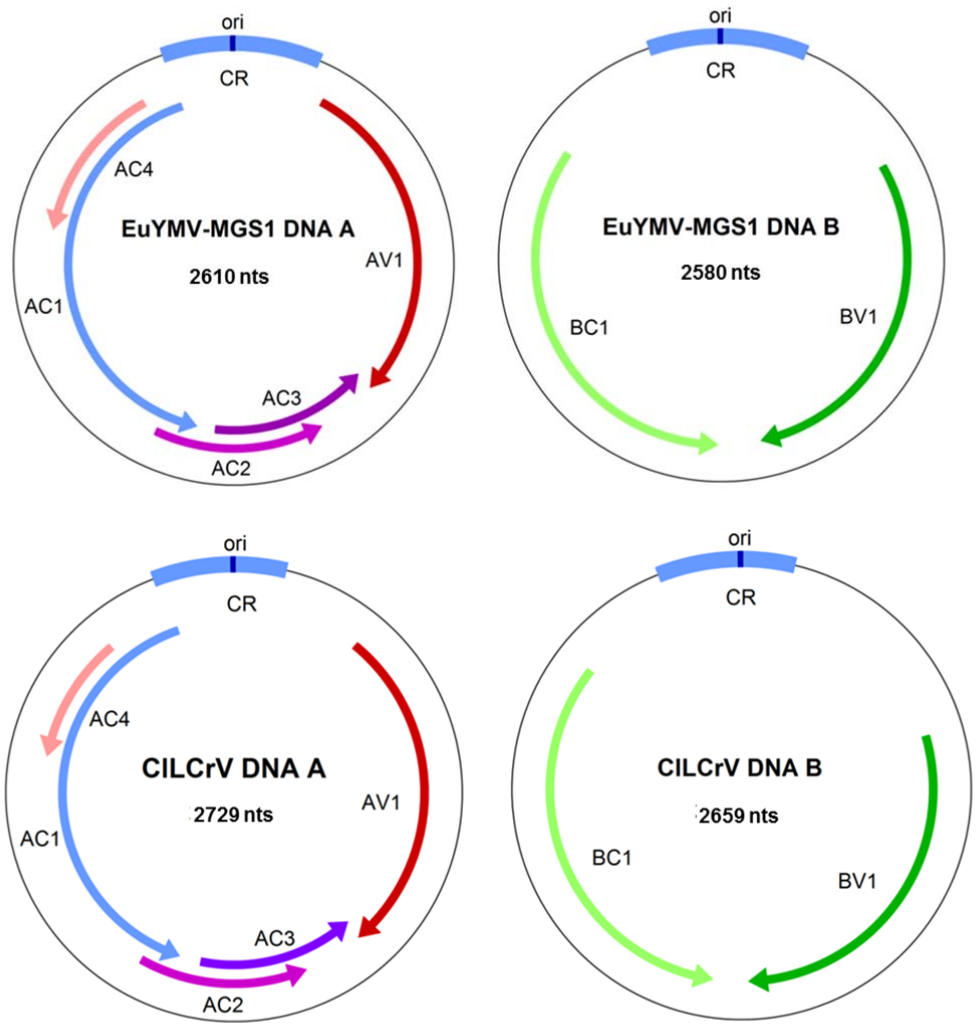

Abbildung 2: Genomorganisation der bipartiten Neuwelt-Geminiviren *Euphorbia yellow mosaic virus* (EuYMV, Isolat MGS1) und *Cleome leaf crumple virus* (ClLCrV) mit DNA A und DNA B. Die offenen Leserahmen sind jeweils in viraler (v) und komplementärer (c) Orientierung dargestellt: AV1 (*coat protein*; 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).

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 Polyadenylierungssignalen 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

Replikationsszyklus nach Übertragung und Disassemblierung der Viruspartikel ist jedoch die Komplementärstrangsynthese (*complementary strand replication;* CSR), bei der das ssDNA-Genom im Nukleus des Wirts zum Doppelstrang umgewandelt wird (Abbildung 3). Dies geschieht bei nahezu allen Geminiviren durch RNA-Primer, die mit Hilfe von Wirtsenzymen *de novo* synthetisiert werden (Saunders *et al.*, 1992). Die Ausnahme bilden Viren der Gattung *Mastrevirus*, bei denen RNA-Primer im Kapsid mitverpackt und übertragen werden (Donson *et al.*, 1987; Donson *et al.*, 1984). Da Geminiviren für keine DNA-Polymerase kodieren, ist die nachfolgende DNA-Synthese ebenso von Wirtsenzymen abhängig wie die Entfernung des RNA-Anteils durch RNase H und die Ligation des neu gebildeten Stranges durch Ligasen. Die dsDNA liegt nach Verpackung mit Wirtshistonen als kovalent geschlossenes zirkuläres (*covalently closed circular;* ccc) DNA-Molekül in Minichromosomen vor und dient in dieser Form als Matrize für Transkription und weitere Replikation (Abouzid *et al.*, 1988; Paprotka *et al.*, 2015; Pilartz & Jeske, 1992, 2003).

Die weitere geminivirale DNA-Amplifikation geschieht über RCR, *recombination-dependent replication* (RDR) und wieder nachfolgende CSR (Jeske, 2009). Bei der RCR bindet das durch den AC1 ORF kodierte *replication initiator protein* (Rep) sequenzspezifisch an wiederholte DNA-Motive (Iterons) in der IR bzw. CR (Arguello-Astorga *et al.*, 1994; Arguello-Astorga & Ruiz-Medrano, 2001). Dadurch unterdrückt es einerseits seine eigene Transkription und induziert andererseits durch einen Einzelstrangschnitt innerhalb der konservierten Nonanukleotid-Sequenz des ori (TATAATT|AC) die virale RCR (Heyraud-Nitschke *et al.*, 1995; Stanley, 1995). Dabei bindet Rep kovalent an das entstandene 5'-Ende der nun offen zirkulär (*open circular*, oc) vorliegenden DNA, während das freie 3'-Ende als Primer für die Replikation durch DNA-Polymerasen des Wirts dient. Währenddessen wird der Eltern-Strang schrittweise verdrängt, vermutlich durch Rep-Oligomere, welche 3'-5'-Helikase-Aktivität aufweisen (Choudhury *et al.*, 2006; Clerot & Bernardi, 2006). Nach einer oder mehreren Replikationsrunden katalysiert Rep durch seine *nicking/closing*-Domäne und die in der kovalenten Bindung gespeicherte Energie die Bildung eines zirkulären Einzelstrangs (Laufs *et al.*, 1995). Die neu entstandenen viralen ssDNA-Zirkel werden entweder in Virionen verpackt oder über CSR zu dsDNA konvertiert.

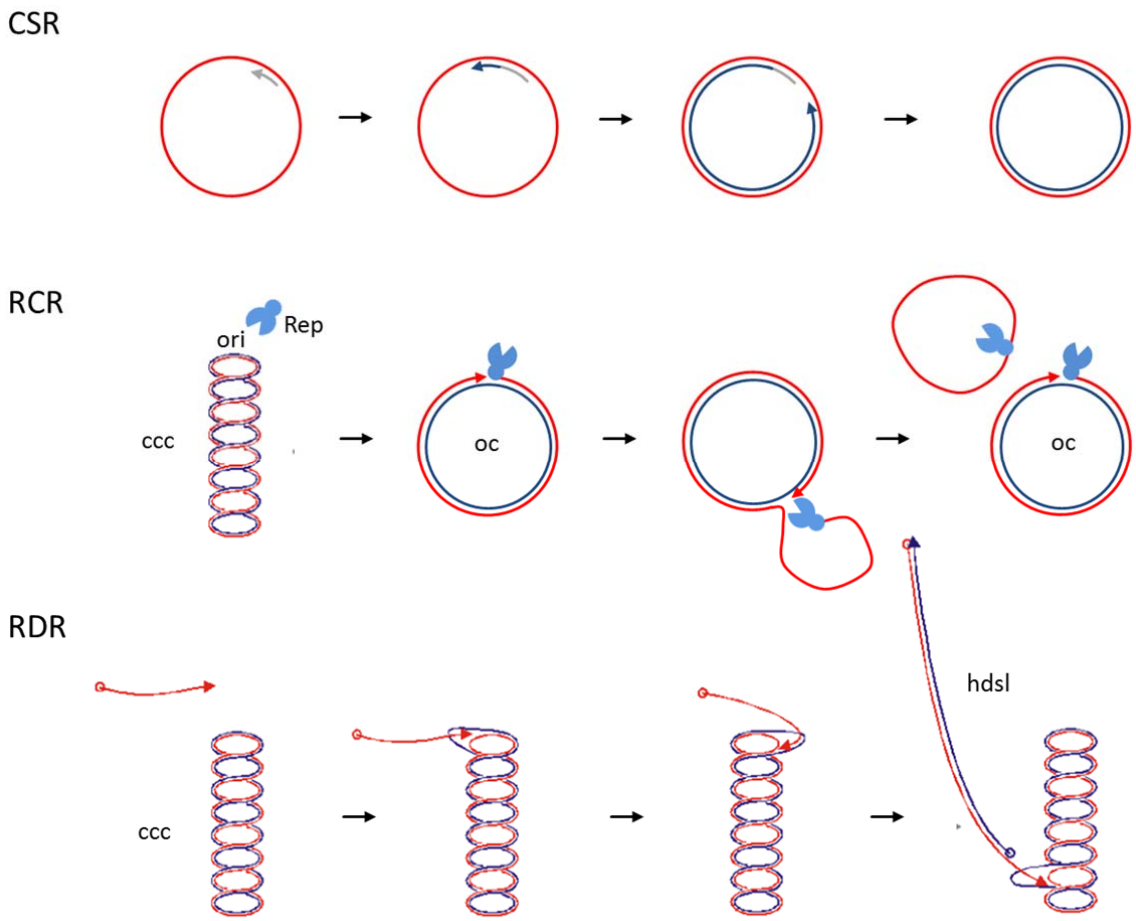

Abbildung 3: Geminivirale Replikation über *complementary strand replication* (CSR), *rolling circle replication* (RCR) und *recombination-dependent replication* (RDR) (Jeske, 2009; Jeske *et al.*, 2001). Durch Elongation der RNA-Primer wird bei der CSR die zirkuläre ssDNA zur dsDNA vervollständigt. Das geschlossen zirkuläre dsDNA-Molekül wird in Wirtshistone verpackt und bildet ein Minichromosom mit *covalently closed circular* (ccc) DNA. Bei der RCR entsteht durch einen Einzelstrangschnitt des viralen Reps im *origin of replication* (ori) eine *open circular* (oc) DNA, deren 3'-Ende verlängert wird, während Rep kovalent am 5'-Ende gebunden bleibt und die neu synthetisierte ssDNA nach einer oder mehreren Runden schließt. Bei der RDR dringen ssDNAs in homologe Sequenzen der cccDNA ein, die verlängert und gleichzeitig zur dsDNA vervollständigt werden. Als Produkt entstehen *heterogeneous double-stranded linear* (hdsl) DNA-Moleküle. Die DNA-Synthese ist in allen Fällen von Wirtsenzymen abhängig.

Der Ablauf der RDR hingegen ähnelt stark dem der HR, speziell dem SDSA-Reparatur-Modell. Kurze virale ssDNA-Stücke oder -Überhänge dringen in die cccDNA-Matrize ein, hybridisieren mit homologen Bereichen und fungieren als Primer für die DNA-Synthese (Erdmann *et al.*, 2010; Jeske *et al.*, 2001; Jovel *et al.*, 2007; Preiss & Jeske, 2003). Diese kurzen, unvollständigen ssDNA-Moleküle können entweder durch wirtsenzymatischen Abbau viraler DNA oder durch Kollision von bidirektionaler Transkription und unidirektionaler Replikation sowie dem infolgedessen ausgelösten vorzeitigen Abbruch der Replikation entstehen (Brewer, 1988). Ein ori als Replikationsstart ist bei der RDR nicht notwendig. Durch die von DNA-Polymerasen des Wirts katalysierte Strangverlängerung kommt es zur *loop migration*. Bereits währenddessen findet am naszierenden linearen Molekül die CSR statt. Da es keinen klar definierten Abbruch der DNA-Synthese gibt und auch hier mehrere Replikationsrunden durchlaufen werden können,

entstehen lineare dsDNA-Produkte von heterogener Länge (*heterogeneous double-stranded linear*; hdsl). Ob hdslDNA-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 (*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 (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 (*replication factor C*), was vermutlich der Zusammensetzung des Replikationskomplexes zur Vorbereitung der Virusreplikation dient (Castillo *et al.*, 2003; Luque *et al.*, 2002).

Neben Rep kodieren Geminiviren, abhängig von ihrer Genomorganisation, für bis zu sieben weitere Proteine. Das Hüllprotein (*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 (Briddon *et al.*, 1990; Höhnle *et al.*, 2001). Das AC2 oder *transcriptional activator protein* (TrAP) erfüllt ebenso wie Rep verschiedene Funktionen. Zum einen kann es die Transkription der Gene AV1 und BV1 transaktivieren (Sunter & Bisaro, 1991, 1992, 1997). Zum anderen wurde für einige Virusisolate eine *silencing suppressor* Funktion beschrieben (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 (Liu *et al.*, 2014; Soitamo *et al.*, 2012; Trinks *et al.*, 2005; Wang *et al.*, 2003) und wirkt hemmend auf die frühe virale Replikation (Krenz *et al.*, 2015). Das AC3 oder *replication enhancer protein* (REn) hingegen verstärkt die virale Replikation. Dies geschieht vermutlich durch Homo-Oligomerisierung und die Interaktion mit Rep, PCNA oder NAC-Transkriptionsfaktoren (Selth *et al.*, 2005; Settlage *et al.*, 2005). Das AC4 Protein ist maßgeblich an der Symptomausprägung beteiligt und wirkt bei einigen Virusisolaten als *silencing suppressor*, allerdings ist seine genaue Funktionsweise noch nicht vollständig geklärt (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 (Fondong, 2013; Jeske, 2009; Rojas *et al.*, 2005). Hierbei dient das *nuclear shuttle protein* (NSP) des BV1 Gens dem Transport zwischen Nukleus und Cytoplasma durch die Kernporen. Das *movement protein* (MP) des BC1 Gens ist in

der Plasmamembran oder in ER-abgeleiteten Vesikeln lokalisiert und bewerkstelligt die Zell-zu-Zell-Übertragung viraler DNA über Plasmodesmata. Zum genauen Ablauf der DNA-Übertragung stehen zum einen das Staffellauf-Modell (*relay race model*) und zum anderen das Paarlauf-Modell (*couple skating model*) zur Diskussion. Im Verlauf des Staffellauf-Modells wird dsDNA vom NSP ins Cytoplasma übermittelt und dort an das MP für den Zell-zu-Zell-Transport übergeben. Beim Paarlauf-Modell dagegen wird virale ssDNA durch das NSP aus dem Nukleus exportiert und ein vollständiger Komplex bestehend aus ssDNA, NSP und MP in die Nachbarzelle übertragen. Diese verschiedenen Transport-Hypothesen basieren auf den unterschiedlichen Bindungspräferenzen des NSP und MP von *Bean dwarf mosaic virus* (BDMV) und *Squash leaf curl virus* (SqLCV). NSP und MP von BDMV binden sowohl ssDNA als auch dsDNA (Rojas *et al.*, 1998). Dagegen ist ssDNA der bevorzugte Bindungspartner des NSP von SqLCV, während das MP nur schwach mit dsDNA, aber nicht mit ssDNA interagiert (Pascal *et al.*, 1994). Weitere Befunde für SqLCV, *Abutilon mosaic virus* (AbMV) und *Cabbage leaf curl virus* (CaLCuV) unterstützen das Paarlauf-Modell (Carvalho *et al.*, 2008; Frischmuth *et al.*, 2007; Sanderfoot *et al.*, 1996; Sanderfoot & Lazarowitz, 1995; Zhang *et al.*, 2001a). Darüber hinaus wurde ein drittes, alternatives intra- und interzelluläres Transport-Modell über Chloroplasten postuliert, welches auf der Interaktion des MP von AbMV mit dem plastidären Chaperon cpHsc70-1 und die durch Virusinfektion induzierte Ausbildung von Stromuli basiert (Krenz *et al.*, 2012; Krenz *et al.*, 2010). Unabhängig vom jeweiligen Transport-Modell ist letztendlich immer noch unklar, welche DNA-Formen transportiert werden. Interessanterweise bilden NSP und MP von AbMV Komplexe mit *supercoiled* Plasmid-DNA (Hehnle *et al.*, 2004) und NSP und MP von BDMV interagieren mit Histon H3 (Zhou *et al.*, 2011).

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.

Auf molekularer Ebene kann dies durch den RDR-Mechanismus realisiert werden. Durch die Ähnlichkeit zum SDSA-Modell wären Wirtsfaktoren des HR-Reparaturwegs ebenso für die viralen Rekombinationsprozesse bestens geeignet. Ein interessanter Hinweis für die direkte Verbindung von HR-Prozessen und Virusvermehrung könnte die Interaktion von Rep mit den HR-Faktoren Rad51 und Rad54 von *A. thaliana* bzw. *S. cerevisiae* darstellen (Kaliappan *et al.*, 2012; Suyal *et al.*, 2013). Allerdings wurde bisher noch keine direkte Funktion dieser Faktoren bei der Virusreplikation *in planta* nachgewiesen. Weiterhin wurde durch eine umfangreiche Transkriptionsanalyse in *A. thaliana* gezeigt, dass einige DSB-Sensor- und -Reparaturfaktoren wie Rad17, Rad50 oder Ku80 durch Geminivirusinfektion signifikant hochreguliert wurden (Ascencio-Ibanez *et al.*, 2008). Da die Transkriptionsprofile nach Virusinfektion und DNA-schädigenden Behandlungen allerdings nur begrenzt überlappten, wird angenommen, dass eine geminivirale Infektion keine allgemeine genotoxische Stressantwort in der Pflanze auslöst. Die Hochregulierung einzelner Faktoren könnte deshalb einen spezifischen viralen Einfluss auf pflanzliche DNA-Reparatursysteme andeuten und die Rolle einzelner Reparaturwege oder -faktoren während der Virusreplikation betonen. Alternativ könnte dadurch auch die Beteiligung der jeweiligen Faktoren an Abwehrmechanismen der Wirtspflanze oder ein unspezifischer Nebeneffekt der Virusinfektion angezeigt werden.

Neben den DSB-Reparaturfaktoren könnten TLS-Polymerasen bei der geminiviralen Replikation, vor allem während der CSR, von Bedeutung sein. Bevor Rep auf den Zellzyklus Einfluss nehmen kann und Replikationsenzyme wie DNA Polymerasen α, ε und δ zur Verfügung stehen (Hanley-Bowdoin *et al.*, 2004; Nagar *et al.*, 1995), muss in der ruhenden Phloemzelle die initiale virale CSR stattfinden, um die virale Proteinexpression überhaupt zu ermöglichen. Dafür kommen ausschließlich DNA-Polymerasen in Frage, die in ausdifferenzierten Geweben exprimiert werden, was auf alle vier der in Pflanzen charakterisierten TLS-Polymerasen zutrifft (Garcia-Ortiz *et al.*, 2004; Nakagawa *et al.*, 2011; Sakamoto *et al.*, 2003; Santiago *et al.*, 2008; Takahashi *et al.*, 2005). Zudem lassen sich die hohen Mutationsraten geminiviraler Genome höchstwahrscheinlich nicht durch Rekombinationsereignisse und nur teilweise durch die Anfälligkeit von ssDNA auf oxidativen Schaden erklären (Monjane *et al.*, 2012). Ein Beitrag der fehleranfälligen TLS-Polymerasen zur geminiviralen CSR wäre daher plausibel.

Diese Arbeit befasst sich mit dem Einfluss der geminiviralen Infektion auf die HR-Reparaturwege der Wirtspflanze und den spezifischen Beitrag von DSB-Reparaturfaktoren und TLS-Polymerasen zur geminiviralen Vermehrung. Hierfür wurde zum einen die Häufigkeit von Rekombinationsereignissen nach geminiviraler Infektion unter Verwendung von *A. thaliana* Rekombinationsreporterlinien gewebespezifisch analysiert. Weiterhin wurde die Auswirkung des *knock-out* verschiedener Kandidatengene der TLS- oder DSB-Reparatur-Wege auf den geminiviralen Infektionsverlauf und die einzelnen Replikationsformen mit Hilfe von *A. thaliana*

T-DNA-Insertionsmutantenlinien untersucht. Die beiden Neuwelt-Begomoviren EuYMV und ClLCrV sind zwei von wenigen *A. thaliana*-infizierenden Geminiviren. Sie induzieren dabei nur relativ milde Symptome und dienten daher als Grundlage dieser Untersuchung.

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

Dieser Teil der Arbeit befasst sich mit der Fragestellung, ob eine geminivirale Infektion zu einer Zunahme von HR-Ereignissen in somatischen Zellen der Wirtspflanze führt. Generell können verschiedene abiotische und biotische Stressoren die Häufigkeit von HR-Ereignissen in somatischen Zellen von Pflanzen erhöhen (Kovalchuk *et al.*, 2003; Lucht *et al.*, 2002; Molinier *et al.*, 2005; Rahavi & Kovalchuk, 2013). Dies geschieht vermutlich als Reaktion auf genotoxischen Stress durch eine induzierte DNA-Schädigung oder die Beeinflussung der damit verbundenen Signalwege. Die Stressoren können die DNA-Schädigung entweder direkt verursachen oder sekundär durch die Entstehung von reaktiven Sauerstoffspezies bedingen. Diese Stressreaktion ist nicht nur auf die betroffenen Gewebe beschränkt und teilweise noch in der nächsten Generation nachweisbar (Boyko & Kovalchuk, 2010; Molinier *et al.*, 2006). Um zu überprüfen, ob die geminivirale Infektion eine unspezifische Stressantwort in der gesamten Pflanze induziert oder einen spezifischen Einfluss im betroffenen Gewebe ausübt, wurde berücksichtigt, dass die meisten Geminiviren auf das Phloem-Gewebe limitiert sind. Daher wurde bei dieser Analyse zwischen HR im Leitgewebe und anderen Geweben differenziert. Zum Nachweis der HR-Ereignisse dienten transgene *A. thaliana* Reporterlinien, die verschiedene Konstrukte des Reportergens der β-Glucuronidase (GUS) enthalten. Die GUS-Sequenz ist dabei aufgetrennt in zwei nichtfunktionale Teile, die aber überlappende, homologe Sequenzen besitzen. Die Aktivität der β-Glucuronidase kann durch HR dieser überlappenden Sequenzen wiederhergestellt werden. Durch histochemische Färbung des Pflanzengewebes mit dem Substrat X-Gluc werden die einzelnen Rekombinationsereignisse als blaue Punkte oder Sektoren sichtbar und quantifizierbar. Vier verschiedene Linien (651, 1445, IC9C, 11) wurden verwendet, die sich jeweils durch die Länge oder die Orientierung der homologen Sequenzüberlappung, den Insertionsort im Genom oder den Ökotyp-Hintergrund unterschieden. Dadurch weisen die einzelnen Linien ein verschiedenes Grundniveau an HR-Ereignissen und ein unterschiedliches Antwortverhalten für bestimmte Stressoren auf (Pecinka *et al.*, 2009).

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 ClLCrV biolistisch inokuliert. Als Vergleichsgruppen für den nachfolgenden

histochemischen Test dienten *mock*-inokulierte Pflanzen, die ausschließlich mit der DNA B des jeweiligen Virusisolats behandelt und daher zwar durch den Partikelbeschuss gleichermaßen mechanisch gestresst, aber nicht infiziert wurden. Der Nachweis der Infektion erfolgte 14 Tage nach der Inokulation (*days post inoculation*, dpi) durch RCA und *restriction fragment length polymorphism* (RFLP). Keine der Kombinationen von transgener Linie und Virusisolat zeigte im histochemischen Test 21 dpi einen signifikanten Unterschied der Rekombinationshäufigkeit zu den *mock*-inokulierten Pflanzen, weder insgesamt noch spezifisch für das Leitgewebe. Hierbei wurden jeweils drei Blätter verschiedenen Alters pro Pflanze verwendet. In einer zweiten Versuchsreihe wurden die Pflanzen bereits im 4-6-Blatt Stadium inokuliert, um die komplette Rosette 21 dpi histochemisch zu testen und so insgesamt mehr HR-Ereignisse auswerten zu können. Dieses Vorgehen reduzierte die Infektionsraten für alle Kombinationen zwar deutlich, allerdings war die Anzahl der EuYMV-MGS1-infizierten Pflanzen der Linien 651 und 1445 ausreichend, um die histochemische Analyse durchzuführen und zuverlässig statistisch auszuwerten. Die Linie 1445 zeigte hierbei eine signifikante Zunahme der Leitbündel-assoziierten HR-Ereignisse durch die EuYMV-MGS1-Infektion, während die Anzahl der HR-Ereignisse in anderen Geweben unverändert blieb. Line 651 zeigte einen ähnlichen, allerdings nicht signifikanten Trend und insgesamt deutlich weniger HR-Ereignisse, unabhängig von der Behandlung. Um einen groben Abgleich mit anderen Stressoren ziehen zu können, wurde ein Hitzestress-Experiment mit der Linie 1445 durchgeführt. Übereinstimmend mit der Literatur (Pecinka *et al.*, 2009), wurde ein etwa dreifacher Anstieg der gesamten HR-Ereignisse durch den Hitzestress ermittelt. Sowohl die HR-Ereignisse der Leitbündel-assoziierten als auch die der anderen Gewebe zeigten eine Zunahme, allerdings war diese nur in der Summe aller HR-Signale signifikant. Zwei unabhängige biologische Wiederholungen des Inokulationsexperiments bestätigten die spezifische, signifikante Zunahme der Leitgewebe-assoziierten HR-Ereignisse für Linie 1445 durch die EuYMV-MGS1-Infektion. Die absoluten Werte variierten zwar von Experiment zu Experiment, die relative Zunahme war allerdings durchgehend stabil bei etwa dem Fünffachen der *mock*-inokulierten Pflanzen. Weiterhin wurde durch *tissue blot* und *in situ* Hybridisierungen bestätigt, dass EuYMV-MGS1 in *A. thaliana* auf das Phloem beschränkt ist.

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-

Häufigkeit in *Nicotiana tabacum* (Kovalchuk *et al.*, 2003) und eine 1,6-fache in *A. thaliana* (Yao *et al.*, 2013; Yao *et al.*, 2011). Dieser Effekt war nicht nur auf das infizierte Gewebe beschränkt, sondern wurde vermutlich durch eine systemische Signalübertragung durch z.B. reaktive Sauerstoffspezies oder Salicylsäure an nicht-infiziertes Pflanzengewebe weitergegeben. In den genannten Studien wurde dies durch Pfropfungsexperimente oder Entfernen der lokal infizierten Blätter, bevor die systemische Virusinfektion eintreten konnte, angedeutet. Die Zunahme der HR-Ereignisse durch EuYMV-MGS1-Infektion war hingegen auf das Leitgewebe beschränkt. Eine geminivirale Infektion löst außerdem wahrscheinlich keine allgemeine genotoxische Stressantwort in der Pflanze aus, da die Transkriptionsprofile nach geminiviraler Infektion und DNA-schädigenden Behandlungen nur begrenzt überlappten (Ascencio-Ibanez *et al.*, 2008). Die Effekte durch Tobamoviren beruhen daher vermutlich auf anderen Mechanismen als die geminiviral induzierten. Die verstärkte HR-Häufigkeit im Leitgewebe nach EuYMV-Infektion könnte durch die Verbindung von Re-Replikation in der infizierten Wirtszelle (Nagar *et al.*, 2002) und der Rekrutierung von HR-Enzymen wie Rad51 und Rad54 durch Rep (Kaliappan *et al.*, 2012; Suyal *et al.*, 2013) schlüssig erklärt werden. Eine allgemeine Verstärkung der HR durch Geminivirusinfektionen könnte darüber hinaus mit den häufigen Rekombinationsereignissen geminiviraler Genome in Zusammenhang stehen (van der Walt *et al.*, 2009).

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* (*Sc*Rad54) 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 *Sc*Rad54 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 ClLCrV eingehend überprüft.

Die systemische Infektion wurde zu drei verschiedenen Zeitpunkten (7, 14, 21 dpi) durch eindimensionale (1D) und zweidimensionale (2D) Agarosegelelektrophorese (AGE) gefolgt von Southern-Hybridisierung analysiert. Jeweils drei unabhängige Inokulationsexperimente wurden mit jeweils zehn inokulierten Pflanzen je Virus und pflanzlichem Genotyp durchgeführt. Die 1D AGE diente der allgemeinen Kontrolle des Infektionsverlaufs, der Infektionsraten und der Menge der vorwiegenden viralen DNA-Formen von ssDNA, cccDNA und ocDNA. Die EuYMV- sowie die ClLCrV-Infektionen waren 7 dpi durch das Auftreten von viraler DNA in einigen inokulierten Pflanzen mit variierenden Intensitäten zwischen einzelnen Proben nachweisbar, jedoch ohne signifikante Unterschiede zwischen *rad54*- und wt-Pflanzen. Das Aufkommen der systemischen Infektion war demzufolge ein stochastischer Prozess. Unabhängig vom Genotyp war die systemische Infektion beider Viren 14 dpi in allen Pflanzen voll etabliert. Die verschiedenen viralen DNA-Formen waren für alle drei ClLCrV-Inokulationsreihen und für zwei von drei EuYMV-Inokulationsreihen zu gleichen Mengen vorhanden. Lediglich bei einer Inokulationsreihe war die EuYMV ocDNA in *rad54*-Pflanzen leicht vermindert im Vergleich zum wt. Die viralen DNA-Titer beider Infektionen waren auch 21 dpi noch hoch und bei keiner Inokulationsreihe war ein Unterschied zwischen den Genotypen festzustellen. Die Infektionsraten beider Viren lagen durchgehend bei 100 %, sowohl für *rad54*- als auch für wt- Pflanzen. Symptome der Virusinfektionen wie leichtes Rollen (EuYMV, ClLCrV) oder eine Gelbfleckung (EuYMV) der Blätter waren sowohl in der Ausprägung als auch im Zeitpunkt des Einsetzens (9-11 dpi) nicht unterscheidbar zwischen beiden pflanzlichen Genotypen.

Durch 2D AGE kombiniert mit Southern-Hybridisierung war es zusätzlich möglich, Intermediate der Virusreplikation sichtbar zu machen. Bei dieser Methode werden die verschiedenen DNA-Formen in der ersten Dimension hauptsächlich nach ihrer Masse aufgetrennt. In der zweiten Dimension wird durch Zugabe des Interkalators Chloroquin die Mobilität von dsDNA zusätzlich verringert, positive superhelikale Überdrehungen werden in die negativ überspiralisierte cccDNA eingefügt, und so die einzelnen Topoisomere der cccDNA sichtbar gemacht (Snapka *et al.*, 1991). Die verschiedenen geminiviralen DNA-Formen und Replikationsintermediate ergeben nach der Auftrennung ein charakteristisches Muster aus Linien, Bögen und Punkten (Jeske *et al.*, 2001; Preiss & Jeske, 2003). Die Intermediate von CSR, RCR und RDR wurden für beide Viren 7 dpi und 14 dpi ohne merkliche Mengenunterschiede zwischen *rad54*- und wt-Pflanzen nachgewiesen. Dabei hatten sich 14 dpi bereits deutlich mehr Endprodukte der verschiedenen Replikationsmodi angehäuft, jedoch ebenso ohne Unterschied zwischen den Genotypen. Die

Proben des Inokulationsexperiments, bei dem bereits während der 1D AGE-Analyse eine leichte Reduktion der EuYMV ocDNA in *rad54*-Pflanzen detektiert wurde, zeigten auch hier eine Reduktion der EuYMV ocDNA der *rad54*-Pflanzen. Bei dieser Inokulationsreihe waren allerdings für beide Genotypen keine Intermediate von CSR oder RCR detektierbar, auf die diese Reduktion zurückzuführen wäre. Die RDR-Intermediate waren hingegen für beide Genotypen zu gleichen Mengen vorhanden. Bei allen Inokulationsreihen wurden 21 dpi gleiche Mengen an RDR-Intermediaten und Endprodukten der Replikation beider Viren sowohl in *rad54-* als auch in wt-Pflanzen nachgewiesen.

Zusammenfassend zeigen diese Ergebnisse, dass Rad54 keine essentielle Rolle bei der geminiviralen Replikation *in planta* spielt. Weder RDR noch RCR von EuYMV und ClLCrV wurden durch den Ausfall von Rad54 in *A. thaliana* beeinträchtigt. Die dazu im Widerspruch stehenden Beobachtungen von Kaliappan *et al.* (2012) mit ToLCNDV-basierten Konstrukten könnten durch einen unterschiedlichen Bedarf der verschiedenen Virusspezies für Rad54 erklärt werden. Allerdings wiesen die Untersuchungen von Kaliappan *et al.* (2012) deutliche Mängel auf, die wahrscheinlich ursächlich für diese abweichenden Beobachtungen waren. Beispielsweise wurde eine Kombination aus transienter Agroinfiltration eines viralen Konstrukts mit zwei oris (*bitmer*) und PCR-Nachweis benutzt, um im infiltrierten Blatt virale, monomere RCR-Produkte nachzuweisen. Dabei wurde außer Acht gelassen, dass das virale *bitmer*-Infiltrationskonstrukt bereits in den *Agrobacterium*-Zellen zu Monomeren freigesetzt wird (Rigden *et al.*, 1996; Selth *et al.*, 2002). Diese bakteriellen Monomere können dann über PCR im infiltrierten Blatt detektiert werden und sind kein Beweis für eine *in planta* RCR. Obwohl hier keine essentielle Funktion von Rad54 für die geminivirale Replikation bestätigt wurde, ist es nicht ausgeschlossen, dass Rad54 durch die Interaktion mit Rep zur Infektion beiträgt. Diese könnte allerdings entbehrlich sein oder durch die Funktionen anderer HR-Enzyme komplementiert werden. Die Kombination aus systemischer EuYMV- oder ClLCrV-Infektion von *knock-out*-Linien der Modellpflanze *A. thaliana*, 1D und 2D AGE hat sich weiterhin als geeignete Methode zur Analyse des Zusammenspiels von Wirtsfaktoren und geminiviraler Replikation erwiesen.

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 *Sc*Rad54, eine Interaktion mit dem Rep des MYMIV nachgewiesen und der Ausfall von *Sc*Rad51 wurde durch *At*Rad51 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 essentiellen Einfluss auf den Infektionsverlauf oder die Akkumulation der verschiedenen viralen DNA-Formen oder Replikationsintermediate. 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 *At*Rad51 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 Matrize 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.

Der Ausfall von Ku80, Teil des DSB-bindenden Ku70/Ku80 Heterodimers und damit einer der Schlüsselfaktoren des NHEJ, verursachte einen außergewöhnlichen Effekt: Zu frühen Zeitpunkten der systemischen Infektion (7 dpi) enthielten *ku80*-Pflanzen signifikant mehr virale DNA (oc, ccc, ssDNA) im Vergleich zu wt-Pflanzen. Ku80 wirkt folglich der Virusvermehrung entgegen und könnte beispielsweise bei einer Abwehrreaktion der Pflanze gegen virale Pathogene beteiligt sein. Mit Fortschreiten der Virusinfektion (14 dpi) schwand dieser Unterschied und *ku80*- und wt-Pflanzen enthielten ähnliche Mengen viraler DNA. Zum späten Zeitpunkt der systemischen Infektion (21 dpi) lagen bei zwei von drei unabhängigen Inokulationsexperimenten einige virale DNA-Formen wie ssDNA oder cccDNA und interessanterweise auch monomere lineare dsDNA wieder erhöht in *ku80*-Pflanzen vor. Bei der 2D AGE-Analyse wurde ebenfalls eine leichte Erhöhung der linearen dsDNA in *ku80*-Pflanzen festgestellt. Die viralen Replikationsintermediate wurden hingegen ohne Unterschiede zwischen *ku80*- und wt-Pflanzen nachgewiesen. Diese Beobachtungen könnten insgesamt darauf hinweisen, dass Ku80 als Teil des NHEJ-Reparaturwegs benutzt wird, um virale lineare dsDNA zu verbinden oder zu zirkularisieren. Diese viralen Reparaturprodukte würden jedoch nicht zwangsläufig die virale Replikation fördern, sondern könnten ihr sogar entgegenwirken und die inhibierende Wirkung von Ku80 auf die EuYMV-Infektion erklären. NHEJ ist ein relativ fehleranfälliger Reparaturweg, und kann Insertionen, Deletionen und dadurch den Verlust genetischer Information verursachen (Mannuss *et al.*, 2012; Puchta, 2005). Im Gegensatz zu eukaryotischen Genomen mit umfangreichen nichtkodierenden Bereichen, würde ein stark verdichtetes Genom wie das geminivirale höchstwahrscheinlich unter dieser Aktivität überproportional leiden. Die Anhäufung fehlerhafter offener Leserahmen und deren Expression könnte wiederum nachfolgende Effekte der Pflanzenabwehr bewirken. Die Vermeidung dessen durch den Ausfall von Ku80 könnte daher für die geminivirale Infektion vorteilhaft sein. Dieser Weg der viralen DNA-Reparatur würde in starkem Kontrast zu der fehlerfreien Alternative über Rad51D und SSA stehen.

In Säugern 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

Herpesvirus 1 identifiziert (Ferguson *et al.*, 2012). Dabei wirkt der DNA-PK-Komplex als *pattern-recognition receptor* (PRR) und aktiviert die angeborene Immunantwort über den *interferon regulatory factor 3* (IRF-3) Transkriptionsfaktor. Der Ku70/Ku80-Komplex in Pflanzen könnte daher ebenso als Sensor für virale DNA im Cytoplasma dienen und eine Abwehrreaktion induzieren. In Pflanzen sind allerdings keine Homologe der DNA-PKcs oder nachfolgende Interferon-ähnliche Antworten charakterisiert. Daher sind weitere Untersuchungen notwendig, um den Mechanismus hinter dem antiviralen Effekt von Ku80 aufzuklären. Vielversprechende Hinweise könnte die Interaktion von Ku70/Ku80 mit der *Werner-like exonuclease* (Wex) in *A. thaliana* liefern (Li *et al.*, 2005). Wex ist das pflanzliche Homolog der Exonuklease-Domäne des humanen Werner-Proteins, welches zur Familie der RecQ-Helikasen gehört (Plchova *et al.*, 2003). Diese können die DSB-Reparatur regulieren, indem sie HR-Intermediate entweder unterbrechen oder sinnvoll auflösen (Knoll & Puchta, 2011). Wex interagiert mit RecQ2, welches D-Loops bei der HR unterbricht (Hartung *et al.*, 2000; Kobbe *et al.*, 2008). Es wäre daher denkbar, dass Ku70/Ku80, Wex und RecQ2 kooperativ der Reparatur über HR entgegenwirken und auf den NHEJ Reparatur-Modus umschalten, was die bereits erwähnten, nachteiligen Effekte für virale Genome zur Folge hätte. Wex und dem Werner-Homolog von *Caenorhabditis elegans* (Mut-7) wurde überdies eine RNase D-Aktivität und eine Funktion während des *posttranscriptional gene silencing* (PTGS) bzw. bei der RNA-Interferenz zugeschrieben (Glazov *et al.*, 2003; Ketting *et al.*, 1999; Tops *et al.*, 2005). Ku70/Ku80 könnte daher eine neue Art von cytoplasmatischem PRR gegen virale DNA in Pflanzen darstellen und über Wex eine nachfolgende PTGS-Antwort als Abwehrreaktion induzieren.

Die Beteiligung von Transläsionssynthese-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 Replikationsungenauigkeit könnte dabei die hohen Mutationsraten geminiviraler 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 (kodiert 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

sichtbar zu machen, wurde mit einzelnen Pflanzenproben der *polh-1*-, der *rev1-2*- und der jeweiligen wt-Linien, die 7 dpi größere Mengen viraler DNA enthielten, eine 2D AGE-Analyse durchgeführt. Sowohl Intermediate der RCR, RDR und CSR als auch die Replikationsprodukte waren ohne oder nur mit geringfügigen Unterschieden zwischen den verschiedenen Proben vorhanden. Die viralen DNA-Gehalte von *rev3-2* und deren wt-Schwester-Pflanzen waren 7 dpi zu gering für eine Auswertung mit 2D AGE. Zusätzlich wurden *polh-1*, *rev3-2* und die zugehörigen wt-Linien biolistisch mit ClLCrV inokuliert. Der Infektionsverlauf oder die viralen DNA-Gehalte waren hier ebenfalls nicht unterscheidbar zwischen den verschiedenen Genotypen.

Da diese Beobachtungen auf redundante Funktionen der einzelnen TLS-Polymerasen hindeuten könnten, wurden zwei Pflanzenlinien mit einem kombinierten *knock-out* von Polη/Polζ nach biolistischer Inokulation mit EuYMV und ClLCrV untersucht. Die Linie *rev3-1 polh-1* wurde von A. Sakamoto zur Verfügung gestellt (Nakagawa *et al.*, 2011; Sakamoto *et al.*, 2003) und die Linie *rev3-2 polh-1* wurde durch Kreuzung der beiden einzelnen *knock-out*-Linien hergestellt. Eine *knock-out*-Linie des *A. thaliana*-Homologs (Ubc2) von Rad6, welches die TLS-Aktivität über Monoubiquitinierung von PCNA reguliert, wurde gleichermaßen überprüft. Auch bei diesen Versuchen waren Infektionsverlauf und die viralen DNA-Gehalte zwischen *knock-out* und wt-Linien identisch.

Im Gegensatz zu den etablierten Linien *polh-1*, *rev3-2* und *rev1-2*, stand für POLK keine charakterisierte *knock-out*-Linie zur Verfügung. Daher wurden die Datenbanken für Insertionsmutantenlinien durchsucht und die Linie Salk_081715 (*polk-1*) identifiziert, welche eine T-DNA-Insertion im Intron zwischen dem 13. und 14. Exon des POLK-Gens trägt. Ein RT (Reverse Transkriptase)-PCR-Experiment mit Primern, die die Insertion flankierten, bestätigte die Abwesenheit eines vollständigen POLK-Transkripts in *polk-1*. Allerdings wurde hierbei sowohl bei wt- als auch in *polk-1*-Pflanzen eine zusätzliche Bande detektiert, die eine neue alternative Spleißvariante von POLK darstellen könnte. Darüber hinaus wurde durch ein weiteres RT-PCR-Experiment mit Primern, die stromaufwärts der Insertion binden, gezeigt, dass verkürzte POLK-Transkripte sowohl in wt- als auch in *polk-1*-Pflanzen vorliegen. Bereits in einer früheren Studie wurden mindestens drei Spleißvarianten von *At*POLK nachgewiesen (Garcia-Ortiz *et al.*, 2004). Im Gegensatz zu der vollständigen Polκ-Form von 671 AS, würden die zwei alternativen Transkripte für N-terminale Polκ-Varianten von 345 AS bzw. 184 AS kodieren. Alle hochkonservierten DNA-Polymerasedomänen wären zwar in den ersten 345 AS erhalten, mutmaßliche Zinkfinger- und PCNA-Bindedomänen hingegen würden verloren gehen (Garcia-Ortiz *et al.*, 2004). Ob diese verkürzten Proteinvarianten tatsächlich Polymerase-Aktivität besitzen und exprimiert werden, ist ungeklärt. Eine rekombinante N-terminale *At*Polκ-Variante von 478 AS wies indes DNA-Polymerase-Aktivität auf (Garcia-Ortiz *et al.*, 2004; Garcia-Ortiz *et al.*, 2007). Um die Expression von *At*Polκ und möglichen *At*Polκ-Varianten in der wt- und *polk-1*-

Linie zu überprüfen, wurde eine *Western blot*-Analyse mit einem Antiserum gegen den N-terminalen Teil des humanen Polκ-Proteins durchgeführt. Hierbei war kein Unterschied der Signale zwischen wt- und *polk-1*-Pflanzen erkennbar. Zwar konnte keine der Banden eindeutig dem vollständigen *At*Polκ-Protein zugeordnet werden, allerdings entsprach ein Signal in etwa der erwarteten Molekülmasse von 38 kD der verkürzten *At*Polκ-Variante von 345 AS. Demzufolge ist es durchaus möglich, dass Polκ-Varianten in der wt- als auch in der *polk-1*-Linie exprimiert werden. Es kann außerdem nicht komplett ausgeschlossen werden, dass diese noch Polymerase-Aktivität besitzen. Nichtsdestotrotz wurden beide Linien biolistisch mit EuYMV und ClLCrV inokuliert. Wie zuvor für die anderen *knock-out*-Linien wurden keine signifikanten Unterschiede der viralen DNA-Akkumulation nachgewiesen. Eine zweite Insertionsmutantenlinie (Flag_566E01: *polk-2*) wurde aufgrund dieser Sachlage identifiziert und analysiert. Diese trägt eine T-DNA-Insertion im Intron zwischen dem 1. und 2. Exon des POLK-Gens. Die Sequenzierung der T-DNA/POLK-Übergänge zeigte, dass durch die T-DNA-Insertion eine Deletion von 12 bp des Introns zwischen Position 181 und 194 von POLK verursacht wurde. Durch eine RT-PCR-Analyse mit Primern, die die Insertion flankierten, wurde nachgewiesen, dass die POLK-Transkription in *polk-2*-Pflanzen stark reduziert, aber vermutlich nicht komplett unterbunden wurde. Die Linie *polk-2* und ihre wt-Schwesterpflanzen wurden, wie nachfolgend erläutert, durch Insektenübertragungsexperimente untersucht.

Unter natürlichen Bedingungen wird virale DNA in einzelsträngiger Form in die Wirtspflanzen eingebracht. Die biolistische Inokulation fand hingegen bisher mit doppelsträngigen RCA-Produkten statt. Da durch diese Inokula möglicherweise der Bedarf für TLS-Polymerasen bei der initialen CSR umgangen werden könnte, wurden alternative Inokulationsmethoden mit EuYMV- und ClLCrV- ssDNA an wt *A. thaliana*- und *Nicotiana benthamiana*-Pflanzen erprobt. Zum einen wurde die virale ssDNA aus Viruspartikeln isoliert und anstelle der RCA-Produkte bei der biolistischen Inokulation eingesetzt. Zum anderen wurden infektiöse *Agrobacterium tumefaciens*-Klone der einzelnen viralen Genom-Komponenten hergestellt. Allerdings lagen die Infektionsraten mit diesen alternativen Inokulationsverfahren in wt *A. thaliana*-Pflanzen jeweils bei 40 % oder darunter. Daher wurde, erstmalig für *Arabidopsis*, die geminivirale Übertragung durch *B. tabaci* etabliert. Sowohl EuYMV als auch ClLCrV waren von und auf *A. thaliana*-Pflanzen übertragbar. Durch EuYMV wurde eine höhere Infektionsrate (100 %) als mit ClLCrV (40%) erzielt. Die nachfolgenden Insektenübertragungs- experimente mit *knock-out*-Linien wurden daher mit EuYMV fortgeführt.

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-Schwesterlinie von *rev3-2* der Übertragung von EuYMV durch *B. tabaci* ausgesetzt. Infektionsverlauf und die viralen DNA-Gehalte waren nicht unterscheidbar zwischen

den verschiedenen Genotypen. Die Linie *polk-2* und ihre wt-Schwesterlinie besitzen einen anderen Ökotyp-Hintergrund und wurden daher in einem separaten Übertragungsexperiment getestet. Die Infektionsraten waren hierbei Genotyp-übergreifend so gering, dass kein zuverlässiger Vergleich der viralen Infektion von wt- und *polk-2*-Linie möglich war. Da allerdings einzelne Individuen der *polk-2*-Linie infiziert waren, wurde deutlich, dass die geminivirale Replikation in der Abwesenheit Polκ stattfinden kann.

Die bisherigen Inokulationsexperimente könnten darauf hindeuten, dass TLS-Polymerasen nicht an der geminiviralen Replikation beteiligt sind. Alternativ wäre es denkbar, dass Auswirkungen auf die virale Replikation durch redundante Aktivitäten der anderen TLS-Polymerasen überdeckt werden und die angewandten Methoden nicht ausreichen, um eventuelle subtile Unterschiede nachzuweisen. Da TLS-Polymerasen hohe Fehlerraten und spezifische Fehlerarten aufweisen (Arana & Kunkel, 2010), wurde eine umfangreiche Sequenzanalyse durchgeführt, um über mögliche Veränderungen der Fehlerraten, -verteilungen oder -spezifitäten eine Beteiligung von TLS-Polymerasen an der geminiviralen Replikation nachweisen zu können. Hierfür wurde die durch RCA vermehrte EuYMV-DNA aus wt- und *rev3-1 polh-1*-Pflanzen des Insektenübertragungsexperiments einem *deep sequencin*g durch das Unternehmen GATC in Konstanz unterzogen. RCA-Produkte des Plasmids pBluescript SK(+) aus *Escherichia coli*, die zuvor beiden viralen Proben beigemischt wurden, dienten hierbei als technische Kontrolle. Je Probe wurden 6 Millionen *reads* à 100 nts erhalten. Die Sequenzdaten wurden über ein Python-Skript mit den Datenbanksequenzen von EuYMV DNA A und DNA B bzw. von pBluescript SK(+) für beide Orientierungen verglichen und auf einzelne Nukleotidaustausche sowie größere Deletionen und Insertionen hin untersucht. Erste Auswertungen der Daten zeigten, dass ein signifikanter differentieller Unterschied der Nukleotidaustauschraten zwischen DNA A und DNA B der wt- und *rev3-1 polh*-1-Pflanzen besteht: Austausche für DNA A wurden durch den Ausfall von Polζ/Polη reduziert, wohingegen sie für DNA B erhöht wurden. Die in beiden Proben enthaltenen Plasmidsequenzen wiesen dagegen keine signifikanten Unterschiede auf und besaßen zudem signifikant geringere Austauschraten als die viralen Sequenzen. Dieser differentielle Einfluss des Genotyps auf die Austauschraten der verschiedenen Genomkomponenten ist daher biologisch relevant und impliziert eine Rolle von Polζ und /oder Polη bei der geminiviralen Infektion.

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 Polκ-Aktivität aufweisen bzw. verkürzte Polκ-Varianten exprimieren könnten, kann keine endgültige Aussage über die Rolle von Polκ getroffen werden. Das Rad6-Homolog Ubc2, das

vermutlich die TLS-Aktivität über die Monoubiquitinierung von PCNA reguliert, war für die geminivirale Replikation ebenfalls nicht notwendig. Jedoch wird durch diese Ergebnisse nicht ausgeschlossen, dass TLS-Polymerasen an der geminiviralen Replikation beteiligt sein können. Sowohl humane als auch TLS-Polymerasen von *S. cerevisiae* binden an unmodifiziertes PCNA (Haracska *et al.*, 2001; Haracska *et al.*, 2002) oder die alternative DNA-Klammer 9-1-1 (Sabbioneda *et al.*, 2005) und wären daher womöglich in der Lage, mit hoher Prozessivität ohne PCNA-Monoubiquitinierung zu replizieren. Da PCNA erst nach der Induktion des S-Phase-ähnlichen Zustands durch Rep in der infizierten Pflanzenzelle exprimiert wird (Nagar *et al.*, 1995), würde die PCNA-Bindung für die initiale CSR ohnehin keine Rolle spielen. Kleine virale Genome können vermutlich selbst mit geringer Prozessivität ohne PCNA repliziert werden. 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.

Darüber hinaus könnten die Aktivitäten einzelner TLS-Polymerasen durch redundante Funktionen der anderen kompensiert werden. Um diese mögliche Redundanz zu überprüfen, wäre es notwendig, weitere doppel-, drei- oder sogar vierfach *knock-out*-Linien zu untersuchen. Allerding ist es möglich, dass mehrfache *knock-out*-Mutanten Wachstums- oder Entwicklungsdefizite aufweisen würden und damit für Inokulationsversuche ungeeignet wären.

Durch die signifikant veränderten Nukleotidaustauschraten der EuYMV-DNA aus wt- und *rev3-1 polh-1*-Pflanzen wurde ein Einfluss von Polζ 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→C Mutationen (Matsuda *et al.*, 2000; Matsuda *et al.*, 2001). Dagegen verursachte Polζ tendenziell A→G und C→G (Zhong *et al.*, 2006) und Polκ T→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→TT Mutationen nachgewiesen, im Rad30-defizienten (Homolog zu Polη) Stamm wurde dagegen ein breiteres Spektrum an Tandem-Mutationen dokumentiert (CC→TT; CC→TA; CC→AT; GC→TT; TC→AT; TC→AΔ). Der kombinierte *knock-out*-Stamm *rev3 rad30* umfasste ausschließlich CC→AA Mutationen.

Bisherige Sequenzanalysen geminiviraler Genome ließen eine Präferenz für C→T, G→A oder G→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→T) oder oxidativ-induzierten Stress (G→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→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

Veröffentlicht in *Virology* (2014), 452-453: 287-296

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

Veröffentlicht als *short communication* in *Journal of General Virology* (2015), 96: 2913-2918

Manuskript 4:

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

Kathrin S. Richter, Heïdi 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

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#Author for correspondence:

Email: holger.jeske@bio.uni-stuttgart.de

Phone: +49-711-685-65070

Figures: 6

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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[1] 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).

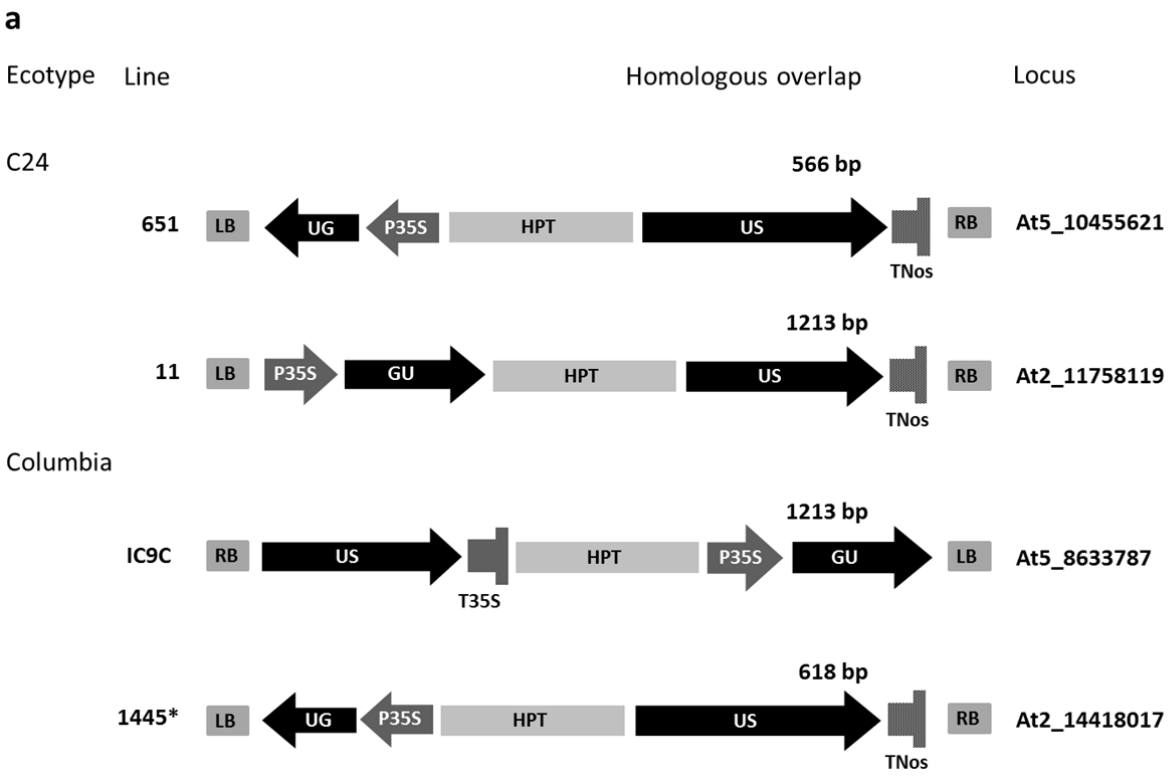
Recombination is an important factor for the evolution and epidemics of geminiviruses (van der Walt *et al.*, 2009, and references therein). At the same time the RDR mode provides an efficient mechanism for early recombination during infection. This motivates our current study on whether host recombination 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 (ClLCrV) 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).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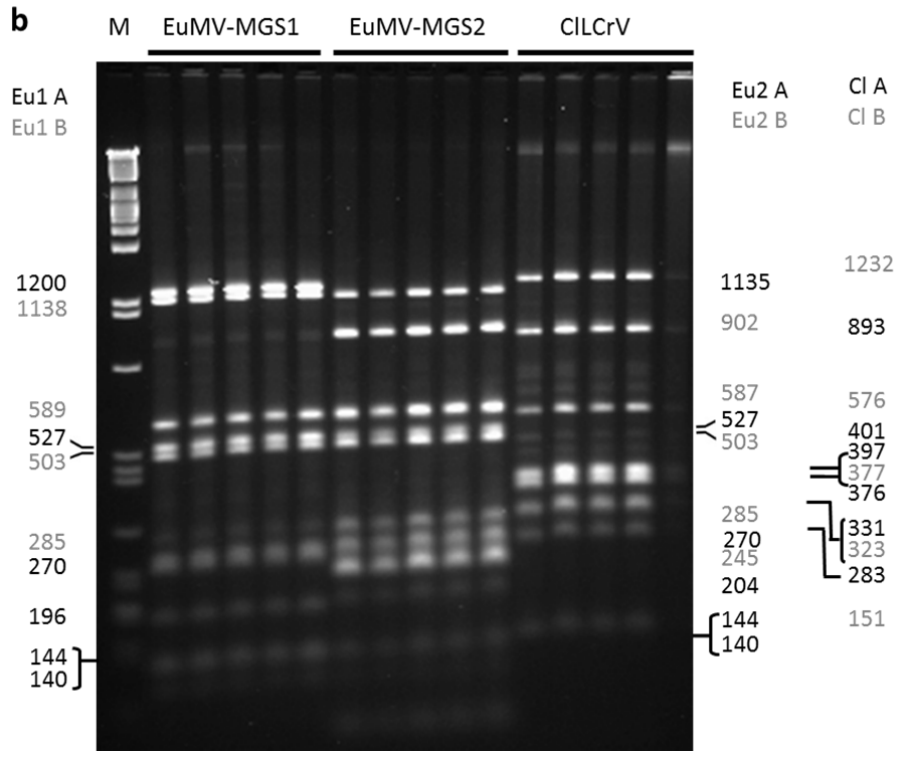
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ClLCrV
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Eu1 B
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Eu2 B
1135
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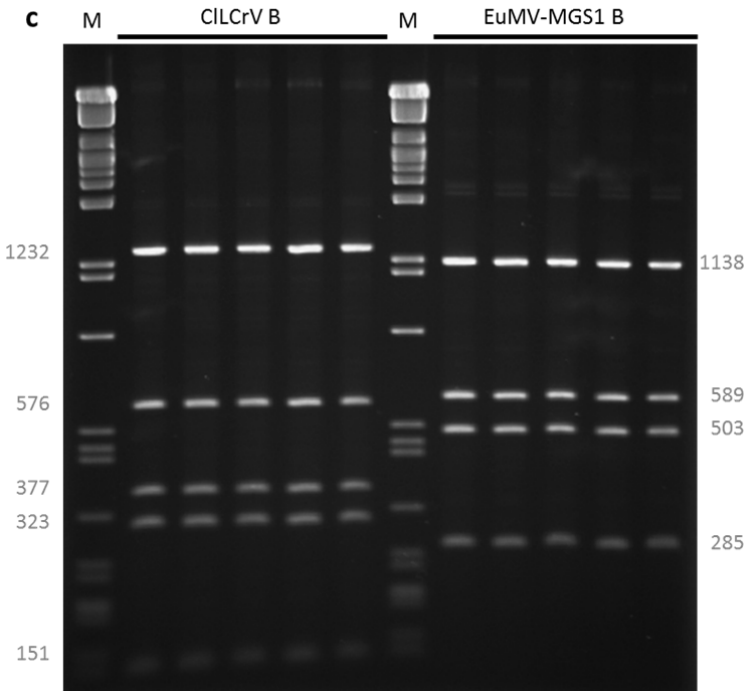

Figure 1: Prerequisites of the analysis. (a) Schematic representation of SHR reporter constructs present in *A. thaliana* transgenic lines 651 (Puchta *et al*., 1995b), 11 (Swoboda *et al*., 1994), IC9C (Molinier *et al*., 2004) and 1445 (Fritsch *et al*., 2004; Gherbi *et al.*, 2001; Pecinka *et al*., 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 (ClLCrV) in the applied inocula. RCA products of viral DNAs from systemically infected wild-type *A. thaliana* plants are shown, which were treated with *Hpa*II (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).

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

Late inoculation				
Line	651	1445	IC9C	11
EuMV-MGS1	10/10	9/10	9/10	9/10
EuMV-MGS2	6/10	5/10	1/10	0/10
ClLCrV	10/10	10/10	8/10	3/10

Early inoculation						
Experiment #	1		2		3	
Line	651	1445	651	1445	651	1445
EuMV-MGS1	5/10	5/10	11/20	15/20	10/20	11/20
EuMV-MGS2	0/10	0/10	-	-	-	-
ClLCrV	0/10	2/10	-	-	-	-

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 ClLCrV 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.

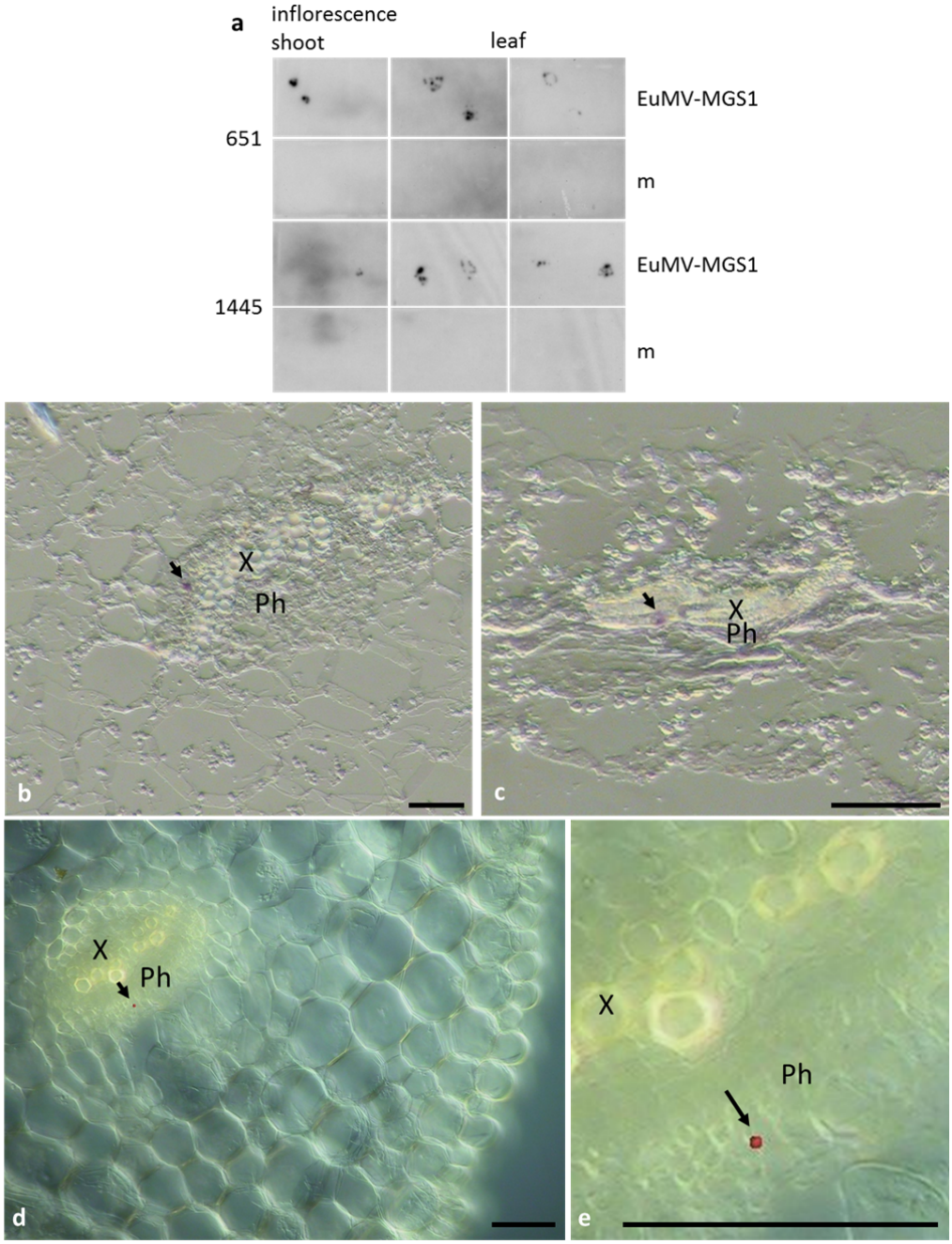

Figure 2: Phloem-limitation of EuMV-MGS1 in *A. thaliana* plants. (a) Tissue blots of infected or mock-inoculated (m) lines 651 and 1445 were probed at 33 dpi. Cross-sections of inflorescence shoots or furled rosette leaves from one plant per line and infection type were dappled onto nylon membranes 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**.**

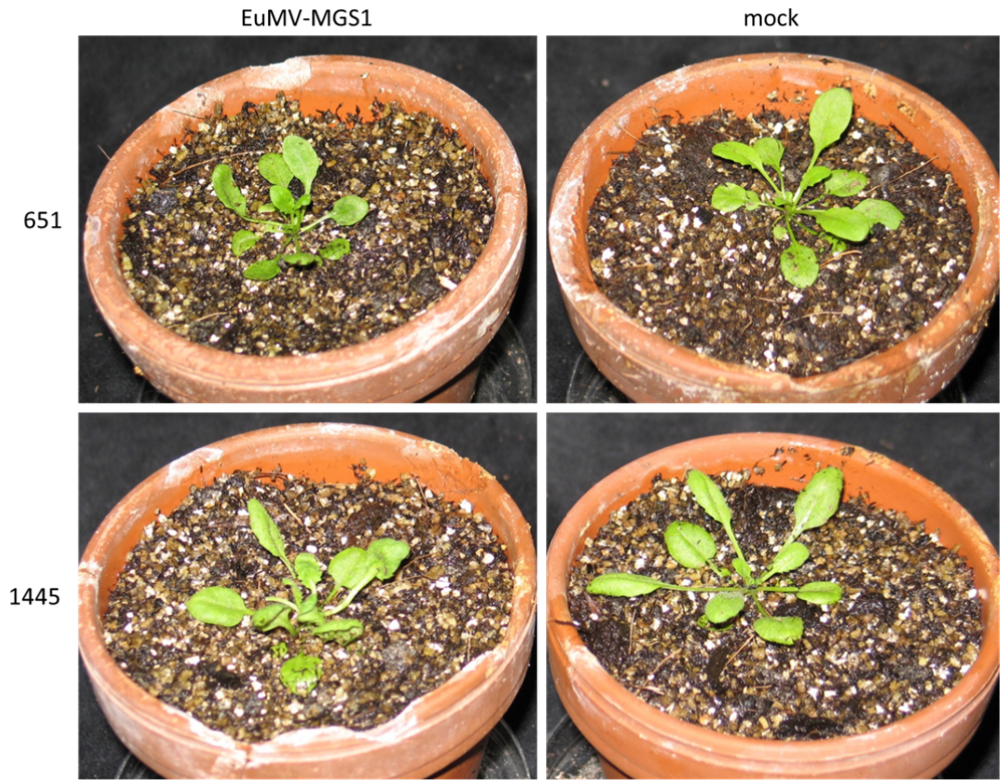

Figure 3: Symptoms after early inoculation at the 4-6 leaf stage. EuMV-MGS1 infected and mock-inoculated *A. thaliana* plants of the C24 line 651 and the Col line 1445 at 16 dpi. Infected and mock-inoculated plants at higher magnification show mild but distinct leaf rolling in line 1445, which was barely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

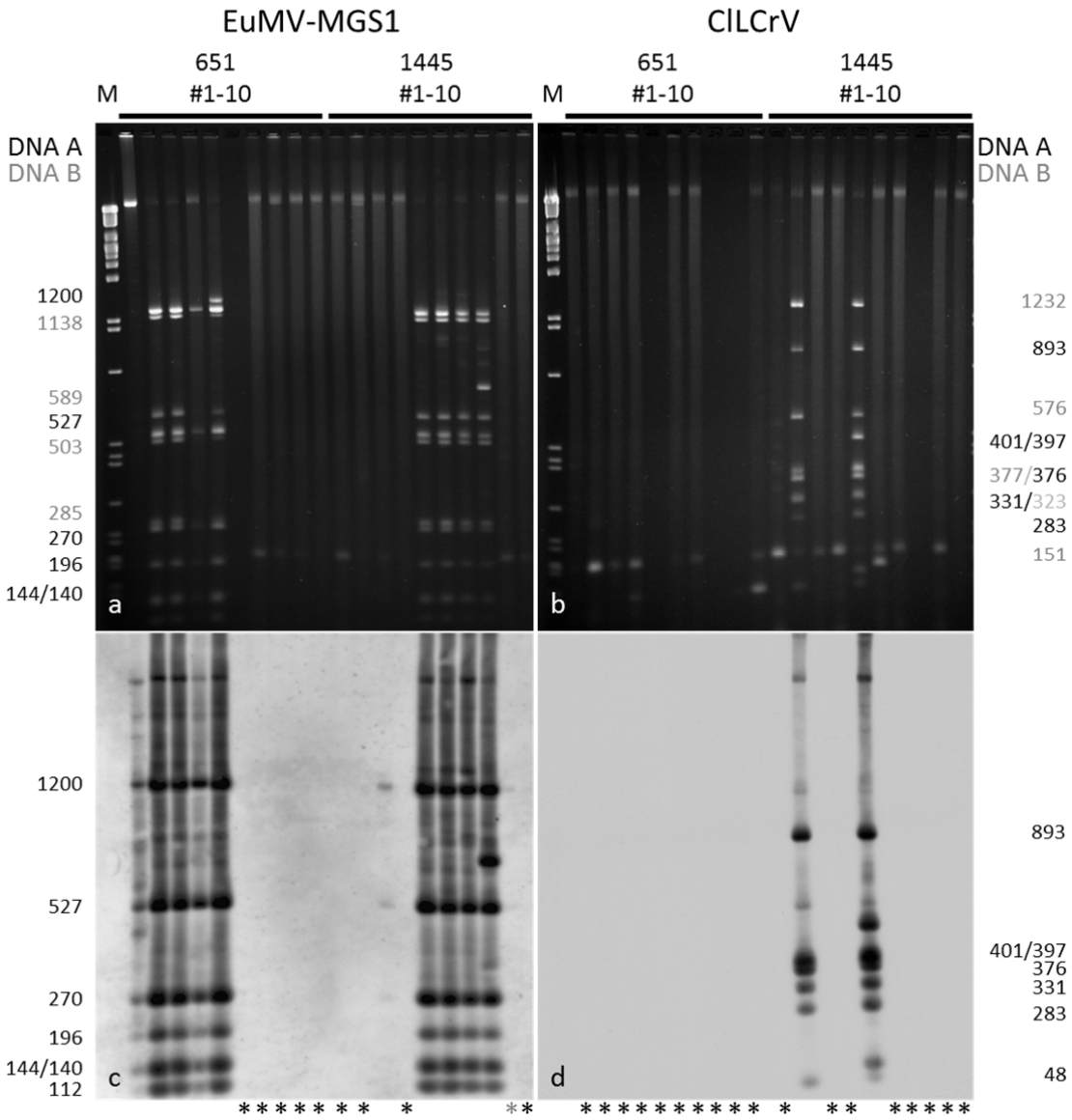

Figure 4: Detection of EuMV-MGS1 and ClLCrV DNA in reporter lines 651 and 1445 at 14 dpi. (a, b) RCA/*Hpa*II RFLP analyses as described in Fig. 1b, (c, d) Southern blot hybridization with full-length EuMV-MGS1 or ClLCrV DNA A probes. Total nucleic acids of rosette leaves from ten plants (#1-10) per virus and plant line revealed either the expected RFLP patterns (bp; black and grey numbers for DNA A and B, respectively) or proved to be uninfected (marked with black asterisks) and were considered similar to mock-inoculated for the GUS assay evaluation. Specimens marked with grey asterisks were not used for GUS experiments. The figure shows the representative result of one infection experiment.

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,

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.

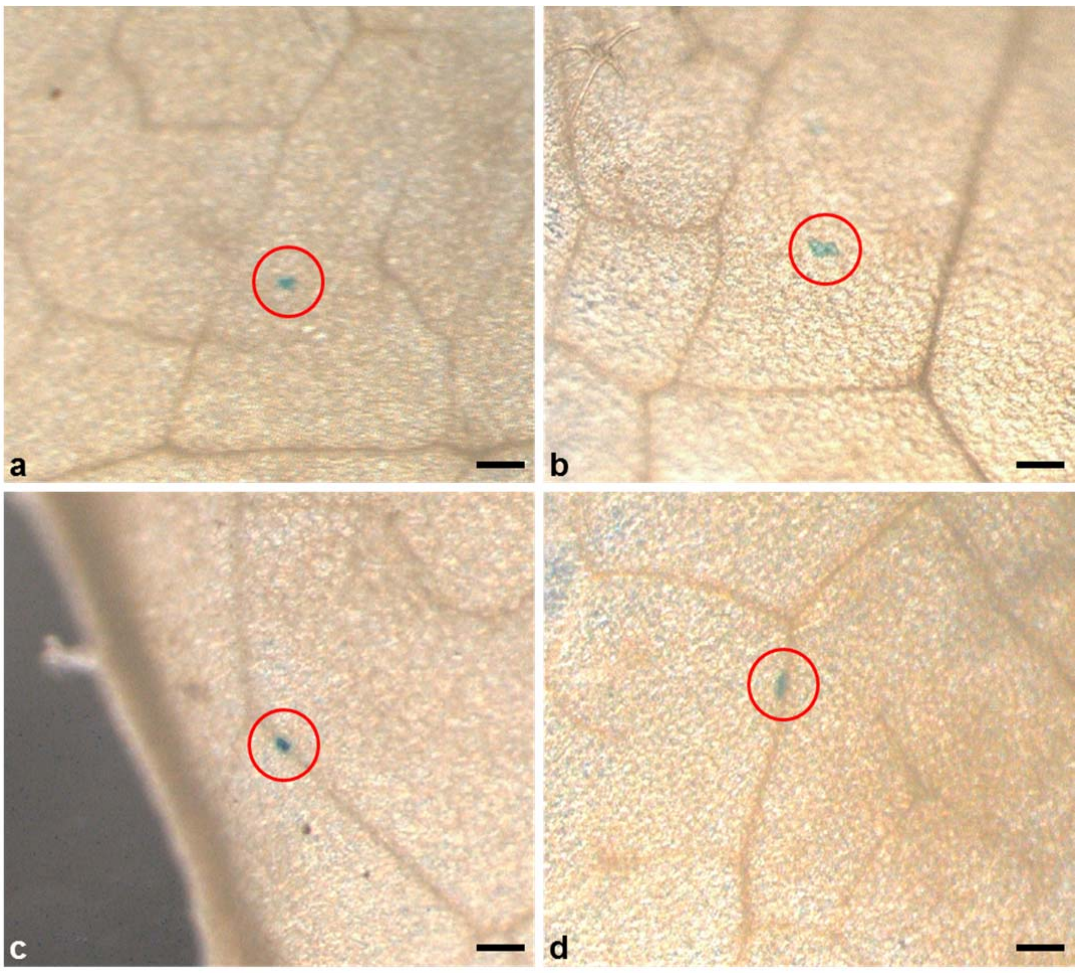

Figure 5: Recombination events detected by GUS activity. Plants of line 1445 after mock-treatment (a, c) or EuMV-MGS1 (b, d) infection at 21 dpi show GUS signals (blue spots) in single cells. GUS activity (encircled) was observed after histochemical staining with X-Gluc by stereomicroscopy in the mesophyll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

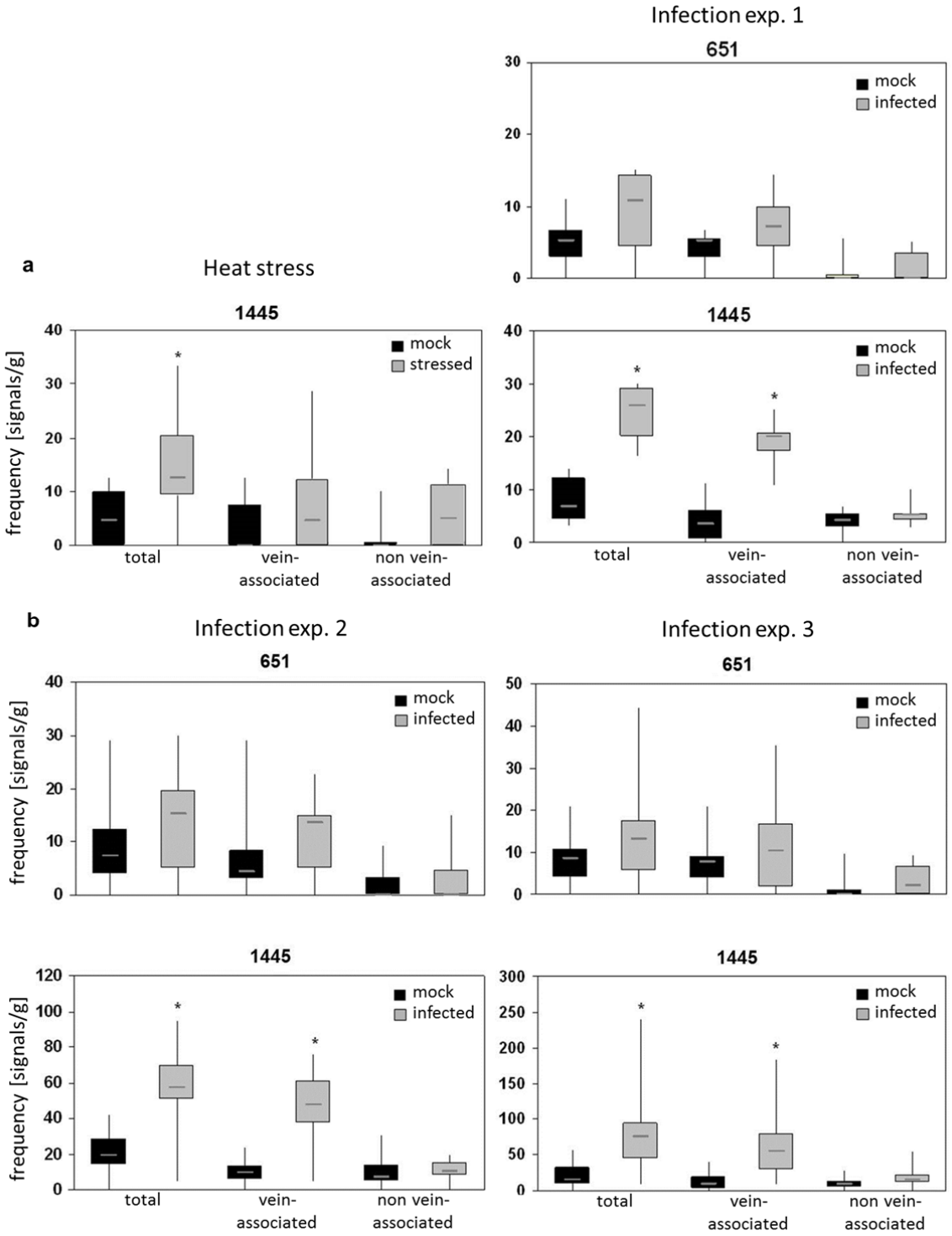

Figure 6: Statistical analysis of recombination events in SHR reporter plants. Box plot graphs of total, vein- and non-vein-associated GUS signal frequencies per gram plant material for heat-stressed, mock-inoculated, or EuMV-MGS1 infected plants of lines 651 and 1445 at 21 dpi are compared. (a-b) Independent 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-

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 (ClLCrV) DNA without satellite DNA were used to inoculate the plants (Paprotka *et al.*, 2010). For mock inoculation DNAs B alone of EuMV-MGS1 or ClLCrV 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 *Xma*I; for ClLCrV *Nsi*I). 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.

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 H_2O and mixed with 10 µl of gold or tungsten suspension. 2 µl of 0.1 M spermidine and 5 µl of 2.5 M $CaCl_2$ 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 (10x), 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 ddH_2O 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 dappled onto N+ membranes (GE Healthcare, Munich, Germany) and DNA was UV-crosslinked to the membrane using UV light of 70,000 mJ/cm^2 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, ClLCrV 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).

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' CTCGTATTTCCCTGCTTCTTG '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.

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References

Alberter, B, Rezaian, MA, Jeske, H (2005) Replicative intermediates of Tomato leaf curl virus and its satellite DNAs. *Virology* **331**: 441-448

Ascencio-Ibanez, JT, Sozzani, R, Lee, TJ, Chu, TM, Wolfinger, RD, Cella, R, Hanley-Bowdoin, L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol* **148**: 436-454

Baubec, T, Pecinka, A, Rozhon, W, Mittelsten Scheid, O (2009) Effective, homogeneous and transient interference with cytosine methylation in plant genomic DNA by zebularine. *Plant J* **57**: 542-554

Boyko, A, Blevins, T, Yao, YL, Golubov, A, Bilichak, A, Ilnytskyy, Y, Hollander, J, Meins, F, Kovalchuk, I (2010) Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of Dicer-like proteins. *Plos One* **5**: e9514

Boyko, A, Filkowski, J, Kovalchuk, I (2005) Homologous recombination in plants is temperature and day-length dependent. *Mutat Res* **572**: 73-83

Boyko, A, Kovalchuk, I (2010) Transgenerational response to stress in *Arabidopsis thaliana*. *Plant Signal Behav* **5**: 995-998

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

Chomczynski, P, Qasba, PK (1984) Alkaline transfer of DNA to plastic membrane. *Biochem Bioph Res Co* **122**: 340-344

Dubrovina, AS, Kiselev, KV, Zhuravlev, YN (2013) The role of canonical and noncanonical pre-mRNA splicing in plant stress responses. *Biomed Res Int* **2013**: 264314

Erdmann, JB, Shepherd, DN, Martin, DP, Varsani, A, Rybicki, EP, Jeske, H (2010) Replicative intermediates of maize streak virus found during leaf development. *J Gen Virol* **91**: 1077-1081

Fernandes, FR, Albuquerque, LC, de Oliveira, CL, Cruz, AR, da Rocha, WB, Pereira, TG, Naito, FY, Dias Nde, M, Nagata, T, Faria, JC, Zerbini, FM, Aragao, FJ, Inoue-Nagata, AK (2011) Molecular and biological characterization of a new Brazilian begomovirus, euphorbia yellow mosaic virus (EuYMV), infecting *Euphorbia heterophylla* plants. *Arch Virol* **156**: 2063-2069

Fritsch, O, Benvenuto, G, Bowler, C, Molinier, J, Hohn, B (2004) The INO80 protein controls homologous recombination in *Arabidopsis thaliana*. *Mol Cell* **16**: 479-485

Ghanim, M, Brumin, M, Popovski, S (2009) A simple, rapid and inexpensive method for localization of Tomato yellow leaf curl virus and Potato leafroll virus in plant and insect vectors. *J Virol Methods* **159**: 311-314

Gherbi, H, Gallego, ME, Jalut, N, Lucht, JM, Hohn, B, White, CI (2001) Homologous recombination in planta is stimulated in the absence of Rad50. *Embo Rep* **2**: 287-291

Gutierrez, C, Ramirez-Parra, E, Castellano, MM, Sanz-Burgos, AP, Luque, A, Missich, R (2004) Geminivirus DNA replication and cell cycle interactions. *Vet Microbiol* **98**: 111-119

Haible, D, Kober, S, Jeske, H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J Virol Methods* **135**: 9-16

Hanley-Bowdoin, L, Settlage, SB, Orozco, BM, Nagar, S, Robertson, D (1999) Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Plant Sci* **18**: 71-106

Hanley-Bowdoin, L, Settlage, SB, Robertson, D (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. *Mol Plant Pathol* **5**: 149-156

Horns, T, Jeske, H (1991) Localization of Abutilon mosaic virus (Abmv) DNA within leaf tissue by *in situ* hybridization. *Virology* **181**: 580-588

Ilnytskyy, Y, Boyko, A, Kovalchuk, I (2004) Luciferase-based transgenic recombination assay is more sensitive than beta-glucoronidase-based. *Mutat Res* **559**: 189-197

Jeske, H, **(2007)**. Replication of geminiviruses and the use of rolling circle amplification for their diagnosis, in: *Tomato yellow leaf curl virus disease*, pp. 141-156. Edited by Czosnek, H. Springer, Dordrecht, Netherlands.

Jeske, H (2009) Geminiviruses. *Curr Top Microbiol* **331**: 185-226

Jeske, H, Lutgemeier, M, Preiss, W (2001) DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *Embo J* **20**: 6158-6167

Jovel, J, Preiss, W, Jeske, H (2007) Characterization of DNA intermediates of an arising geminivirus. *Virus Res* **130**: 63-70

Kaliappan, K, Choudhury, NR, Suyal, G, Mukherjee, SK (2012) A novel role for RAD54: this host protein modulates geminiviral DNA replication. *Faseb J* **26**: 1142-1160

Kimura, S, Sakaguchi, K (2006) DNA repair in plants. *Chem Rev* **106**: 753-766

Kittelmann, K, Rau, P, Gronenborn, B, Jeske, H (2009) Plant geminivirus Rep protein induces rereplication in fission yeast. *J Virol* **83**: 6769-6778

Kleinow, T, Nischang, M, Beck, A, Kratzer, U, Tanwir, F, Preiss, W, Kepp, G, Jeske, H (2009) Three C-terminal phosphorylation sites in the Abutilon mosaic virus movement protein affect symptom development and viral DNA accumulation. *Virology* **390**: 89-101

Kovalchuk, I, Kovalchuk, O, Kalck, V, Boyko, V, Filkowski, J, Heinlein, M, Hohn, B (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. *Nature* **423**: 760-762

Lucht, JM, Mauch-Mani, B, Steiner, HY, Metraux, JP, Ryals, J, Hohn, B (2002) Pathogen stress increases somatic recombination frequency in *Arabidopsis*. *Nature Genet* **30**: 311-314

Molinier, J, Oakeley, EJ, Niederhauser, O, Kovalchuk, I, Hohn, B (2005) Dynamic response of plant genome to ultraviolet radiation and other genotoxic stresses. *Mutat Res* **571**: 235-247

Molinier, J, Ries, G, Bonhoeffer, S, Hohn, B (2004) Interchromatid and interhomolog recombination in *Arabidopsis thaliana*. *Plant Cell* **16**: 342-352

Molinier, J, Ries, G, Zipfel, C, Hohn, B (2006) Transgeneration memory of stress in plants. *Nature* **442**: 1046-1049

Nagar, S, Hanley-Bowdoin, L, Robertson, D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**: 2995-3007

Paprotka, T, Metzler, V, Jeske, H (2010) The first DNA 1-like alpha satellites in association with New World begomoviruses in natural infections. *Virology* **404**: 148-157

Pecinka, A, Rosa, M, Schikora, A, Berlinger, M, Hirt, H, Luschnig, C, Mittelsten Scheid, O (2009) Transgenerational stress memory is not a general response in Arabidopsis. *PLoS One* **4**: e5202

Pohl, D, Wege, C (2007) Synergistic pathogenicity of a phloem-limited begomovirus and tobamoviruses, despite negative interference. *J Gen Virol* **88**: 1034-1040

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Puchta, H, Hohn, B (2012) *In planta* somatic homologous recombination assay revisited: A successful and versatile, but delicate tool. *Plant Cell* **24**: 4324-4331

Puchta, H, Swoboda, P, Gal, S, Blot, M, Hohn, B (1995a) Somatic intrachromosomal homologous recombination events in populations of plant siblings. *Plant Mol Biol* **28**: 281-292

Puchta, H, Swoboda, P, Hohn, B (1995b) Induction of intrachromosomal homologous recombination in whole plants. *Plant J* **7**: 203-210

Ries, G, Heller, W, Puchta, H, Sandermann, H, Seidlitz, HK, Hohn, B (2000) Elevated UV-B radiation reduces genome stability in plants. *Nature* **406**: 98-101

Ruschhaupt, M, Martin, DP, Lakay, F, Bezuidenhout, M, Rybicki, EP, Jeske, H, Shepherd, DN (2013) Replication modes of Maize streak virus mutants lacking RepA or the RepA-pRBR interaction motif. *Virology* **442**: 173-179

Sambrook, J, Russell, D, 2001. Molecular Cloning: A Laboratory Manual., 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sardo, L, Wege, C, Kober, S, Kocher, C, Accotto, GP, Noris, E (2011) RNA viruses and their silencing suppressors boost Abutilon mosaic virus, but not the Old World Tomato yellow leaf curl Sardinia virus. *Virus Res* **161**: 170-180

Stainton, D, Kraberger, S, Walters, M, Wiltshire, EJ, Rosario, K, Halafihi, M, Lolohea, S, Katoa, I, Faitua, TH, Aholelei, W, Taufa, L, Thomas, JE, Collings, DA, Martin, DP, Varsani, A (2012) Evidence of inter-component recombination, intra-component recombination and reassortment in banana bunchy top virus. *J Gen Virol* **93**: 1103-1119

Sun, XQ, Zhang, YL, Yang, SH, Chen, JQ, Hohn, B, Tian, DC (2008) Insertion DNA promotes ectopic recombination during meiosis in *Arabidopsis*. *Mol Biol Evol* **25**: 2079-2083

Swoboda, P, Gal, S, Hohn, B, Puchta, H (1994) Intrachromosomal homologous recombination in whole plants. *Embo J* **13**: 484-489

Tinland, B, Hohn, B, Puchta, H (1994) *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant-cell nucleus. *Proc Natl Acad Sci U S A* **91**: 8000-8004

Ülker, B, Hommelsheim, CM, Berson, T, Thomas, S, Chandrasekar, B, Olcay, AC, Berendzen, KW, Frantzeskakis, L (2012) Reevaluation of the reliability and usefulness of the somatic homologous recombination reporter lines. *Plant Cell* **24**: 4314-4323

van der Walt, E, Rybicki, EP, Varsani, A, Polston, JE, Billharz, R, Donaldson, L, Monjane, AL, Martin, DP (2009) Rapid host adaptation by extensive recombination. *J Gen Virol* **90**: 734-746

Wege, C, **(2007)**. Movement and localization of Tomato yellow leaf curl viruses in the infected plant, in: *Tomato yellow leaf curl virus disease*, pp. 185-206. Edited by Czosnek, H. Springer, Dordrecht, Netherlands.

Wege, C, (2009). Mixed infections of geminiviruses and unrelated RNA viruses or viroids in tomato: A multitude of effects with a highly probable impact on epidemiology and agriculture., Crop plant resistance to biotic and abiotic factors Proceedings of the 3rd International Symposium on Plant Protection and Plant Health in Europe. *Deutsche Phytomedizinische Gesellschaft*, Braunschweig, Germany, Berlin, Germany

Wege, C, Saunders, K, Stanley, J, Jeske, H (2001) Comparative analysis of tissue tropism of bipartite geminiviruses. *J Phytopathol* **149**: 359-368

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, 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

Yao, YL, Bilichak, A, Golubov, A, Kovalchuk, I (2011) Local infection with oilseed rape mosaic virus promotes genetic rearrangements in systemic *Arabidopsis* tissue. *Mutat Res* **709-10**: 7-14

Zhang, SC, Wege, C, Jeske, H (2001) 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

Supplementary data

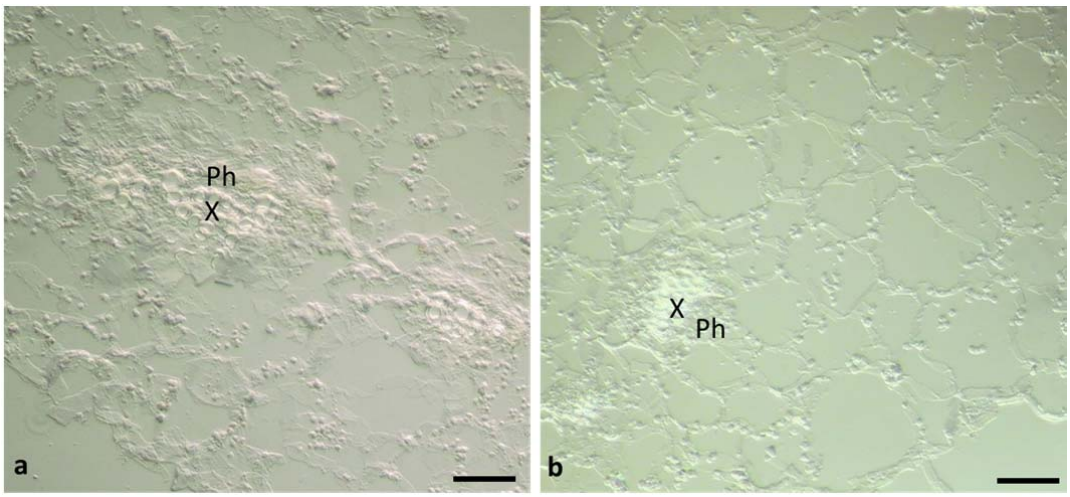


Figure S1: Mock-control of *in situ* hybridization for lines 1445 (a) and 651 (b) after development with NBT/BCIP as in Figs. 2b, c.

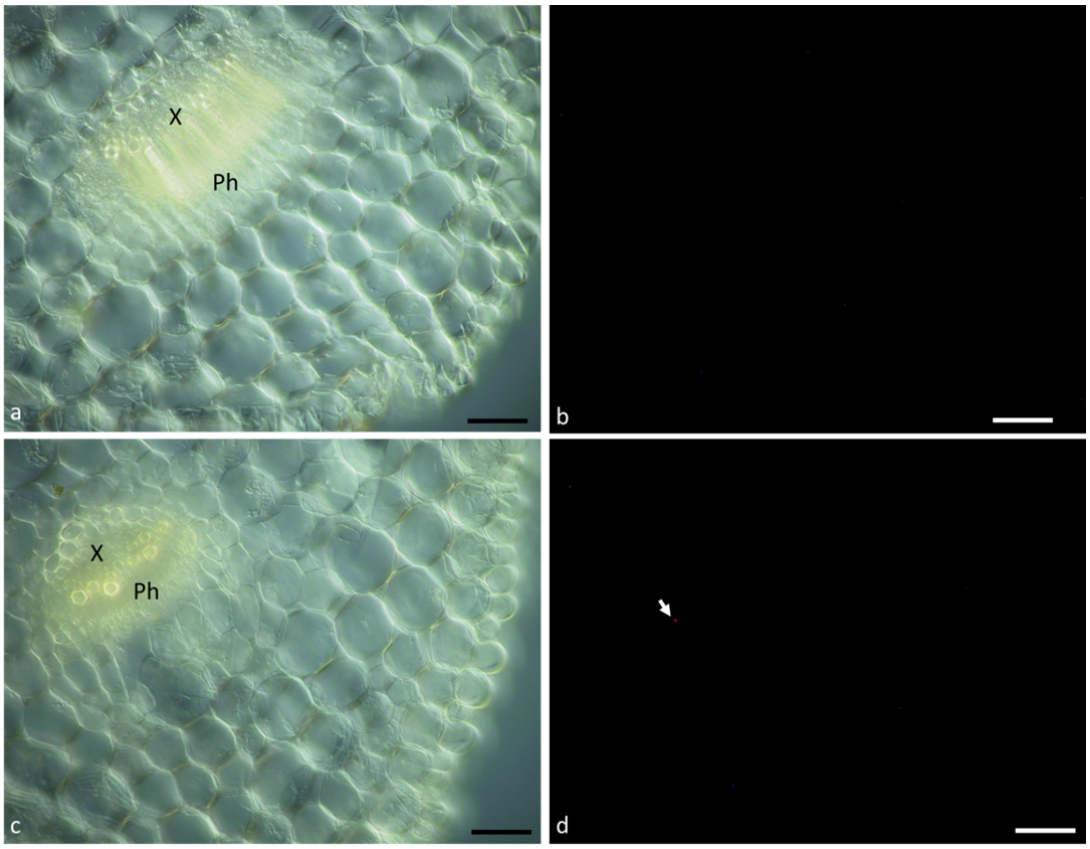


Figure S2: FISH detection of viral DNA in line 1445 using a 5'Cy3-labeled primer hybridizing within the AV1 ORF of EuMV-MGS1 for mock-inoculated (a, b) or infected (c, d) plants (21 dpi). DIC images (a, c) and corresponding fluorescent images (b, d) of petioles. Virus-specific signal (arrow) were detected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

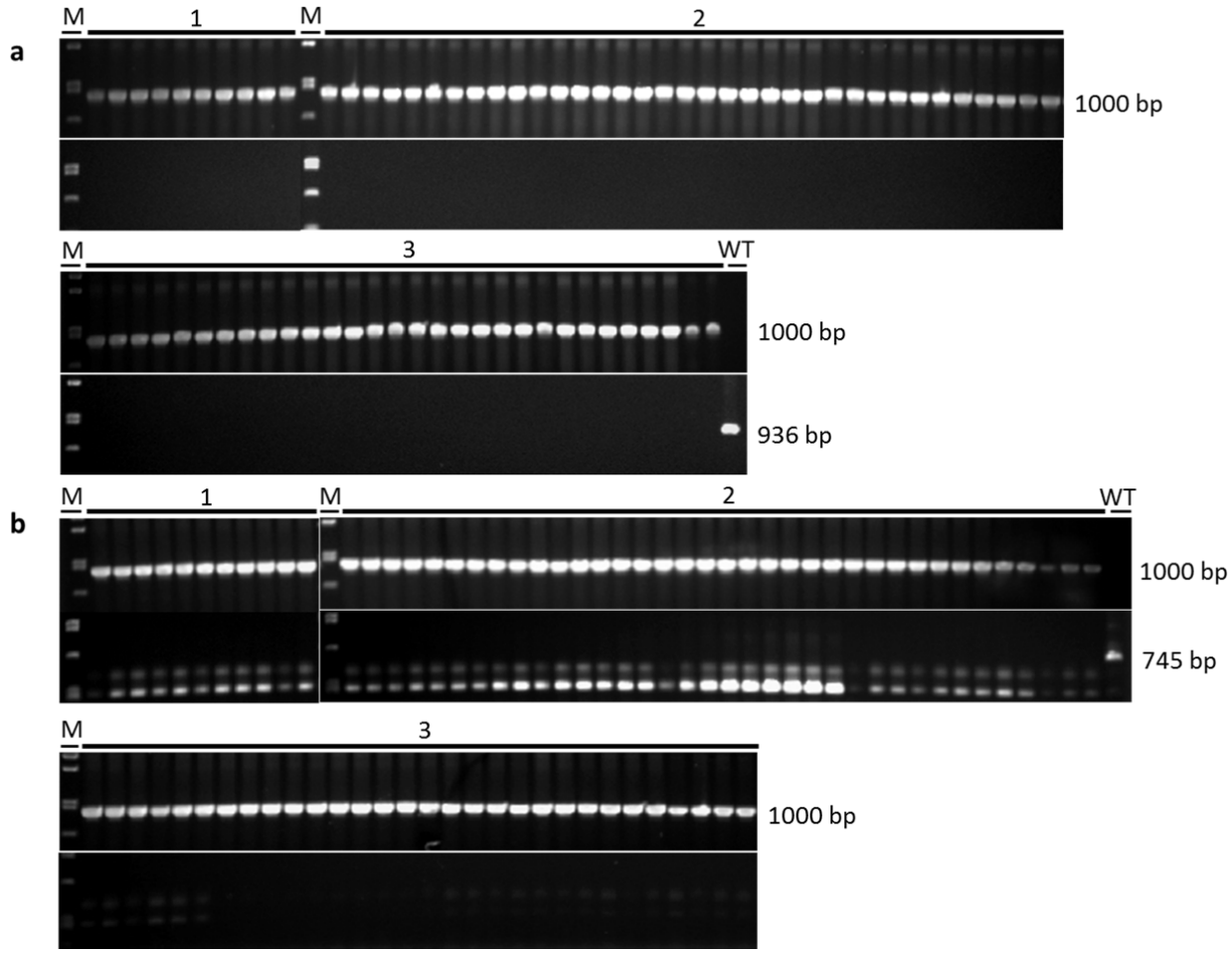
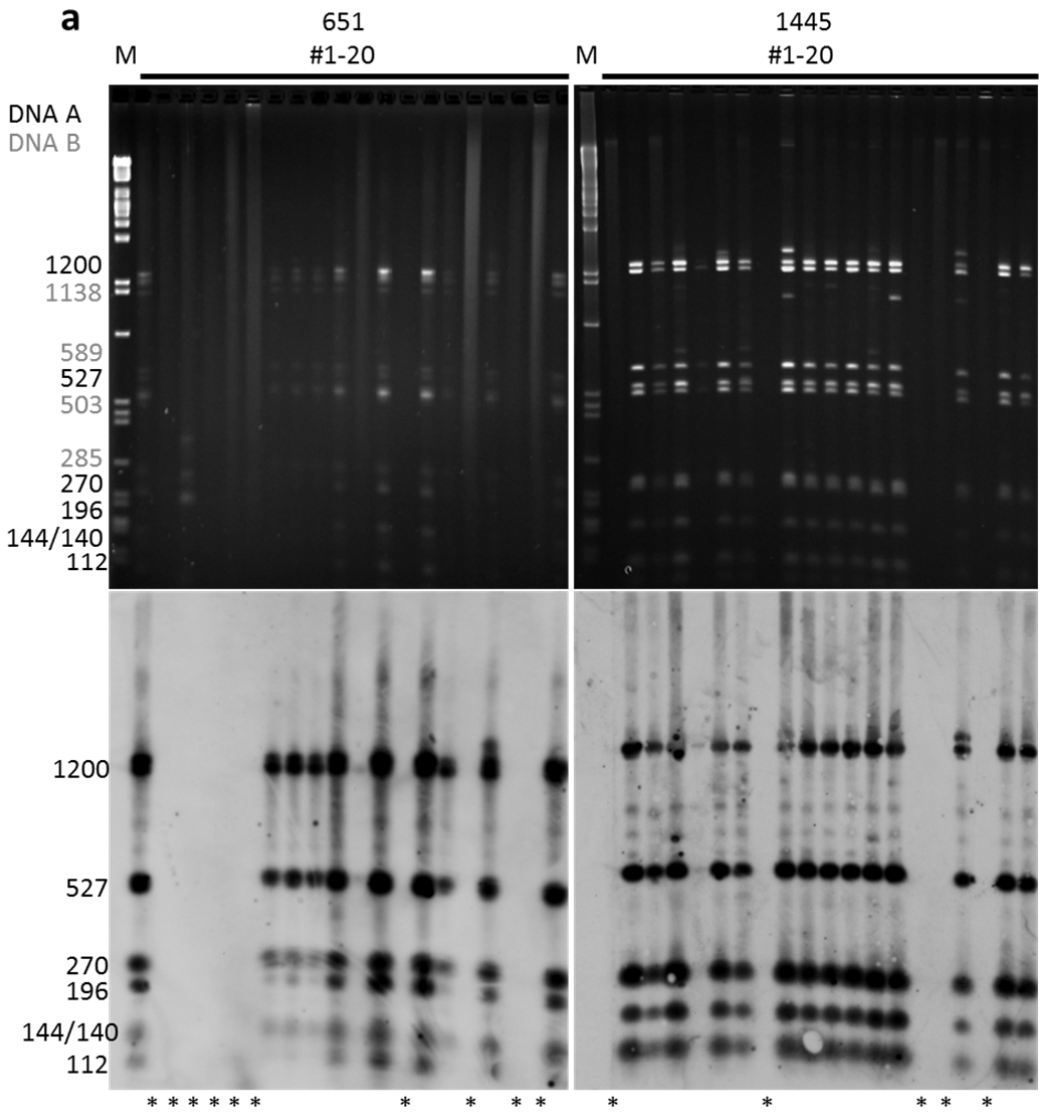


Figure S3: Genotyping of *A. thaliana* plant lines used. Lines 651 (a), 1445 (b), and two wild type (WT) control plants (C24 for line 651 or Col for line 1445) were tested separately by PCR with either the uidA (GUS) primer pair (respective upper depiction) or primer pairs with binding sites overlapping 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 *Pst*I 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.


a
651
#1-20
M
1445
#1-20
M
DNA A
DNA B
1200
1138
589
527
503
285
270
196
144/140
112
1200
527
270
196
144/140
112

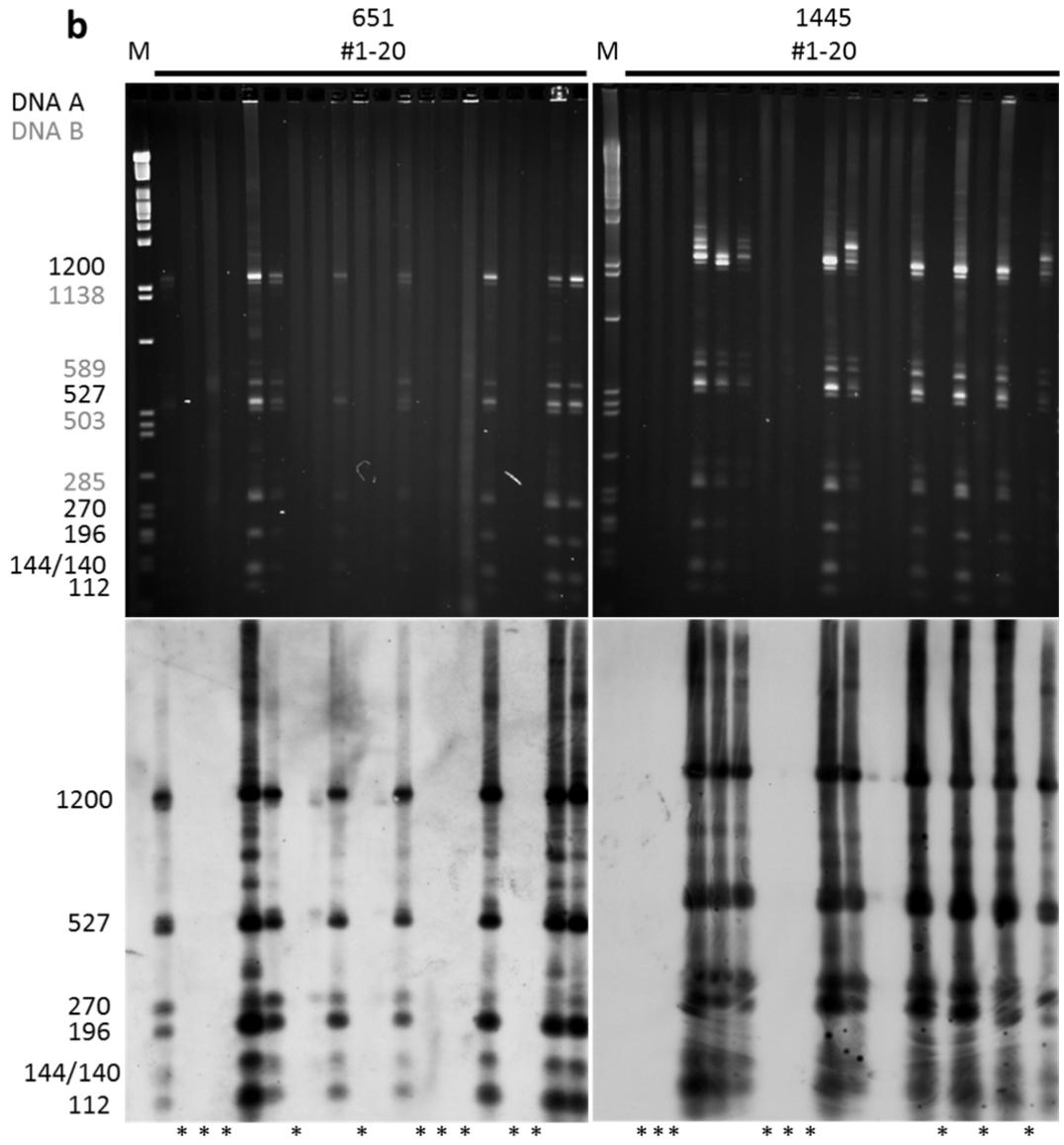


Figure S4: Detection of EuMV-MGS1 DNA as described in Fig. 4 for the second (a) and third (b) biological replicate.

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

Primers for: uidA, 1445wt, 651wt, IC9Cwt, or 11wt

Step	Temp.	Time	Repeats
1.	96°C	3 min	
2.	95°C	30 s	
3.	56°C	30 s	50
4.	72°C	2 min 30 s	
5.	72°C	5 min	
6.	4°C	pause	

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

Names	Sequences	Genomic locus*	Expected fragment length [bp]	
			wt	transgenic
uidA-F	GCAATTGCTGTGCCAGGCAGTTT			
uidA-R	CCTGTAAGTGCGCTTGCTGAGTT		-	~1000
1445wt-F	CAACTCTTTAAATCGTCTATCGTA	At2_14417446-14417469		
1445-R	GATATTGGTGACGGGATGAT	At2_14418191-14418172	745	-
651wt-F	GCAGCAAATGGAGAAAAAG	At5_10455304-10455322		
651wt-R	ACACGATGTCTACATGCTG	At5_10456240-10456222	936	-
IC9Cwt-F	ACTGAGTTTGGTAACCTGTG	At5_8633613-8633632		
IC9Cwt-R	CCAGAAGTAAAAGTGAAGTTC	At5_8633908-8633888	295	-
11wt-F	TGCTGGTGAACACGTAAAGC	At2_11757844-11757863		
11wt-R	CAGTCGGATGGTTCGTTTCT	At2_11758400-11758381	556	-

* 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

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#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 remodeller 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

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 *Saccharomyces cerevisiae* (*Sc*Rad54) 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 *S. cerevisiae* and 55 % to the human homologs suggest a functional conservation of *Arabidopsis thaliana* Rad54 (*At*Rad54) (Klutstein *et al.*, 2008; Osakabe *et al.*, 2006). Equally, an interaction of *At*Rad51 and *At*Rad54 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 *S. cerevisiae* cells. In that regard *Sc*Rad54 has been shown to interact with the MYMIV Rep protein and to enhance its nicking, ATPase and helicase activity *in vitro* (Kaliappan *et al.*, 2012). The authors also have suggested that *Sc*Rad54 is required for RCR of geminivirus-based constructs in yeast and *in vitro*. Moreover, a transient agroinfiltration test with an artificial construct derived from Tomato leaf curl New Delhi virus

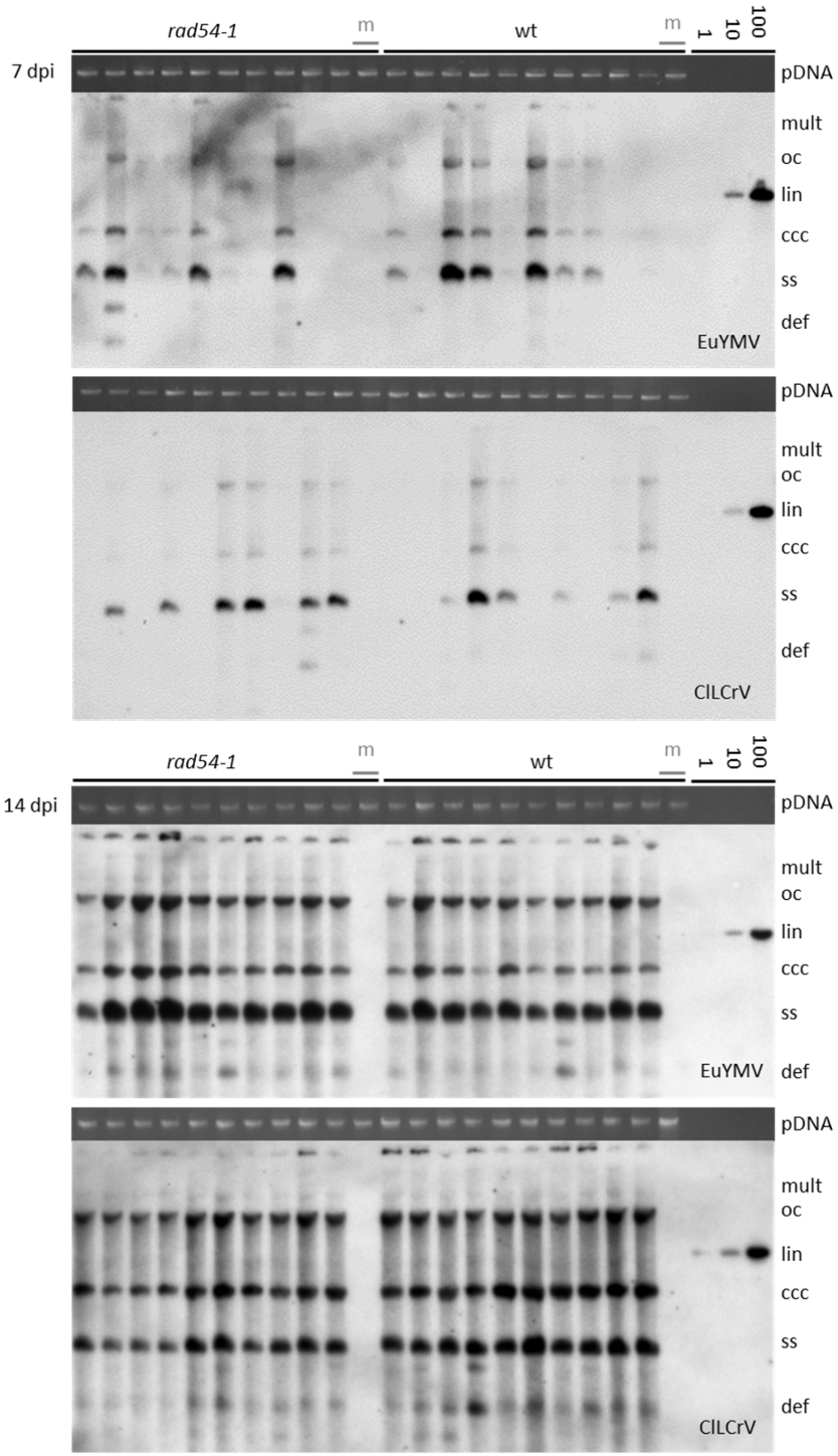
(ToLCNDV) in Rad54-deficient and wildtype (wt) *A. thaliana* was used to propose a necessary role for Rad54 in geminiviral RCR in plants. However, the authors merely used PCR for the detection of viral progeny DNA, which does not allow a differentiation between distinct DNA forms or the respective replication 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 *At*Rad54 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, ClLCrV) (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 *At*Rad54 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 ClLCrV 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.


rad54-1
m
wt
m
1
10
100
7 dpi
pDNA
mult
oc
lin
ccc
ss
def
EuYMV
pDNA
mult
oc
lin
ccc
ss
def
ClLCrV
rad54-1
m
wt
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14 dpi
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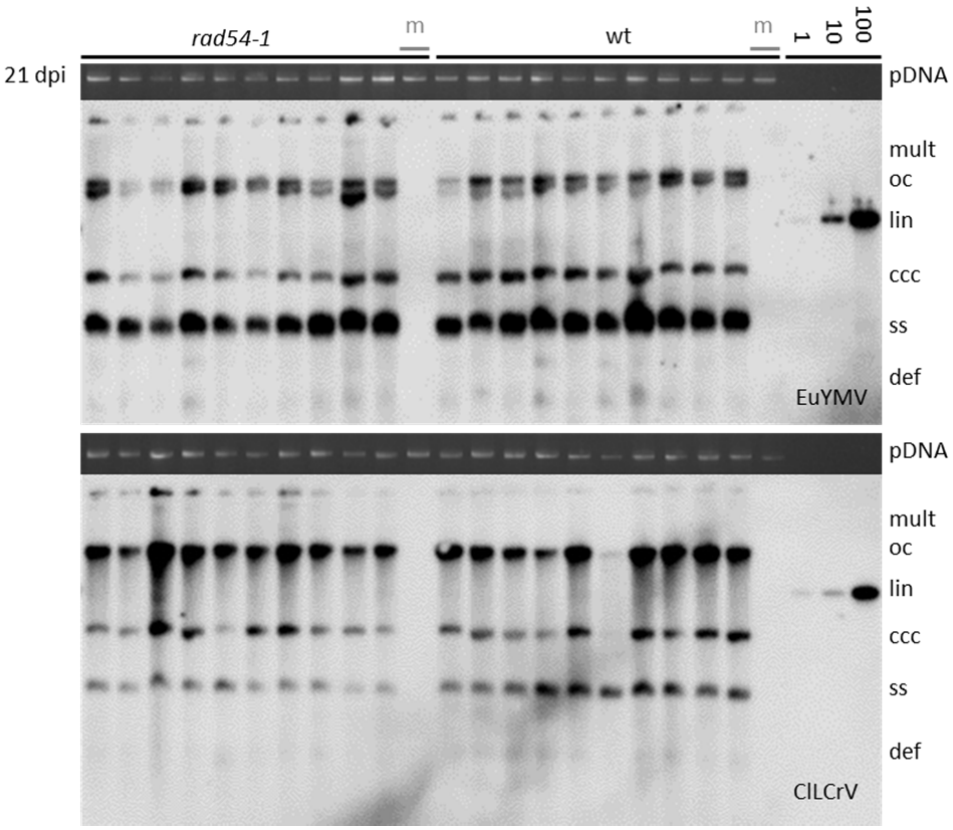

Figure 1: Viral DNA titers of EuYMV or ClLCrV in *rad54-1* or wt *A. thaliana* plants during the time course of systemic infection at 7, 14 and 21 dpi. Virus DNA was detected by Southern Blot hybridization of 1D gel-separated nucleic acids from ten independent plants each. One additional plant per genotype was inoculated with EuYMV or ClLCrV DNA B only and served as mock control (m). 500 ng of total nucleic acids were loaded per lane and migrated in agarose gels (1.4 %) in the presence of 5 µg/ml ethidium bromide. Hybridization was carried out with DIG-labeled full-length DNA A probes of EuYMV or ClLCrV, respectively. Genomic plant DNA (pDNA) served as a loading control and 100, 10, and 1 pg of virus linear dsDNA as a hybridization standard are shown for comparison. DNA forms indicate: multimeric (>1x; mult); open circular (oc); double-stranded linear (lin); covalently closed circular (ccc); single-stranded linear and circular (ss); defective (<1x; def).

At 7 dpi, viral DNAs with distinct forms like ssDNA, covalently closed circular (ccc) and open circular (oc) DNA have emerged for EuYMV and ClLCrV, 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 ClLCrV 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.

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 ClLCrV compared to EuYMV infection at 21 dpi. Like other geminiviruses (Bach & Jeske, 2014; Patil & Dasgupta, 2006), EuYMV and especially ClLCrV produced occassionally 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 *At*Rad54 did not result in an apparent phenotype during plant growth, development or reproduction. After infection, mild symptoms like leaf rolling (EuYMV, ClLCrV) or yellow mottling (EuYMV) appeared first at 9-11 dpi with no difference in severity or timing between plant lines (Fig. 2).

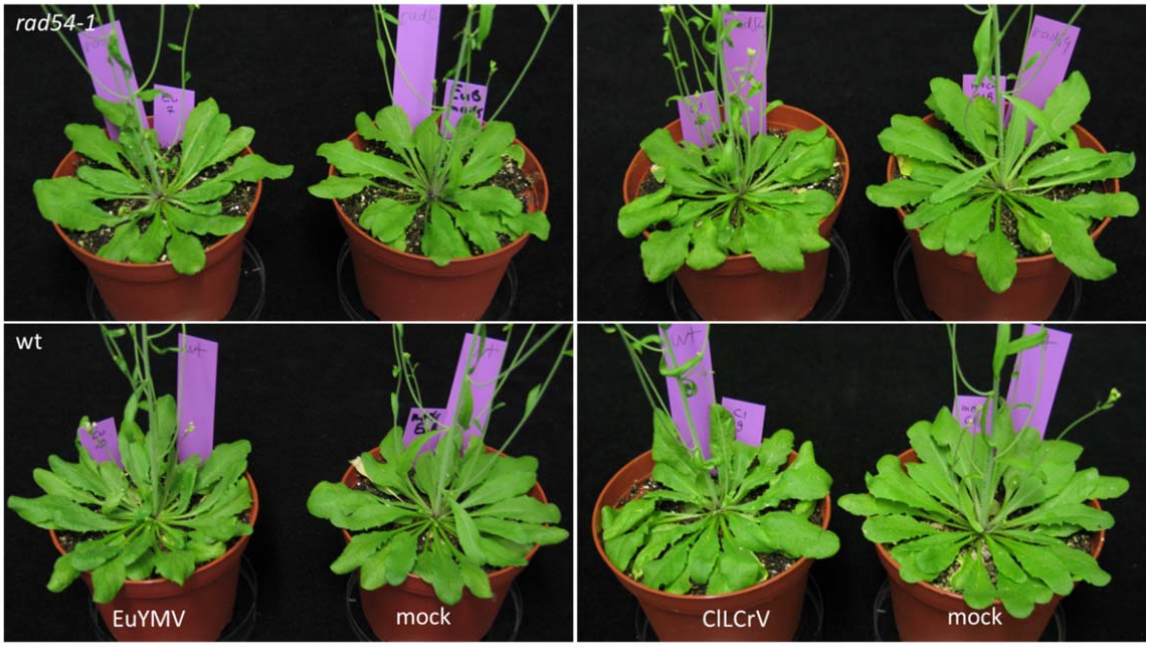

Figure 2: Symptoms of EuYMV and ClLCrV infections at 21 dpi of *rad54-1* and wt *A. thaliana*. Plants are shown in comparison to uninfected mock controls as described in Fig. 1. EuYMV and ClLCrV infected specimens showed leaf rolling or mild mottling (only for EuYMV) without differences between genotypes; *rad54-1* plants had no apparent phenotype and appeared similar to wt plants.

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 (hdsl) 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 (hdsl 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.

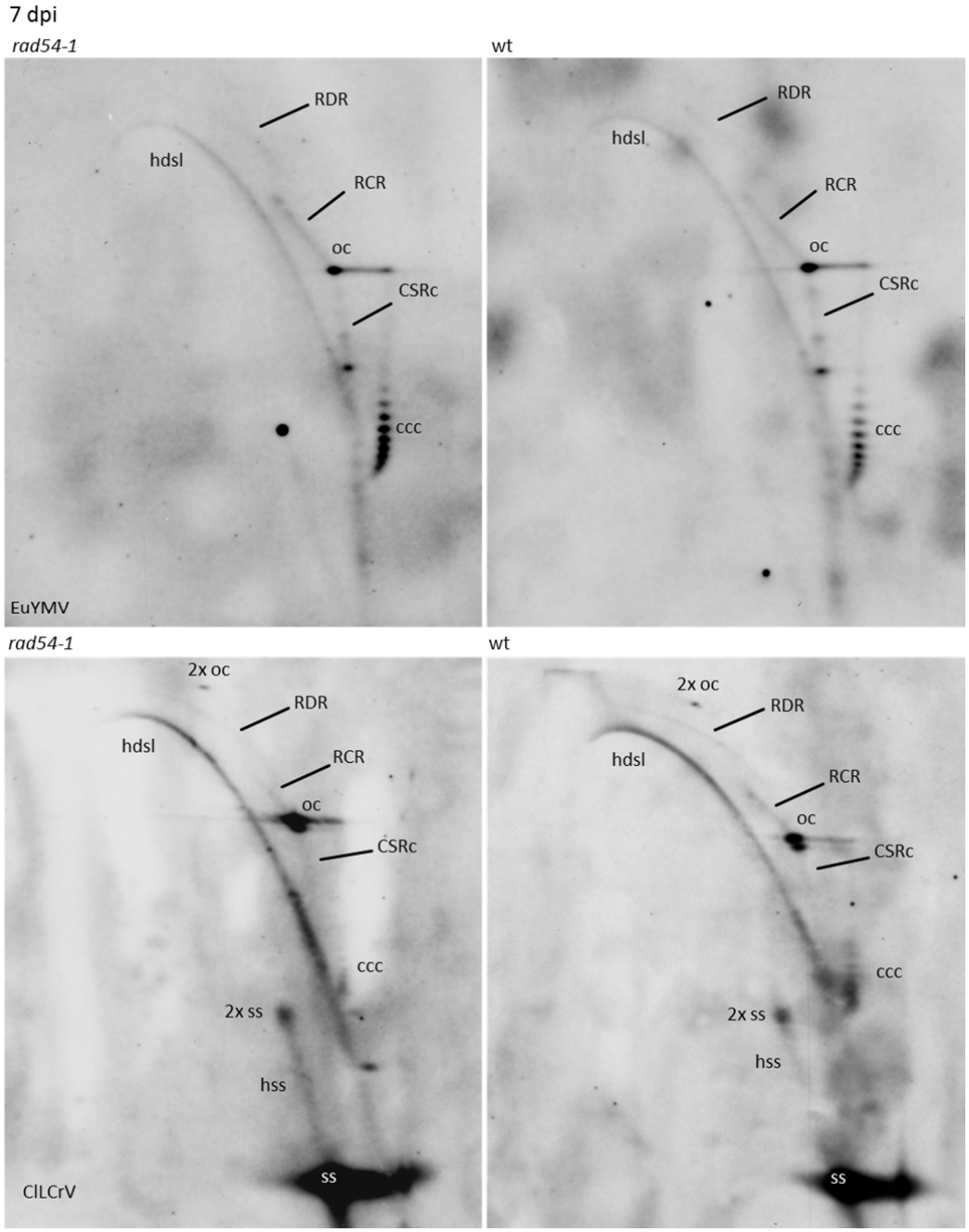

Figure 3: Examination of replicative intermediates at 7 dpi. Virus DNA was detected after 2D gel electrophoresis of individual samples of EuYMV and ClLCrV infected *rad54-1* and wt plants. DIG-labeled full-length DNA A probes of the respective virus were used for hybridization. Separation was performed 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.

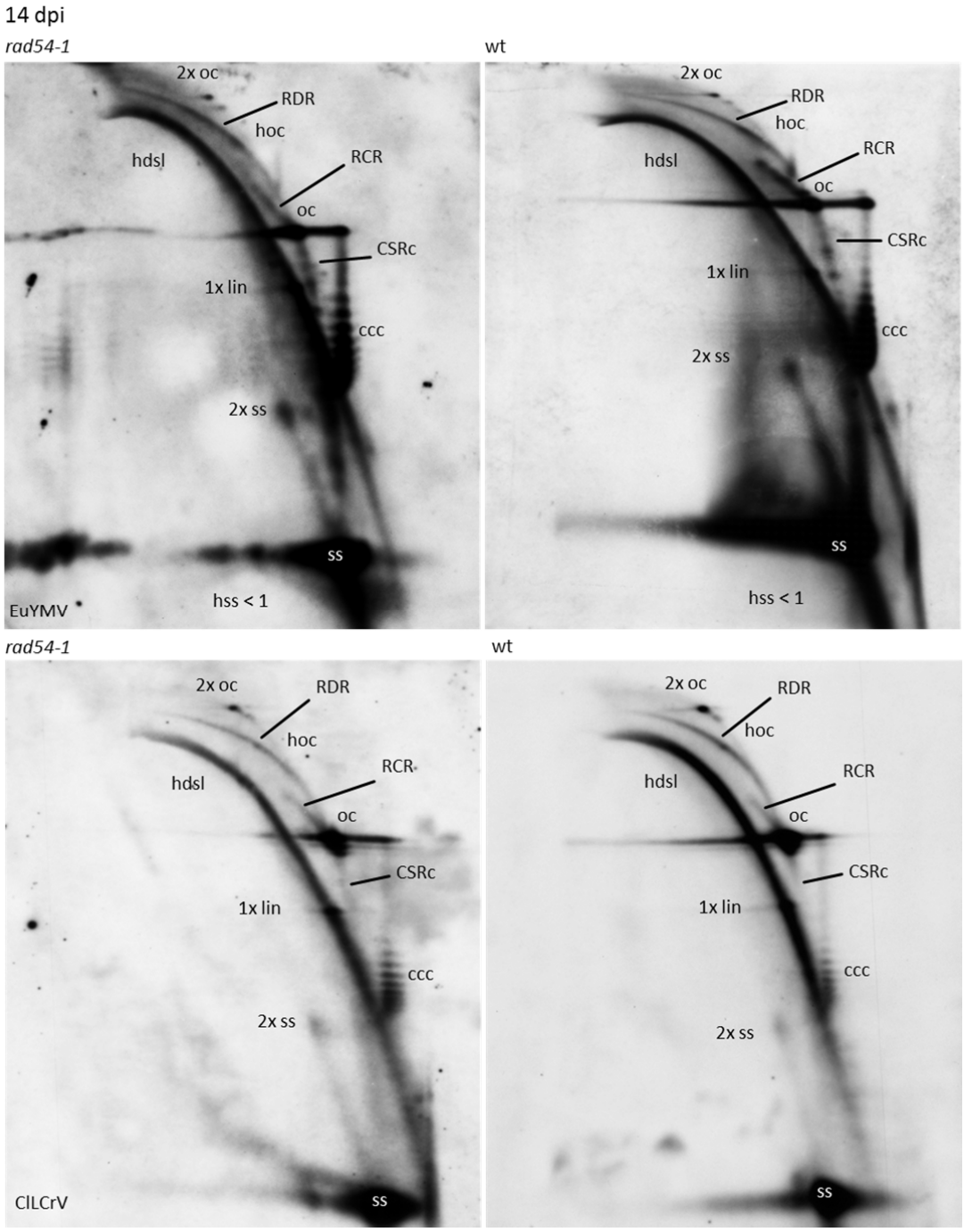

Figure 4: Examination of replicative intermediates at 14 dpi. Analysis as described in Fig. 3, but with pooled ten samples of EuYMV and ClLCrV from one inoculation experiment per genotype and virus infection. 400 ng of DNA was loaded per gel after RNAse A digestion. Additional DNA forms indicated: hoc (heterogeneous open circular); 1x lin (monomer linear double-stranded).

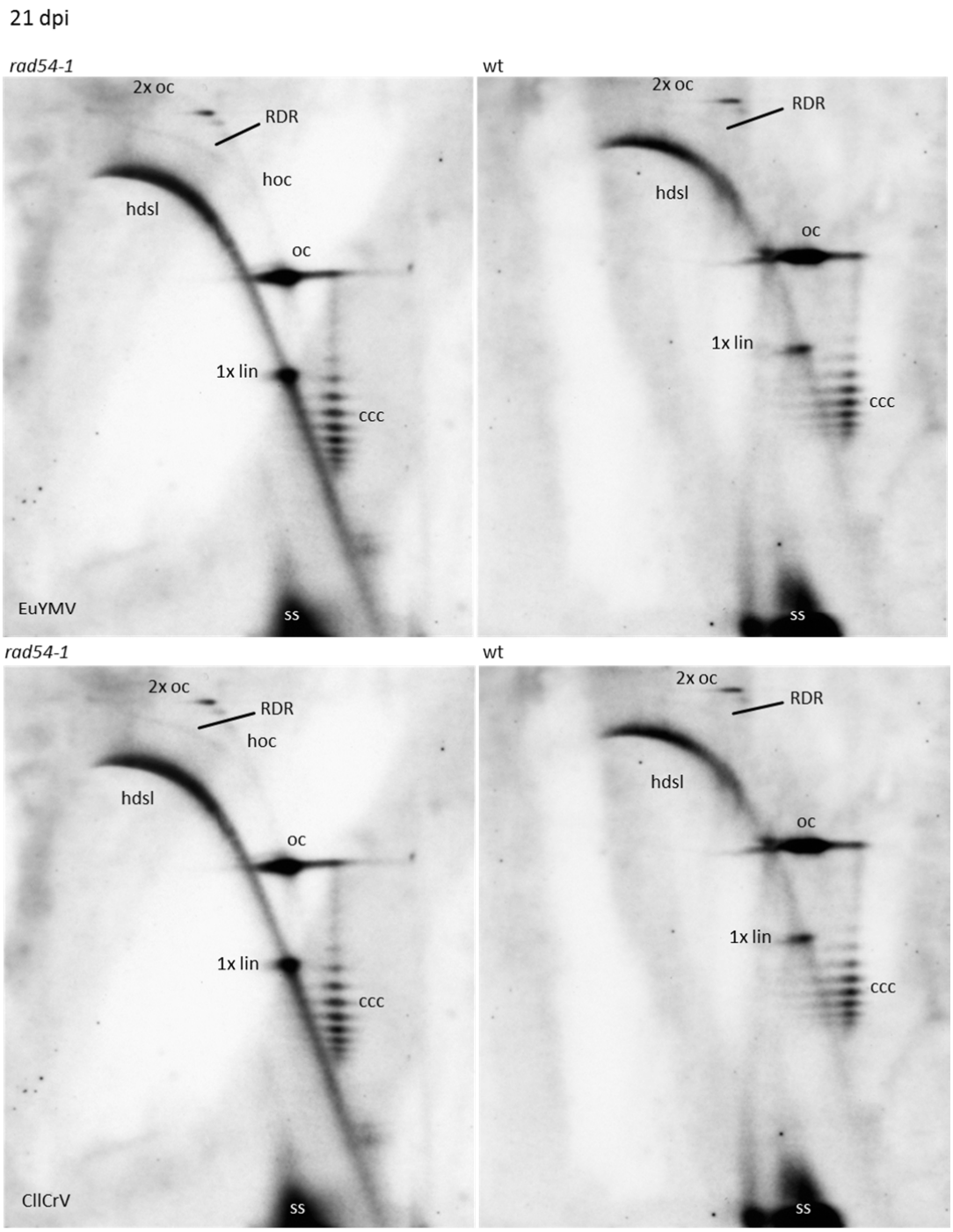

Figure 5: Examination of replicative intermediates at 21 dpi. Analysis of pooled samples as described in Fig. 4.

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 *Sc*Rad54 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 hdslDNA, 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 *At*Rad54 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 *At*Rad54 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 *At*RAD54 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 *At*Rad54 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

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

A. 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 (ClLCrV; DNA A: FN435999 and DNA B: FN436000) (Paprotka *et al.*, 2010). Renaming of EuMV to EuYMV was implemented according to Fernandes *et al.* (2011).

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'-TTACTCAAGTTTCCTTGGGGG-3'; Rad54-RT-LP: 5'-TCCTTCCATGGGGATGTTAG-3'; Rad54-RP: 5'-CTCGTTGAGAGATAACGGCTG-3') or to the T-DNA insertion (Salk-LBb1.3: 5'-ATTTTGCCGATTT CGGAAC-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'-

CCTCCTATTGCGTTGGCTATTG-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 H_2O. 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).

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References

Agarwal, S, van Cappellen, WA, Guenole, A, Eppink, B, Linsen, SE, Meijering, E, Houtsmuller, A, Kanaar, R, Essers, J (2011) ATP-dependent and independent functions of Rad54 in genome maintenance. *J Cell Biol* **192**: 735-750

Alberter, B, Rezaian, AM, Jeske, H (2005) Replicative intermediates of ToLCV and its satellite DNAs. *Virology* **331**: 441-448

Alonso, JM, Stepanova, AN, Leisse, TJ, Kim, CJ, Chen, H, Shinn, P, Stevenson, DK, Zimmerman, J, Barajas, P, Cheuk, R, Gadrinab, C, Heller, C, Jeske, A, Koesema, E, Meyers, CC, Parker, H, Prednis, L, Ansari, Y, Choy, N, Deen, H, Geralt, M, Hazari, N, Hom, E, Karnes, M, Mulholland, C, Ndubaku, R, Schmidt, I, Guzman, P, Aguilar-Henonin, L, Schmid, M, Weigel, D, Carter, DE, Marchand, T, Risseeuw, E, Brogden, D, Zeko, A, Crosby, WL, Berry, CC, Ecker, JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653-657

Ascencio-Ibanez, JT, Sozzani, R, Lee, TJ, Chu, TM, Wolfinger, RD, Cella, R, Hanley-Bowdoin, L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol* **148**: 436-454

Bach, J, Jeske, H (2014) Defective DNAs of beet curly top virus from long-term survivor sugar beet plants. *Virus Res* **183**: 89-94

Bleuyard, JY, Gallego, ME, Savigny, F, White, CI (2005) Differing requirements for the *Arabidopsis* Rad51 paralogs in meiosis and DNA repair. *Plant J* **41**: 533-545

Bugreev, DV, Mazina, OM, Mazin, AV (2006) Rad54 protein promotes branch migration of Holliday junctions. *Nature* **442**: 590-593

Ceballos, SJ, Heyer, WD (2011) Functions of the Snf2/Swi2 family Rad54 motor protein in homologous recombination. *Biochim Biophys Acta* **1809**: 509-523

Chapman, JR, Taylor, MR, Boulton, SJ (2012) Playing the end game: DNA double-strand break repair pathway choice. *Mol Cell* **47**: 497-510

Cheung, AK (2012) Replicative intermediates of porcine circovirus in animal tissue cultured cells or in bacteria undergoing copy-release replication. *Virology* **434**: 38-42

Chomczynski, P, Qasba, PK (1984) Alkaline transfer of DNA to plastic membrane. *Biochem Biophys Res Commun* **122**: 340-344

Choudhury, NR, Malik, PS, Singh, DK, Islam, MN, Kaliappan, K, Mukherjee, SK (2006) The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. *Nucleic Acids Res* **34**: 6362-6377

Clerot, D, Bernardi, F (2006) DNA helicase activity is associated with the replication initiator protein rep of tomato yellow leaf curl geminivirus. *J Virol* **80**: 11322-11330

Da Ines, O, Degroote, F, Amiard, S, Goubely, C, Gallego, ME, White, CI (2013) Effects of XRCC2 and RAD51B mutations on somatic and meiotic recombination in *Arabidopsis thaliana*. *Plant J* **74**: 959-970

Erdmann, JB, Shepherd, DB, Martin, DP, Varsani, A, Rybicki, EP, Jeske, H (2010) Replicative intermediates of Maize streak virus found during leaf development. *J Gen Virol* **91**: 1077-1081

Fernandes, FR, Albuquerque, LC, de Oliveira, CL, Cruz, AR, da Rocha, WB, Pereira, TG, Naito, FY, Dias Nde, M, Nagata, T, Faria, JC, Zerbini, FM, Aragao, FJ, Inoue-Nagata, AK (2011) Molecular and biological characterization of a new Brazilian begomovirus, euphorbia yellow mosaic virus (EuYMV), infecting *Euphorbia heterophylla* plants. *Arch Virol* **156**: 2063-2069

Golub, EI, Kovalenko, OV, Gupta, RC, Ward, DC, Radding, CM (1997) Interaction of human recombination proteins Rad51 and Rad54. *Nucleic Acids Res* **25**: 4106-4110

Green, MR, Sambrook, J, 2012. Molecular Cloning: A Laboratory Manual, 4th ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Gutierrez, C, Ramirez-Parra, E, Mar Castellano, M, Sanz-Burgos, AP, Luque, A, Missich, R (2004) Geminivirus DNA replication and cell cycle interactions. *Vet Microbiol* **98**: 111-119

Haible, D, Kober, S, Jeske, H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J Virol Methods* **135**: 9-16

Hanley-Bowdoin, L, Bejarano, ER, Robertson, D, Mansoor, S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat Rev Microbiol* **11**: 777-788

Hipp, K, Rau, P, Schafer, B, Gronenborn, B, Jeske, H (2014) The RXL motif of the African cassava mosaic virus Rep protein is necessary for rereplication of yeast DNA and viral infection in plants. *Virology* **462-463**: 189-198

Jasin, M, Rothstein, R (2013) Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol* **5**: a012740

Jeske, H, **(2007)**. Replication of geminiviruses and the use of rolling circle amplification for their diagnosis, in: *Tomato yellow leaf curl virus disease*, pp. 141-156. Edited by Czosnek, H. Springer, Dordrecht.

Jeske, H, **(2009)**. Geminiviruses, in: *Torque Teno Virus: The Still Elusive Human Pathogens*, pp. 185-226. Edited by zur Hausen, H., de Villiers, E.-M. Springer, Berlin.

Jeske, H, Lütgemeier, M, Preiss, W (2001) Distinct DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic geminivirus. *EMBO J* **20**: 6158-6167

Jiang, H, Xie, Y, Houston, P, Stemke-Hale, K, Mortensen, UH, Rothstein, R, Kodadek, T (1996) Direct association between the yeast Rad51 and Rad54 recombination proteins. *J Biol Chem* **271**: 33181-33186

Kaliappan, K, Choudhury, NR, Suyal, G, Mukherjee, SK (2012) A novel role for RAD54: this host protein modulates geminiviral DNA replication. *FASEB J* **26**: 1142-1160

Klutstein, M, Shaked, H, Sherman, A, Avivi-Ragolsky, N, Shema, E, Zenvirth, D, Levy, AA, Simchen, G (2008) Functional conservation of the yeast and *Arabidopsis* RAD54-like genes. *Genetics* **178**: 2389-2397

Knoll, A, Fauser, F, Puchta, H (2014) DNA recombination in somatic plant cells: mechanisms and evolutionary consequences. *Chromosome Res* **22**: 191-201

Kong, LJ, Orozco, BM, Roe, JL, Nagar, S, Ou, S, Feiler, HS, Durfee, T, Miller, AB, Gruissem, W, Robertson, D, Hanley-Bowdoin, L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J* **19**: 3485-3495

Laufs, J, Traut, W, Heyraud, F, Matzeit, V, Rogers, SG, Schell, J, Gronenborn, B (1995) *In vitro* cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proc Natl Acad Sci U S A* **92**: 3879-3883

Lazarowitz, SG, Pinder, AJ, Damsteegt, VD, Rogers, SG (1989) Maize streak virus genes essential for systemic spread and symptom development. *EMBO J* **8**: 1023-1032

Lefeuvre, P, Martin, DP, Hoareau, M, Naze, F, Delatte, H, Thierry, M, Varsani, A, Becker, N, Reynaud, B, Lett, JM (2007) Begomovirus 'melting pot' in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. *J Gen Virol* **88**: 3458-3468

Li, X, Heyer, WD (2008) Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* **18**: 99-113

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

Li, X, Zhang, XP, Solinger, JA, Kiianitsa, K, Yu, X, Egelman, EH, Heyer, WD (2007) Rad51 and Rad54 ATPase activities are both required to modulate Rad51-dsDNA filament dynamics. *Nucleic Acids Res* **35**: 4124-4140

Maher, RL, Branagan, AM, Morrical, SW (2011) Coordination of DNA replication and recombination activities in the maintenance of genome stability. *J Cell Biochem* **112**: 2672-2682

Matulova, P, Marini, V, Burgess, RC, Sisakova, A, Kwon, Y, Rothstein, R, Sung, P, Krejci, L (2009) Cooperativity of Mus81.Mms4 with Rad54 in the resolution of recombination and replication intermediates. *J Biol Chem* **284**: 7733-7745

Mazin, AV, Alexeev, AA, Kowalczykowski, SC (2003) A novel function of Rad54 protein. Stabilization of the Rad51 nucleoprotein filament. *J Biol Chem* **278**: 14029-14036

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

Mazina, OM, Mazin, AV (2004) Human Rad54 protein stimulates DNA strand exchange activity of hRad51 protein in the presence of Ca2+. *J Biol Chem* **279**: 52042-52051

Mazina, OM, Mazin, AV (2008) Human Rad54 protein stimulates human Mus81-Eme1 endonuclease. *Proc Natl Acad Sci U S A* **105**: 18249-18254

Mestiri, I, Norre, F, Gallego, ME, White, CI (2014) Multiple host-cell recombination pathways act in Agrobacterium-mediated transformation of plant cells. *Plant J* **77**: 511-520

Nagar, S, Hanley-Bowdoin, L, Robertson, D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**: 2995-3007

Osakabe, K, Abe, K, Yoshioka, T, Osakabe, Y, Todoriki, S, Ichikawa, H, Hohn, B, Toki, S (2006) Isolation and characterization of the RAD54 gene from *Arabidopsis thaliana*. *Plant J* **48**: 827-842

Padidam, M, Sawyer, S, Fauquet, CM (1999) Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**: 218-225

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

Paprotka, T, Metzler, V, Jeske, H (2010) The first DNA 1-like alpha-satellites in association with New World begomoviruses in natural infections. *Virology* **404**: 148-157

Patil, BL, Dasgupta, I (2006) Defective interfering DNAs of plant viruses. *Crit Rev Plant Sci* **24**: 1–18

Petukhova, G, Stratton, S, Sung, P (1998) Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. *Nature* **393**: 91-94

Pilartz, M, Jeske, H (2003) Mapping of abutilon mosaic geminivirus minichromosomes. *J Virol* **77**: 10808-10818

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Puchta, H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J Exp Bot* **56**: 1-14

Raghavan, V, Malik, PS, Choudhury, NR, Mukherjee, SK (2004) The DNA-A component of a plant geminivirus (Indian mung bean yellow mosaic virus) replicates in budding yeast cells. *J Virol* **78**: 2405-2413

Richter, KS, Kleinow, T, Jeske, H (2014) Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Virology* **452-453**: 287-296

Rigden, JE, Dry, IB, Krake, LR, Rezaian, MA (1996) Plant virus DNA replication processes in Agrobacterium: insight into the origins of geminiviruses? *Proc Natl Acad Sci U S A* **93**: 10280-10284

Rocha, CS, Castillo-Urquiza, GP, Lima, AT, Silva, FN, Xavier, CA, Hora-Junior, BT, Beserra-Junior, JE, Malta, AW, Martin, DP, Varsani, A, Alfenas-Zerbini, P, Mizubuti, ES, Zerbini, FM (2013) Brazilian begomovirus populations are highly recombinant, rapidly evolving, and segregated based on geographical location. *J Virol* **87**: 5784-5799

Ruschhaupt, M, Martin, DP, Lakay, F, Bezuidenhout, M, Rybicki, EP, Jeske, H, Shepherd, DN (2013) Replication modes of Maize streak virus mutants lacking RepA or the RepA-pRBR interaction motif. *Virology* **442**: 173-179

Sahu, PP, Sharma, N, Puranik, S, Muthamilarasan, M, Prasad, M (2014) Involvement of host regulatory pathways during geminivirus infection: a novel platform for generating durable resistance. *Funct Integr Genomics* **14**: 47-58

Santiago, MJ, Alejandre-Duran, E, Munoz-Serrano, A, Ruiz-Rubio, M (2008) Two translesion synthesis DNA polymerase genes, AtPOLH and AtREV1, are involved in development and UV light resistance in *Arabidopsis*. *J Plant Physiol* **165**: 1582-1591

Selth, LA, Randles, JW, Rezaian, MA (2002) *Agrobacterium tumefaciens* supports DNA replication of diverse geminivirus types. *FEBS Lett* **516**: 179-182

Snapka, RM, Permana, PA, Marquit, G, Shin, CG (1991) Two-dimensional agarose gel electrophoresis of simian virus 40 DNA replication intermediates. *Methods* **3**: 73-82

Solinger, JA, Kiianitsa, K, Heyer, WD (2002) Rad54, a Swi2/Snf2-like recombinational repair protein, disassembles Rad51:dsDNA filaments. *Mol Cell* **10**: 1175-1188

Solinger, JA, Lutz, G, Sugiyama, T, Kowalczykowski, SC, Heyer, WD (2001) Rad54 protein stimulates heteroduplex DNA formation in the synaptic phase of DNA strand exchange via specific interactions with the presynaptic Rad51 nucleoprotein filament. *J Mol Biol* **307**: 1207-1221

Stanley, J (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology* **206**: 707-712

Stenger, DC, Revington, GN, Stevenson, MC, Bisaro, DM (1991) Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proc Natl Acad Sci U S A* **88**: 8029-8033

Swagemakers, SM, Essers, J, de Wit, J, Hoeijmakers, JH, Kanaar, R (1998) The human RAD54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase. *J Biol Chem* **273**: 28292-28297

Symington, LS (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* **66**: 630-670

van der Walt, E, Rybicki, EP, Varsani, A, Polston, JE, Billharz, R, Donaldson, L, Monjane, AL, Martin, DP (2009) Rapid host adaptation by extensive recombination. *J Gen Virol* **90**: 734-746

Varsani, A, Shepherd, DN, Monjane, AL, Owor, BE, Erdmann, JB, Rybicki, EP, Peterschmitt, M, Briddon, RW, Markham, PG, Oluwafemi, S, Windram, OP, Lefeuvre, P, Lett, JM, Martin,

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

Wang, YX, Xiao, R, Wang, HF, Cheng, ZH, Li, WX, Zhu, GF, Wang, Y, Ma, H (2014) The *Arabidopsis* RAD51 paralogs RAD51B, RAD51D and XRCC2 play partially redundant roles in somatic DNA repair and gene regulation. *New Phytol* **201**: 292-304

West, SC (2003) Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol* **4**: 435-445

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

Supplementary data

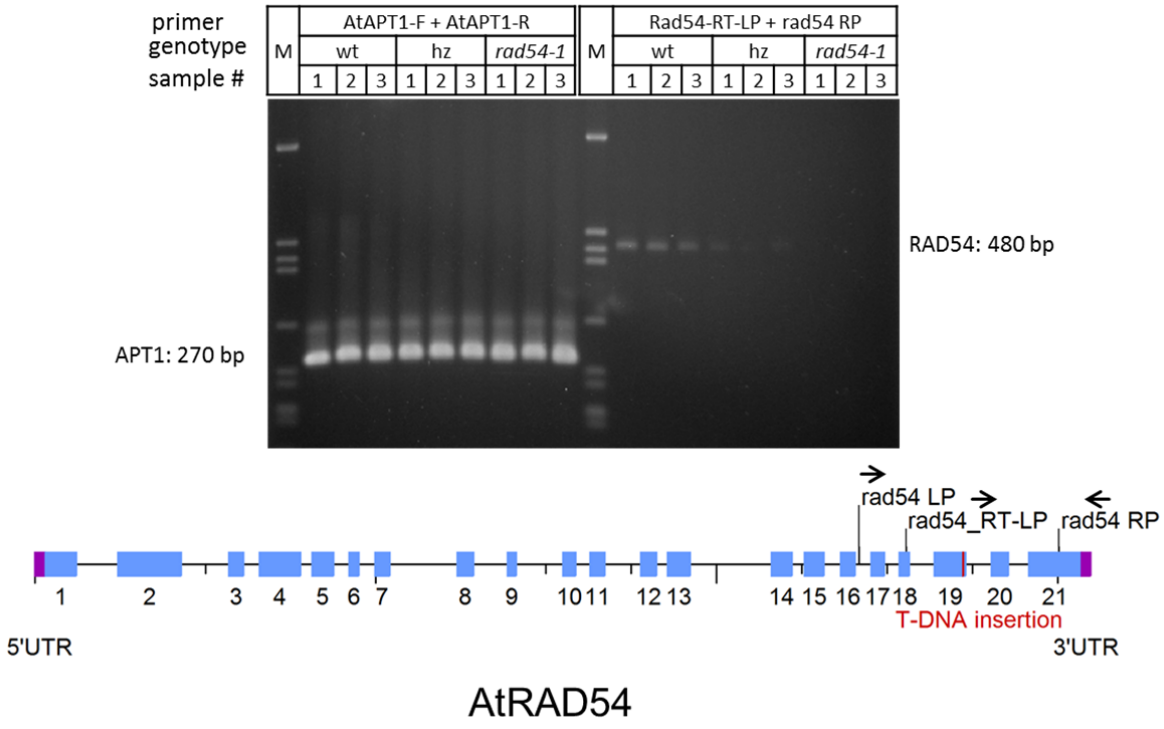

Figure S1: Detection of RAD54 and APT1 transcripts by RT-PCR. Three individual samples from wt, hemizygous (hz) or *rad54-1* plants were tested for presence of RAD54 transcripts. Amplification products of APT1 transcripts were used as internal control. Homozygous *rad54-1* plants contained no expected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

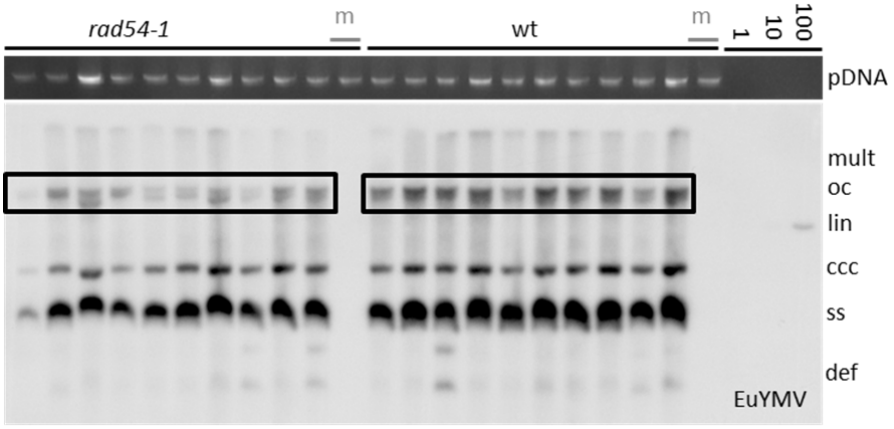

Figure S2: Southern Blot hybridization of 1D gel-separated EuMV DNA from each ten inoculated rad54 or wt *A. thaliana* plants at 14 dpi from one inoculation experiment diverging from the other two experiments. OcDNA of most *rad54-1* plants appeared slightly reduced compared to wt plants. See Fig. 1 for techniques and abbreviations.

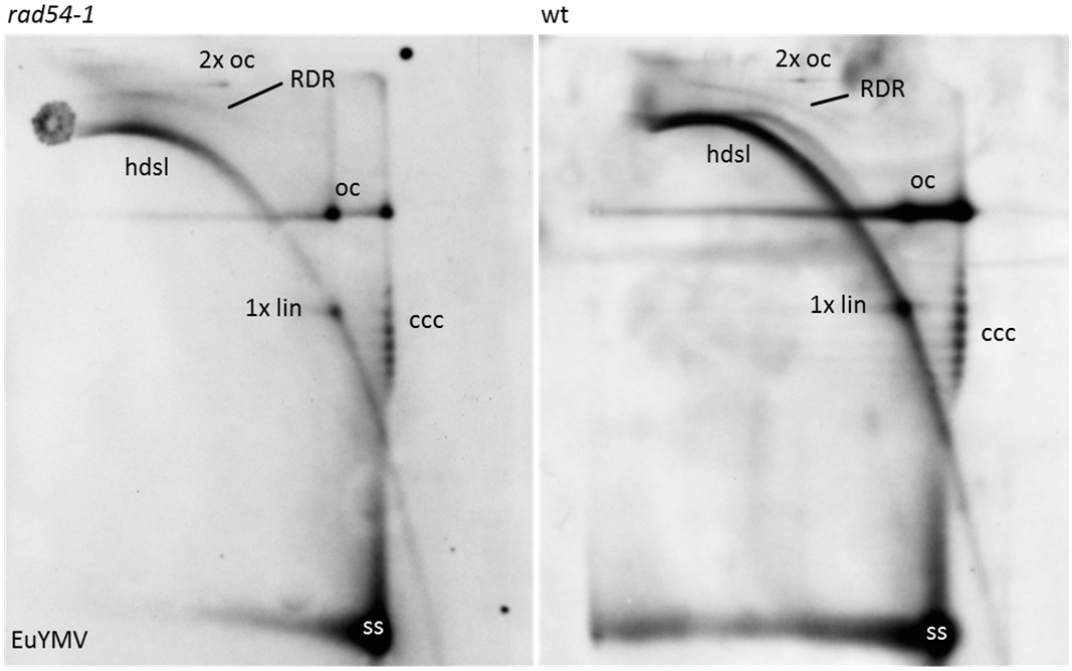

Figure S3: 2D gel analysis of pooled samples of EuMV infected *rad54-1* and wt plants at 14 dpi from the one diverging inoculation experiment. OcDNA and also hdslDNA appeared slightly reduced in *rad54* samples as already visible in Fig. S2. All ten samples from one inoculation experiment per genotype 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

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#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).

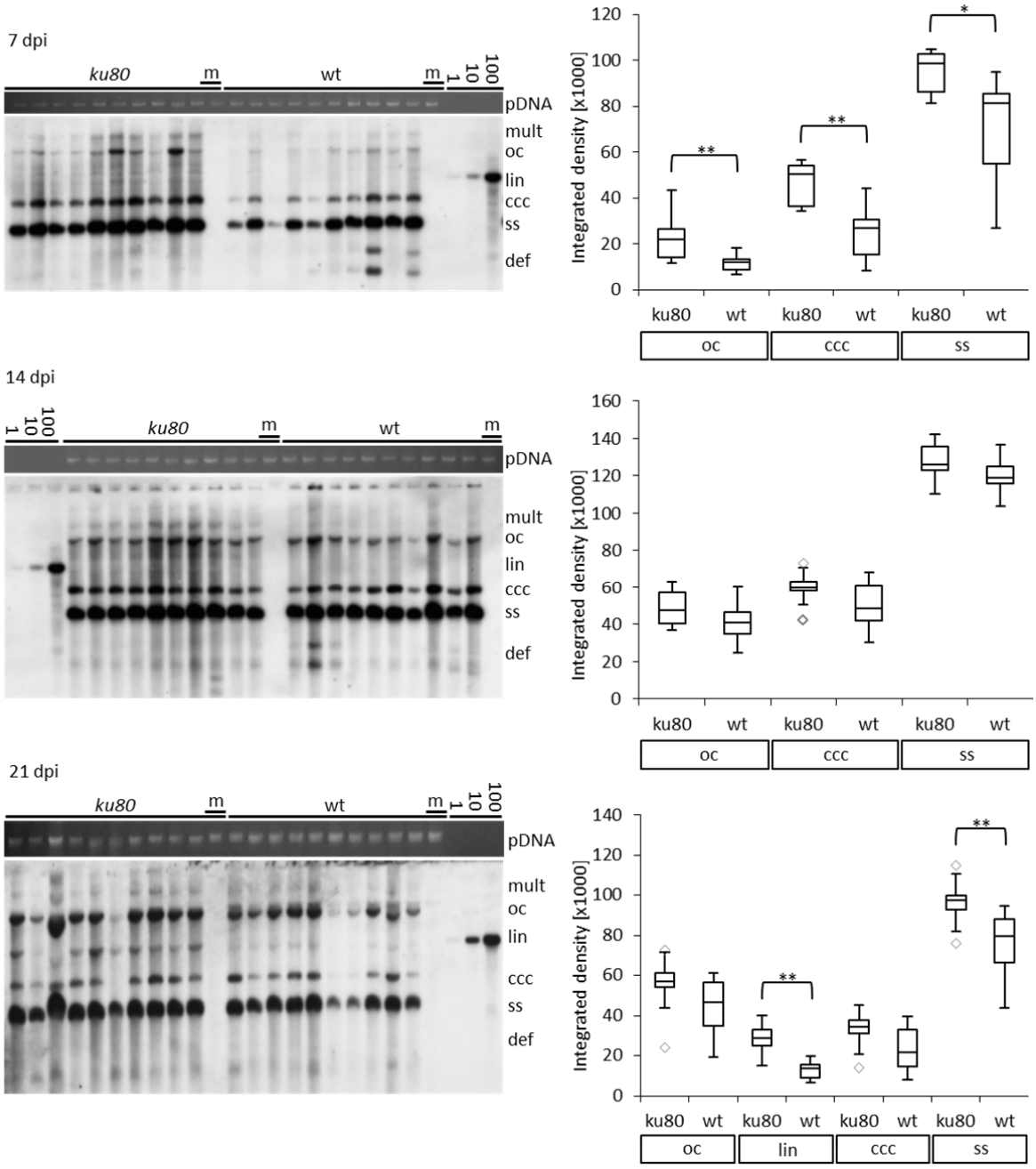

Figure 1: Emergence of EuYMV DNA forms in *ku80* and wt *A. thaliana* plants at 7, 14 and 21 dpi. Total nucleic acids (500 ng each) from ten inoculated plants per genotype were separated in 1.4 % agarose gels in the presence of 5 µg/ml ethidium bromide. Genomic plant DNA (pDNA) is shown as loading control. 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),

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 whisker 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).

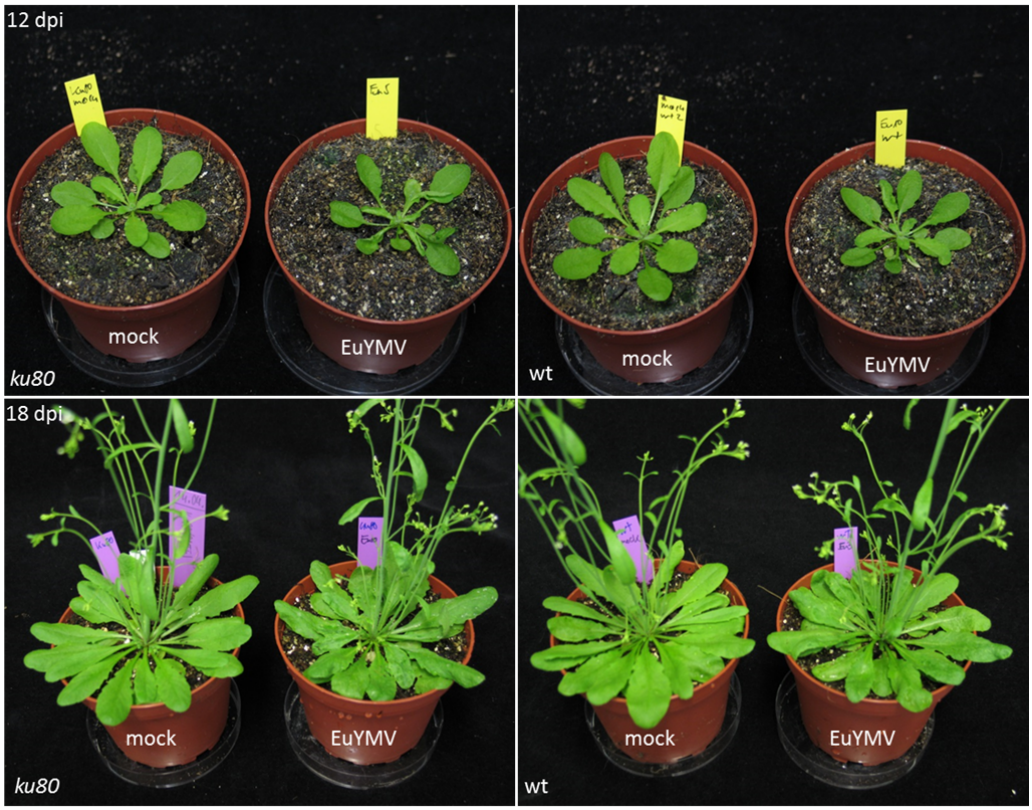

Figure 2: Symptoms of EuYMV-infected plants at 12 and 18 dpi in comparison to mock-inoculated *ku80* and wt *A. thaliana*. EuYMV infection caused mild leaf rolling, crumpling and mottling without apparent differences between genotypes. Knock-out of Ku80 did not cause an apparent phenotype as described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

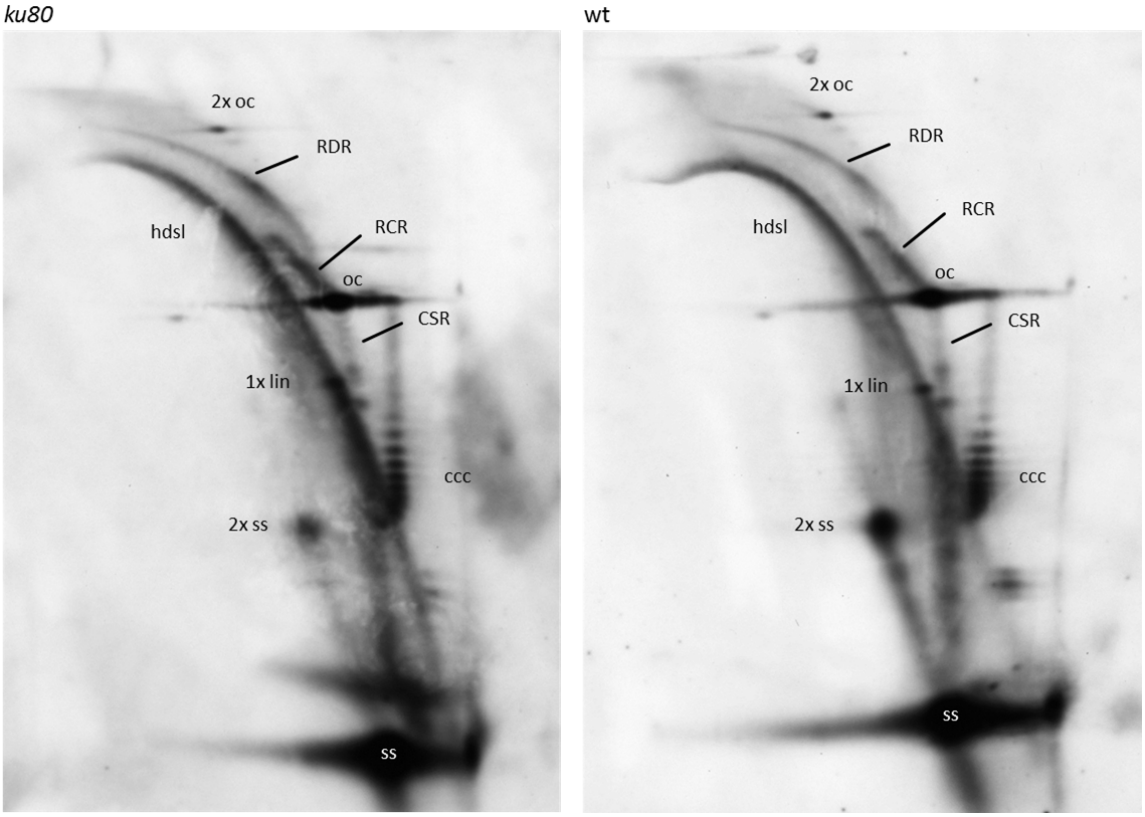

Figure 3: Intermediates and products of EuYMV replication at 7 dpi in *ku80* and wt plants. Nucleic acids of ten samples per genotype (see Fig. 1a) were pooled, digested with RNase A, and DNA (400 ng per gel in total) was separated in 2D gels as described by Richter *et al*. (2015). Viral DNA was detected 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 (Plchova *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.

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References

Ascencio-Ibanez, JT, Sozzani, R, Lee, TJ, Chu, TM, Wolfinger, RD, Cella, R, Hanley-Bowdoin, L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol* **148**: 436-454

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

Gallego, ME, Bleuyard, JY, Daoudal-Cotterell, S, Jallut, N, White, CI (2003a) Ku80 plays a role in non-homologous recombination but is not required for T-DNA integration in *Arabidopsis*. *Plant J* **35**: 557-565

Gallego, ME, Jalut, N, White, CI (2003b) Telomerase dependence of telomere lengthening in Ku80 mutant *Arabidopsis*. *Plant Cell* **15**: 782-789

Glazov, E, Phillips, K, Budziszewski, GJ, Schob, H, Meins, F, Jr., Levin, JZ (2003) A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*. *Plant J* **35**: 342-349

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

Hanley-Bowdoin, L, Bejarano, ER, Robertson, D, Mansoor, S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat Rev Microbiol* **11**: 777-788

Hartung, F, Plchova, H, Puchta, H (2000) Molecular characterisation of RecQ homologues in *Arabidopsis thaliana*. *Nucleic Acids Res* **28**: 4275-4282

Hartung, F, Puchta, H (2006) The RecQ gene family in plants. *J Plant Physiol* **163**: 287-296

Hipp, K, Rau, P, Schafer, B, Gronenborn, B, Jeske, H (2014) The RXL motif of the African cassava mosaic virus Rep protein is necessary for rereplication of yeast DNA and viral infection in plants. *Virology* **462-463C**: 189-198

Jeske, H, **(2007)**. Replication of geminiviruses and the use of rolling circle amplification for their diagnosis, in: *Tomato yellow leaf curl virus disease*, pp. 141-156. Edited by Czosnek, H. Springer, Dordrecht.

Jeske, H, **(2009)**. Geminiviruses, in: *Torque Teno Virus: The Still Elusive Human Pathogens*, pp. 185-226. Edited by zur Hausen, H., de Villiers, E.-M. Springer, Berlin.

Jeske, H, Lutgemeier, M, Preiss, W (2001) DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO J* **20**: 6158-6167

Ketting, RF, Haverkamp, TH, van Luenen, HG, Plasterk, RH (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**: 133-141

Kittelmann, K, Rau, P, Gronenborn, B, Jeske, H (2009) Plant geminivirus rep protein induces rereplication in fission yeast. *J Virol* **83**: 6769-6778

Knoll, A, Puchta, H (2011) The role of DNA helicases and their interaction partners in genome stability and meiotic recombination in plants. *J Exp Bot* **62**: 1565-1579

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

Li, B, Conway, N, Navarro, S, Comai, L (2005) A conserved and species-specific functional interaction between the Werner syndrome-like exonuclease atWEX and the Ku heterodimer in *Arabidopsis*. *Nucleic Acids Res* **33**: 6861-6867

Mannuss, A, Trapp, O, Puchta, H (2012) Gene regulation in response to DNA damage. *Biochim Biophys Acta* **1819**: 154-165

Nagar, S, Hanley-Bowdoin, L, Robertson, D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**: 2995-3007

Paprotka, T, Deuschle, K, Pilartz, M, Jeske, H (2015) Form follows function in geminiviral minichromosome architecture. *Virus Res* **196**: 44-55

Plchova, H, Hartung, F, Puchta, H (2003) Biochemical characterization of an exonuclease from *Arabidopsis thaliana* reveals similarities to the DNA exonuclease of the human Werner syndrome protein. *J Biol Chem* **278**: 44128-44138

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Puchta, H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J Exp Bot* **56**: 1-14

Richter, KS, Ende, L, Jeske, H (2015) Rad54 is not essential for any geminiviral replication mode in planta. *Plant Mol Biol* **87**: 193-202

Richter, KS, Kleinow, T, Jeske, H (2014) Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Virology* **452-453**: 287-296

Szittya, G, Burgyan, J (2013) RNA interference-mediated intrinsic antiviral immunity in plants. *Curr Top Microbiol* **371**: 153-181

Tamura, K, Adachi, Y, Chiba, K, Oguchi, K, Takahashi, H (2002) Identification of Ku70 and Ku80 homologues in *Arabidopsis thaliana*: evidence for a role in the repair of DNA double-strand breaks. *Plant J* **29**: 771-781

Tops, BB, Tabara, H, Sijen, T, Simmer, F, Mello, CC, Plasterk, RH, Ketting, RF (2005) RDE-2 interacts with MUT-7 to mediate RNA interference in *Caenorhabditis elegans*. *Nucleic Acids Res* **33**: 347-355

Trinks, D, Rajeswaran, R, Shivaprasad, PV, Akbergenov, R, Oakeley, EJ, Veluthambi, K, Hohn, T, Pooggin, MM (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J Virol* **79**: 2517-2527

West, CE, Waterworth, WM, Story, GW, Sunderland, PA, Jiang, Q, Bray, CM (2002) Disruption of the *Arabidopsis At*Ku80 gene demonstrates an essential role for *At*Ku80 protein in efficient repair of DNA double-strand breaks *in vivo*. *Plant J* **31**: 517-528

Zellinger, B, Akimcheva, S, Puizina, J, Schirato, M, Riha, K (2007) Ku suppresses formation of telomeric circles and alternative telomere lengthening in *Arabidopsis*. *Mol Cell* **27**: 163-169

Zipfel, C (2014) Plant pattern-recognition receptors. *Trends Immunol* **35**: 345-351

Zvereva, AS, Pooggin, MM (2012) Silencing and innate immunity in plant defense against viral and non-viral pathogens. *Viruses* **4**: 2578-2597

Supplementary data

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

Gene	Line no.	Gene no.
RAD51	GK_134A01	At5g20850
RAD51B	Salk_024755C	At2g28560
RAD51C	Salk_021960	At2g45280
RAD51D	Sail_564_A06	At1g07745
RAD54	Salk_038057C	At3g19210
XRCC2	Salk_029106	At5g64520
XRCC3	Salk_045564	At5g57450
RAD17	Salk_009384C	At5g66130
MUS81	GK_113F11	At4g30870
RAD52-1	Sail_25_H08	At1g71310
POLH	Salk_129731	At5g44740
REV3	Salk_029237	At1g67500
REV1	Salk_005721C	At5g44750
POLK	Salk_081715*	At1g49980
“	Flag_566E01*	“
RAD5A	Salk_047150	At5g22750
UBC2	Salk_060994	At2g02760

*Knock-out not unequivocally confirmed

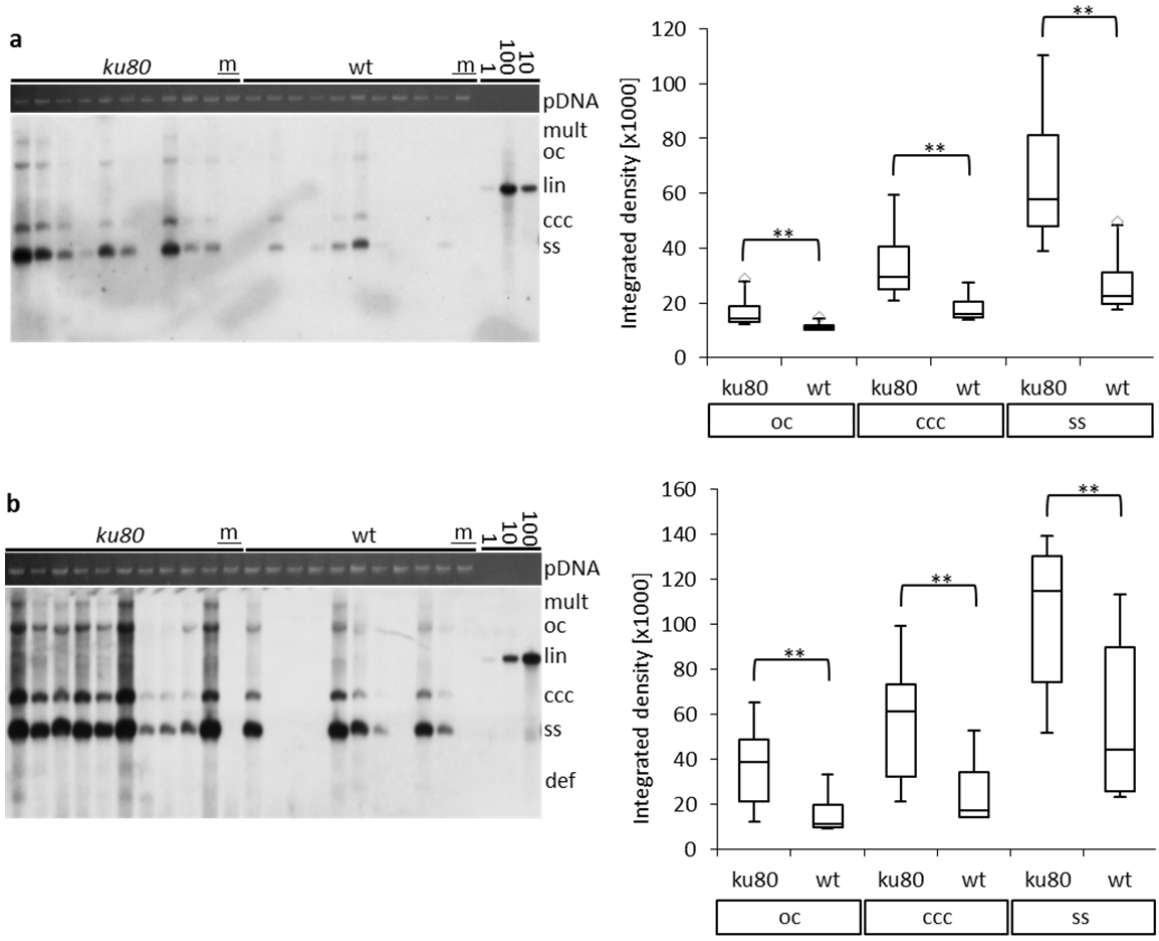

Figure S1: Emergence of EuYMV DNA forms in *ku80* and wt *A. thaliana* plants at 7 dpi for a second (b) and third (c) biological replicate. Viral DNA was separated, detected and analyzed as described for Fig. 1.

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

Kathrin S. Richter[1], Heïdi Serra[2], Charles I. White[2], and Holger Jeske[1#]

[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.

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 remodeller 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.*,

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

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 *At*Rad51 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 (hdsl) DNA, which is the output of RDR, as well as ocDNA, ssDNA and cccDNA were equally prevalent.

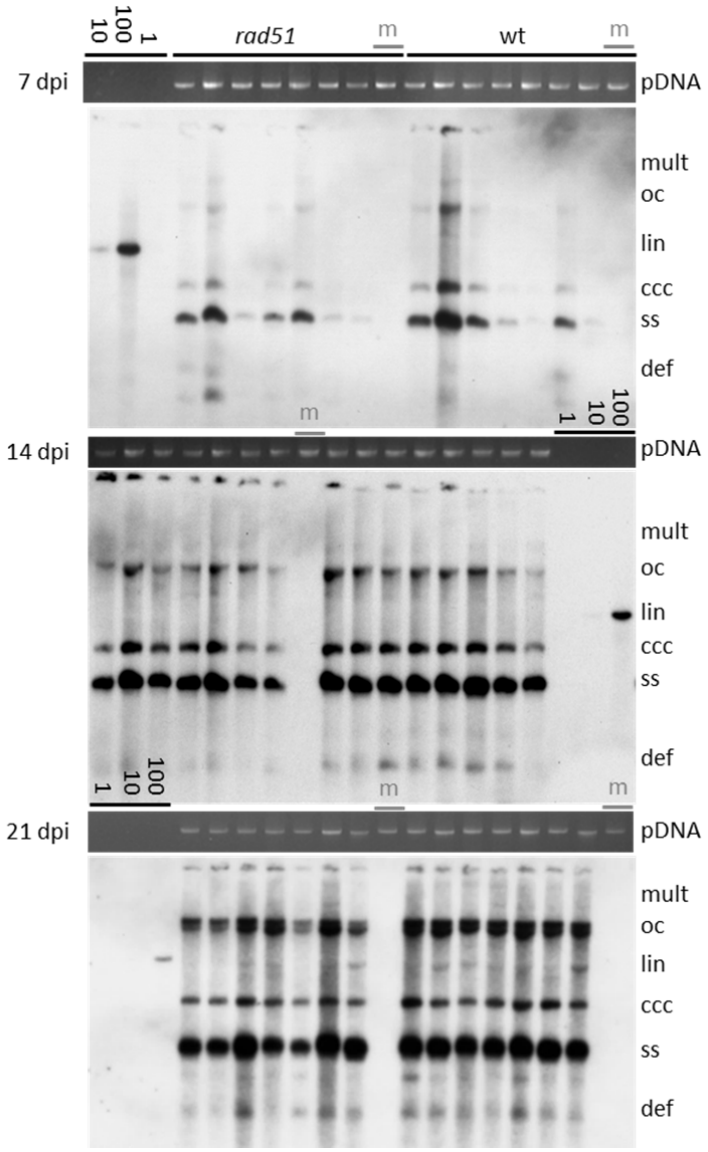

Figure 1: EuYMV DNA emergence in *rad51* and wt *A. thaliana* plants at 7, 14 and 21 dpi. Seven plants per genotype were inoculated and total nucleic acids (500 ng each) were separated in agarose gels (1.4 %) with 5 µg/ml ethidium bromide. Mock controls (m) were inoculated with EuYMV DNA B alone. Virus 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).

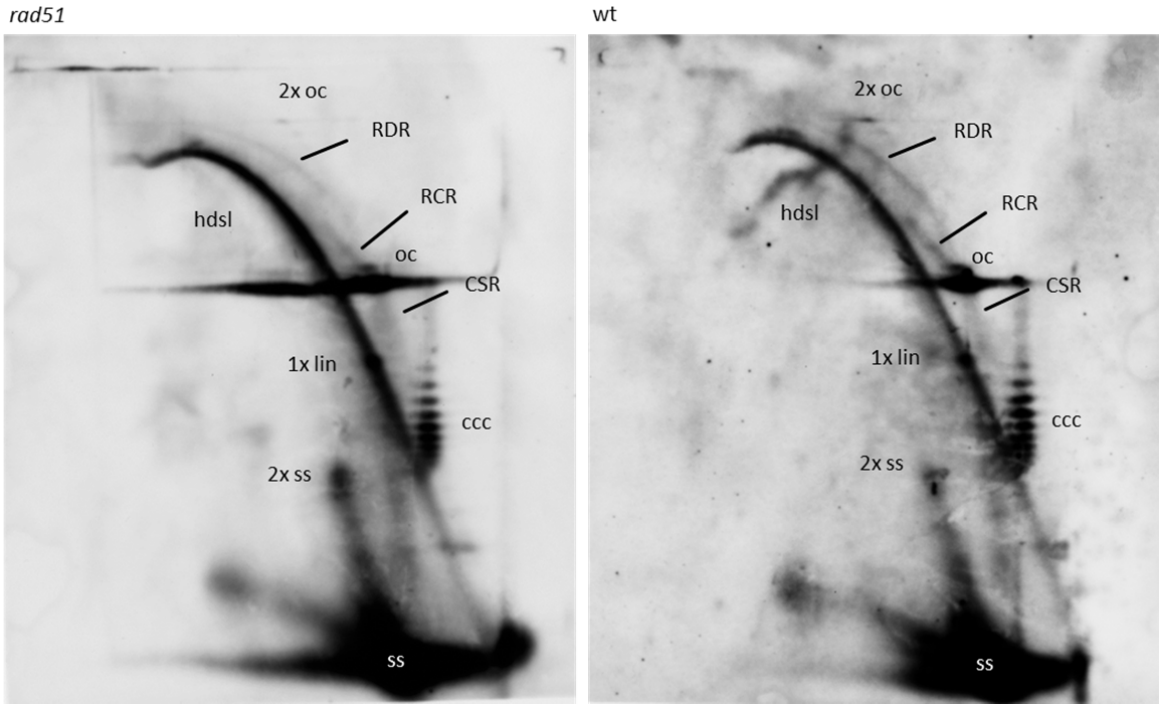

Figure 2: Replicative intermediates and end products of EuYMV from *rad51* and wt plants at 14 dpi. Nucleic acids of the samples described in Fig. 1 were pooled, digested with RNase A, DNA (300 ng per gel in total) was separated in 2D gels and viral DNA detected by hybridization. In addition to the DNA forms described in Fig. 1, CSR (complementary strand replication on circular templates), RCR (rolling circle replication), RDR (recombination-dependent replication), hdsl (heterogeneous double-stranded linear) are indicated. 1x or 2x signify monomeric or dimeric genomic length forms.

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*, *xrcc2* or *xrcc3* 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).

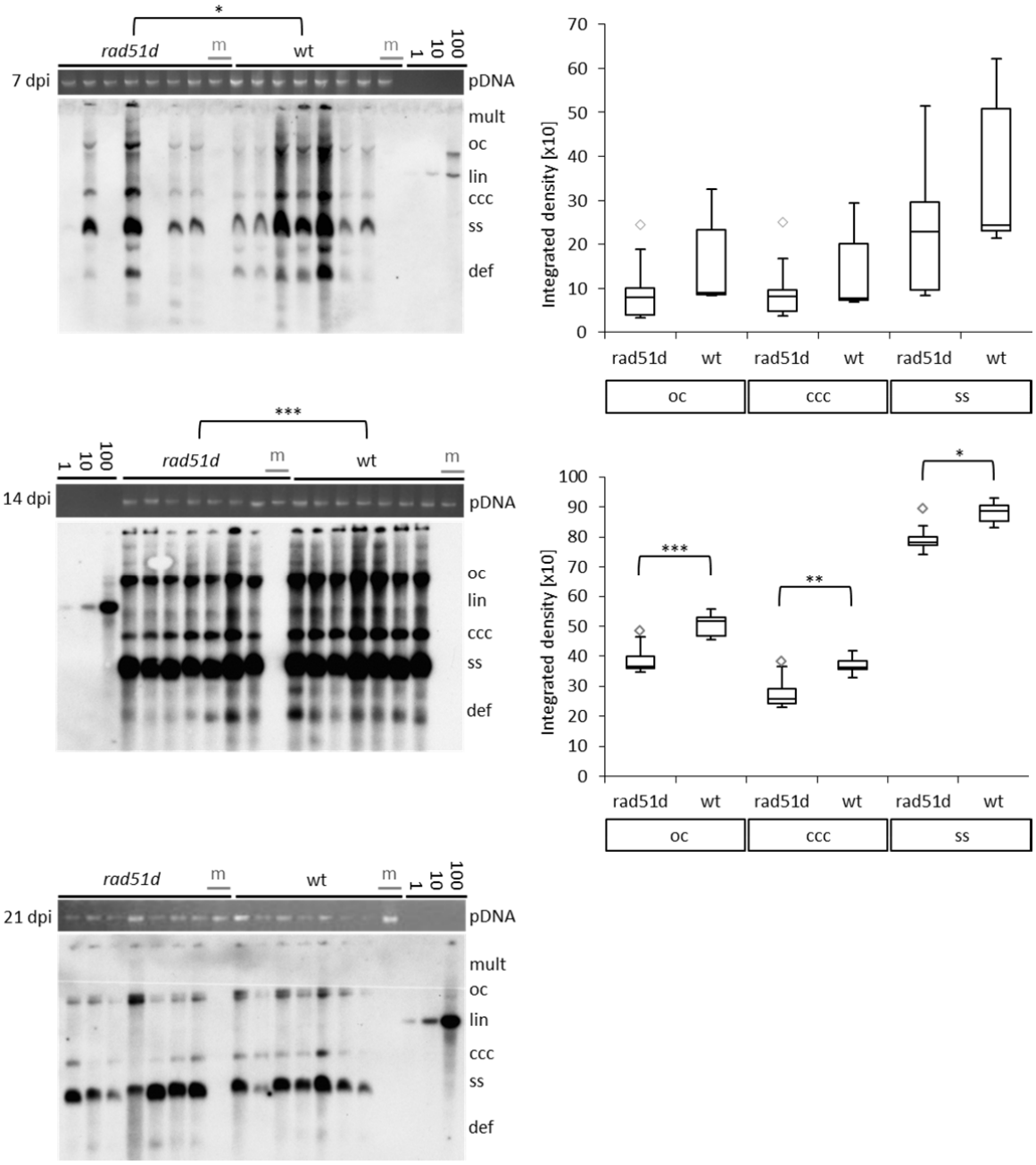

Figure 3: EuYMV DNA emergence in *rad51d* and wt *A. thaliana* plants at 7, 14 and 21 dpi as described in Fig. 1. The box plot graph depicts integrated densities of viral DNA bands of ocDNA, cccDNA and ssDNA from each sample for 7 and 14 dpi: Box ≙ 50 % of data between upper and lower quartiles; line ≙ 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).

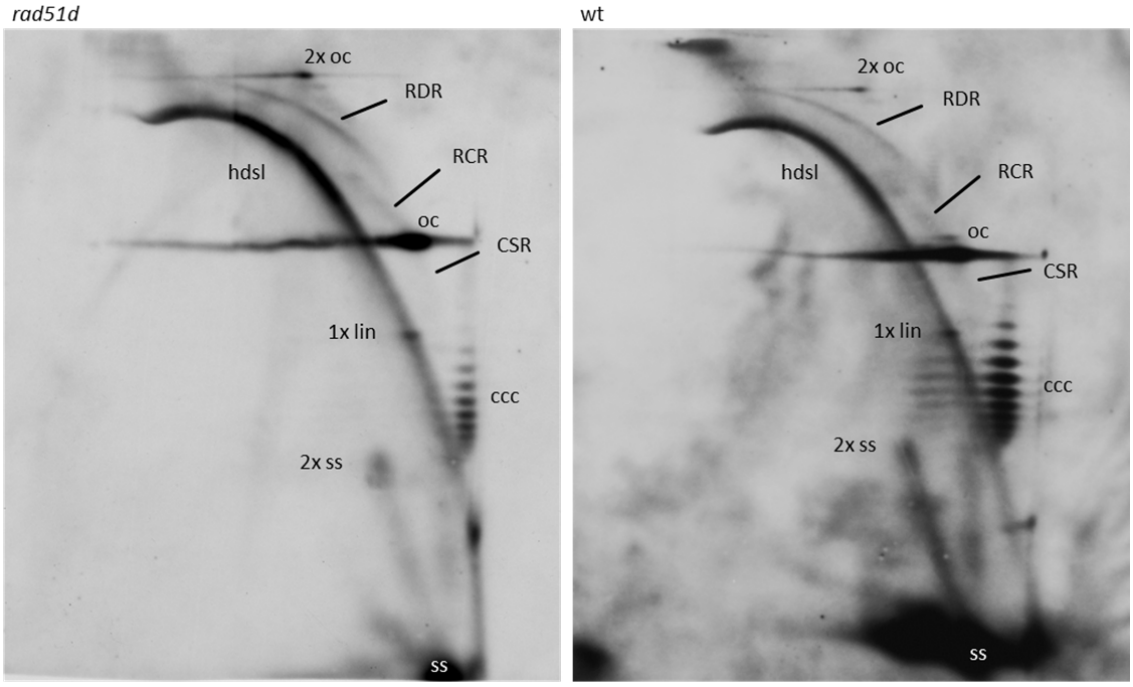

Figure 4: Replicative intermediates and end products, described in Fig. 2, at 14 dpi in *rad51d* and wt plants with the samples shown in Fig. 3.

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).

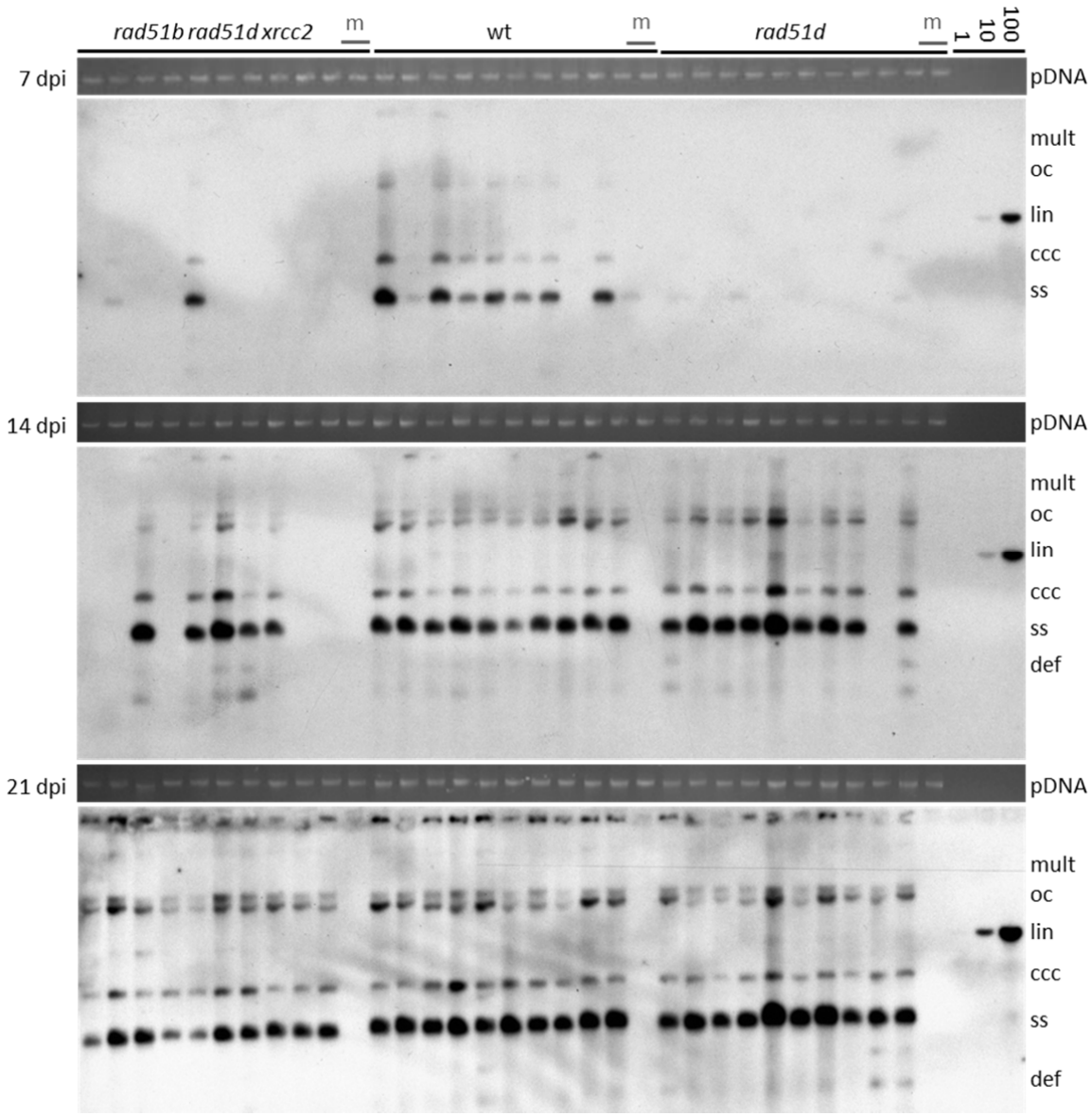

Figure 5: EuYMV DNA emergence in *rad51b rad51d xrcc2*, wt and *rad51d* plants at 7, 14 and 21 dpi. Ten plants per genotype were inoculated and analyzed as described in Fig. 1.

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.

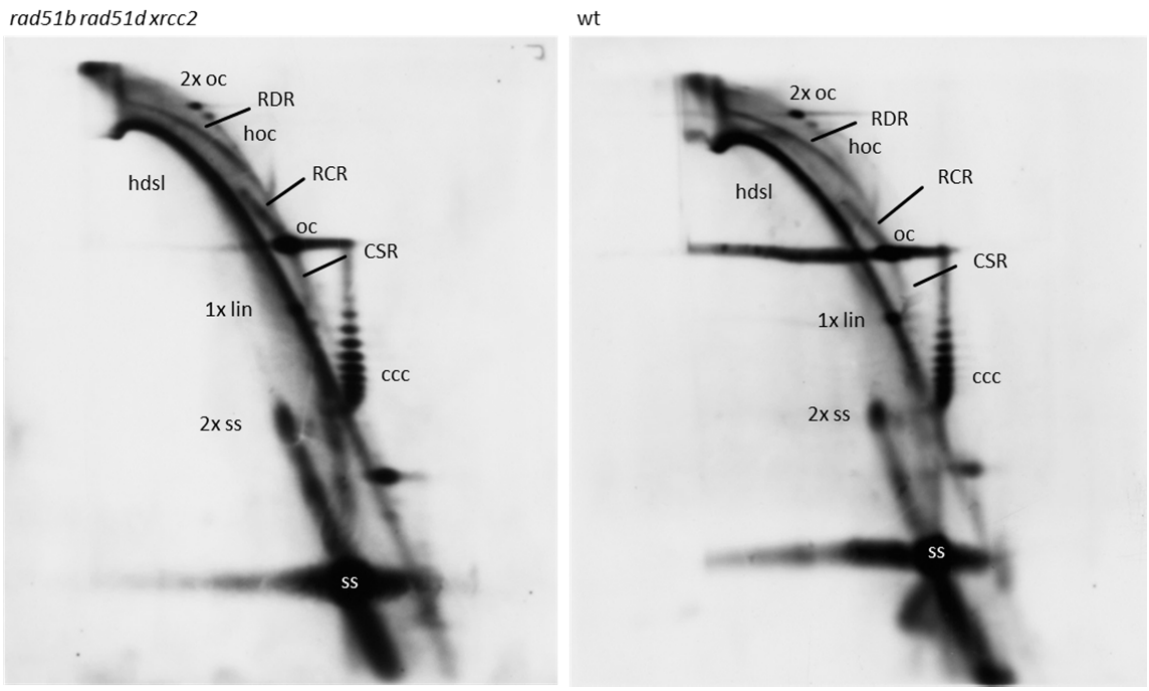

Figure 6: Replicative intermediates and end products, described in Fig. 2, at 14 dpi in *rad51b rad51d xrcc2* and wt plants from the samples shown in Fig. 5 (500 ng DNA per gel).

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 *rad51d* and triple mutant plants compared to wt plants, but not between *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 *rad51d* plants was delayed or not manifested at all.

Since Rad52 of *S. cerevisiae* is involved in SSA in addition to recruiting Rad51 to ssDNA overhangs (San Filippo *et al.*, 2008; Symington, 2002), a *rad52-1* line with nearly abolished transcription of RAD52-1 mRNA (Samach *et al.*, 2011) was tested. The 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 *A. thaliana*, respectively. However, viral DNA accumulation or intermediate composition in *rad52-1* plants showed no difference compared to 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.

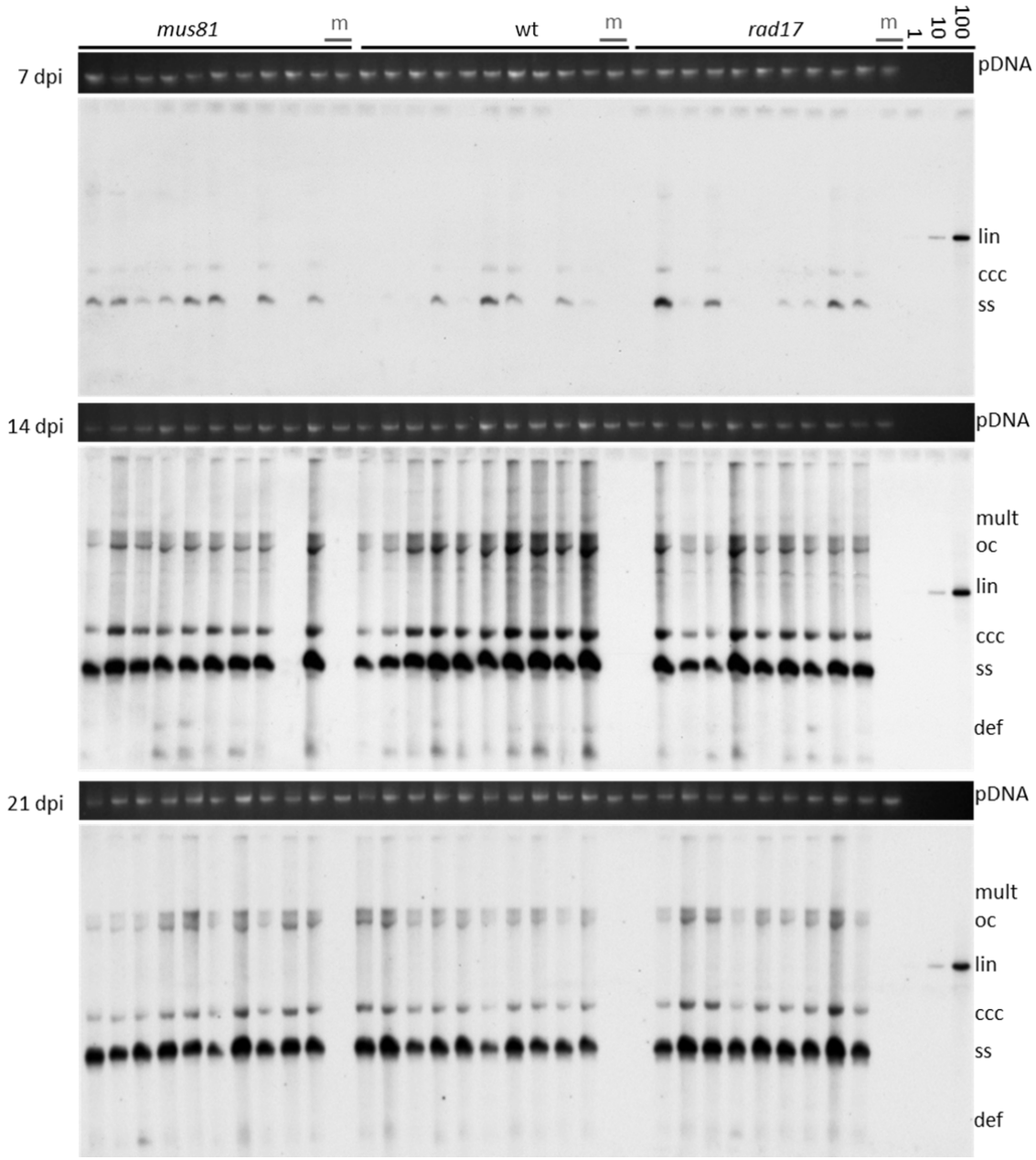

Figure 7: EuYMV DNA emergence in *mus81*, wt and *rad17* plants at 7, 14 and 21 dpi. Ten plants per genotype were inoculated and analyzed as described in in Fig. 1.

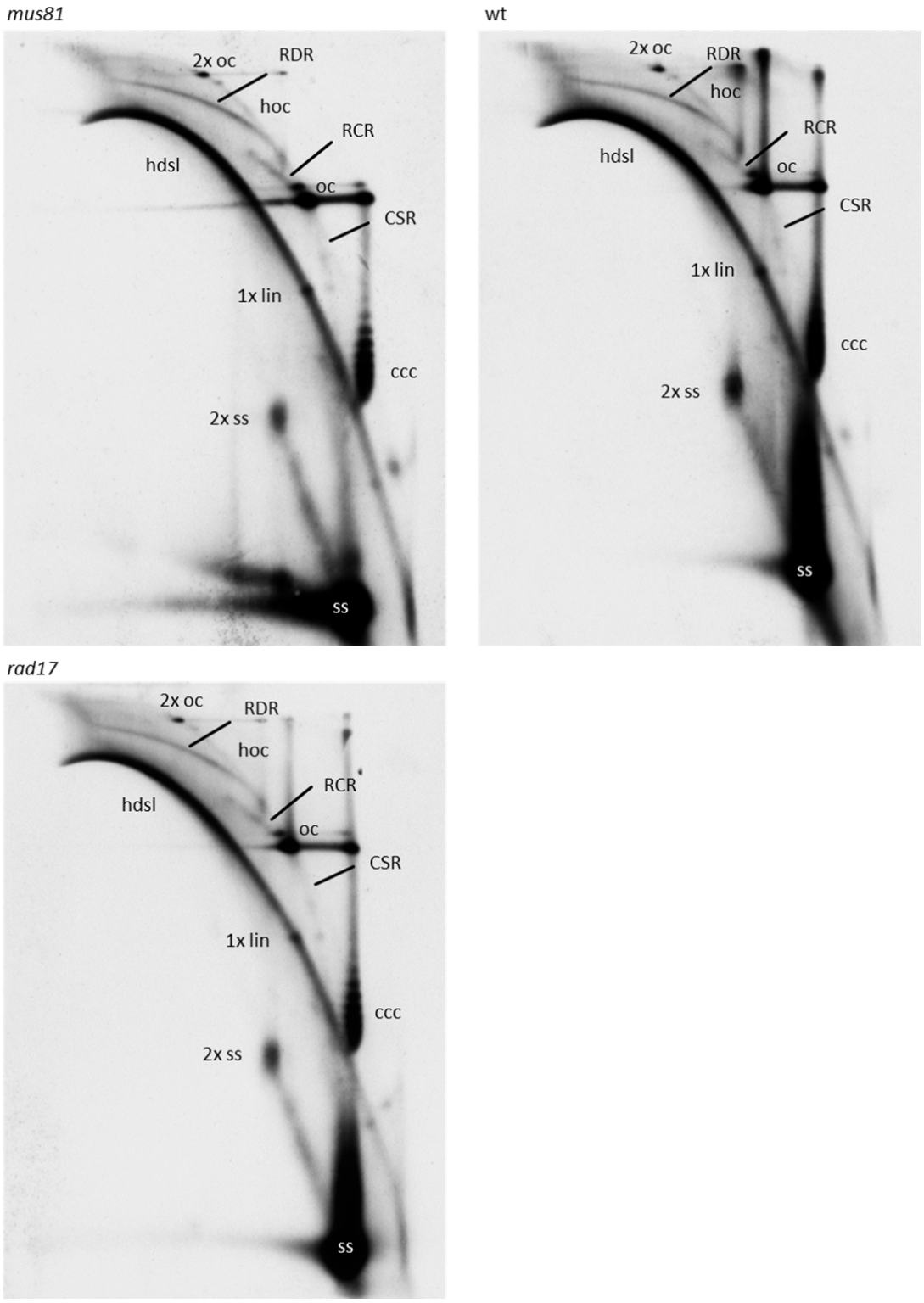

Figure 8: Replicative intermediates and end products, described in Fig. 2, at 14 dpi in *mus81*, wt and *rad17* plants with the samples shown in 7 (500 ng DNA per gel).

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, *At*RAD51 was found to interact with the MYMIV Rep by yeast two-hybrid and co-immunoprecipitation assays, and to complement *Sc*Rad51 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: *Sc*Rad54 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 Sni1 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 xrcc2*), 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.

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

Knock-out	Line	Gene no.	Sequences
Rad51	GK_134A01	At5g20850	LP: 5'CTCCCCTTCCAGAGAAATCTG
			RP: 5'ATGCCAAGGTTGACAAGATTG
Rad51B	Salk_024755C	At2g28560	LP: 5'AAGGACCAGTCGGATGGTTAC
			RP: 5'CGTTATCAGCTTCGTTTCCAG
Rad51C	Salk_021960	At2g45280	LP: 5'TCACAGAGGAGGAAGCATTTG
			RP: 5'TTTTTGGCAAGCTTCATGAAC
Rad51D	Sail_564_A06	At1g07745	LP: 5'GGCTTTCTTTGTGGGTTTCTC
			RP: 5'GCAGAATATTATGCCACACGG
Xrcc2	Salk_029106	At5g64520	LP: 5'TTTACATCTGGCGATTTTTGC
			RP: 5'ATCATCATTGGCATTGGAGAC
Xrcc3	Salk_045564	At5g57450	LP: 5'TGAAGATAGCAACCAAGTGGG
			RP: 5'AAGACACAGCTCTGCCTTCAG
Rad50	Flag_019F04	At2g31970	LP: 5'CTGTGCATTCGTTGAAATGTG
			RP: 5'CACGAGCAATGGTAGTCAGTG
Rad52	Sail_25_H08	At5g47870	LP: 5'AGGCAAGAAGGCAAAGAACTC
			RP: 5'CAAGCACTTTCGTTTTCAGATG
Rad17	Salk_009384C	At5g66130	LP: 5'CAGTCTGGTCAGAAGAGTCCG
			RP: 5'ATGTTTTGGCTTGTCACCTTG
Mus81	GK_113F11	At4g30870	LP: 5'GTCGGAAAATCTGGAGAGGAC
			RP: 5'TGATTCATACCCAACAGGAGC
	Insertion lines	Primer name	Sequences
	Salk	LBb1.3	5'ATTTTGCCGATTTCGGAAC
	Sail	LB3	5'TAGCATCTGAATTTCATAACCAATCTC
	GK	LBo8409	5'ATATTGACCATCATACTCATTGC
	Flag	Tag5	5'CTACAAATTGCCTTTTCTTATCGAC

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).

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References

Abe, K, Osakabe, K, Nakayama, S, Endo, M, Tagiri, A, Todoriki, S, Ichikawa, H, Toki, S (2005) *Arabidopsis* RAD51C gene is important for homologous recombination in meiosis and mitosis. *Plant Physiol* **139**: 896-908

Arguello-Astorga, G, Lopez-Ochoa, L, Kong, LJ, Orozco, BM, Settlage, SB, Hanley-Bowdoin, L (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma-related protein. *J Virol* **78**: 4817-4826

Ascencio-Ibanez, JT, Sozzani, R, Lee, TJ, Chu, TM, Wolfinger, RD, Cella, R, Hanley-Bowdoin, L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol* **148**: 436-454

Bach, J, Jeske, H (2014) Defective DNAs of beet curly top virus from long-term survivor sugar beet plants. *Virus Res* **183**: 89-94

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, Savigny, F, White, CI (2005) Differing requirements for the *Arabidopsis* Rad51 paralogs in meiosis and DNA repair. *Plant J* **41**: 533-545

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

Bleuyard, JY, White, CI (2004) The *Arabidopsis* homologue of Xrcc3 plays an essential role in meiosis. *EMBO J* **23**: 439-449

Böttcher, B, Unseld, S, Ceulemans, H, Russell, RB, Jeske, H (2004) Geminate structures of African cassava mosaic virus. *J Virol* **78**: 6758-6765

Choudhury, NR, Malik, PS, Singh, DK, Islam, MN, Kaliappan, K, Mukherjee, SK (2006) The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. *Nucleic Acids Res* **34**: 6362-6377

Chun, J, Buechelmaier, ES, Powell, SN (2013) Rad51 paralog complexes BCDX2 and CX3 act at different stages in the BRCA1-BRCA2-dependent homologous recombination pathway. *Mol Cell Biol* **33**: 387-395

Clerot, D, Bernardi, F (2006) DNA helicase activity is associated with the replication initiator protein rep of tomato yellow leaf curl geminivirus. *J Virol* **80**: 11322-11330

Cloud, V, Chan, YL, Grubb, J, Budke, B, Bishop, DK (2012) Rad51 is an accessory factor for Dmc1-mediated joint molecule formation during meiosis. *Science* **337**: 1222-1225

Da Ines, O, Degroote, F, Amiard, S, Goubely, C, Gallego, ME, White, CI (2013a) Effects of XRCC2 and RAD51B mutations on somatic and meiotic recombination in *Arabidopsis thaliana*. *Plant J* **74**: 959-970

Da Ines, O, Degroote, F, Goubely, C, Amiard, S, Gallego, ME, White, CI (2013b) Meiotic recombination in Arabidopsis is catalysed by DMC1, with RAD51 playing a supporting role. *PLoS Genet* **9**: e1003787

Doutriaux, MP, Couteau, F, Bergounioux, C, White, C (1998) Isolation and characterisation of the RAD51 and DMC1 homologs from *Arabidopsis thaliana*. *Mol Gen Genet* **257**: 283-291

Durrant, WE, Wang, S, Dong, X (2007) *Arabidopsis* SNI1 and RAD51D regulate both gene transcription and DNA recombination during the defense response. *Proc Natl Acad Sci U S A* **104**: 4223-4227

Gallego, ME, Jeanneau, M, Granier, F, Bouchez, D, Bechtold, N, White, CI (2001) Disruption of the *Arabidopsis* RAD50 gene leads to plant sterility and MMS sensitivity. *Plant J* **25**: 31-41

Geuting, V, Kobbe, D, Hartung, F, Durr, J, Focke, M, Puchta, H (2009) Two distinct MUS81-EME1 complexes from *Arabidopsis* process Holliday junctions. *Plant Physiol* **150**: 1062-1071

Gregorio-Jorge, J, Bernal-Alcocer, A, Banuelos-Hernandez, B, Alpuche-Solis, AG, Hernandez-Zepeda, C, Moreno-Valenzuela, O, Frias-Trevino, G, Arguello-Astorga, GR (2010) Analysis of a new strain of Euphorbia mosaic virus with distinct replication specificity unveils a lineage of begomoviruses with short Rep sequences in the DNA-B intergenic region. *Virol J* **7**: 275

Gutierrez, C, Ramirez-Parra, E, Mar Castellano, M, Sanz-Burgos, AP, Luque, A, Missich, R (2004) Geminivirus DNA replication and cell cycle interactions. *Vet Microbiol* **98**: 111-119

Haible, D, Kober, S, Jeske, H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J Virol Methods* **135**: 9-16

Hanley-Bowdoin, L, Bejarano, ER, Robertson, D, Mansoor, S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat Rev Microbiol* **11**: 777-788

Hartung, F, Suer, S, Bergmann, T, Puchta, H (2006) The role of *At*MUS81 in DNA repair and its genetic interaction with the helicase *At*RecQ4A. *Nucleic Acids Res* **34**: 4438-4448

Heitzeberg, F, Chen, IP, Hartung, F, Orel, N, Angelis, KJ, Puchta, H (2004) The Rad17 homologue of *Arabidopsis* is involved in the regulation of DNA damage repair and homologous recombination. *Plant J* **38**: 954-968

Heyer, WD, Ehmsen, KT, Liu, J (2010) Regulation of homologous recombination in eukaryotes. *Annu Rev Genet* **44**: 113-139

Hipp, K, Rau, P, Schafer, B, Gronenborn, B, Jeske, H (2014) The RXL motif of the African cassava mosaic virus Rep protein is necessary for rereplication of yeast DNA and viral infection in plants. *Virology* **462-463C**: 189-198

Jasin, M, Rothstein, R (2013) Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol* **5**: a012740

Jeske, H, **(2009)**. Geminiviruses, in: *Torque Teno Virus: The Still Elusive Human Pathogens*, pp. 185-226. Edited by zur Hausen, H., de Villiers, E.-M. Springer, Berlin.

Jeske, H, Lutgemeier, M, Preiss, W (2001) DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO J* **20**: 6158-6167

Johnson, RD, Symington, LS (1995) Functional differences and interactions among the putative RecA homologs Rad51, Rad55, and Rad57. *Mol Cell Biol* **15**: 4843-4850

Kaliappan, K, Choudhury, NR, Suyal, G, Mukherjee, SK (2012) A novel role for RAD54: this host protein modulates geminiviral DNA replication. *FASEB J* **26**: 1142-1160

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

Kong, LJ, Orozco, BM, Roe, JL, Nagar, S, Ou, S, Feiler, HS, Durfee, T, Miller, AB, Gruissem, W, Robertson, D, Hanley-Bowdoin, L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J* **19**: 3485-3495

Krogh, BO, Symington, LS (2004) Recombination proteins in yeast. *Annu Rev Genet* **38**: 233-271

Kurumizaka, H, Ikawa, S, Nakada, M, Eda, K, Kagawa, W, Takata, M, Takeda, S, Yokoyama, S, Shibata, T (2001) Homologous-pairing activity of the human DNA-repair proteins Xrcc3.Rad51C. *Proc Natl Acad Sci U S A* **98**: 5538-5543

Kurumizaka, H, Ikawa, S, Nakada, M, Enomoto, R, Kagawa, W, Kinebuchi, T, Yamazoe, M, Yokoyama, S, Shibata, T (2002) Homologous pairing and ring and filament structure formation activities of the human Xrcc2*Rad51D complex. *J Biol Chem* **277**: 14315-14320

Lamarche, BJ, Orazio, NI, Weitzman, MD (2010) The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett* **584**: 3682-3695

Laufs, J, Traut, W, Heyraud, F, Matzeit, V, Rogers, SG, Schell, J, Gronenborn, B (1995) In vitro cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proc Natl Acad Sci U S A* **92**: 3879-3883

Lefeuvre, P, Moriones, E (2015) Recombination as a motor of host switches and virus emergence: geminiviruses as case studies. *Curr Opin Virol* **10C**: 14-19

Li, W, Chen, C, Markmann-Mulisch, U, Timofejeva, L, Schmelzer, E, Ma, H, Reiss, B (2004) The *Arabidopsis At*RAD51 gene is dispensable for vegetative development but required for meiosis. *Proc Natl Acad Sci U S A* **101**: 10596-10601

Li, WX, Yang, XH, Lin, ZG, Timofejeva, L, Xiao, R, Makaroff, CA, Ma, H (2005) The *At*RAD51C gene is required for normal meiotic chromosome synapsis and double-stranded break repair in *Arabidopsis*. *Plant Physiology* **138**: 965-976

Liu, Y, Masson, JY, Shah, R, O'Regan, P, West, SC (2004) RAD51C is required for Holliday junction processing in mammalian cells. *Science* **303**: 243-246

Masson, JY, Stasiak, AZ, Stasiak, A, Benson, FE, West, SC (2001a) Complex formation by the human RAD51C and XRCC3 recombination repair proteins. *Proc Natl Acad Sci U S A* **98**: 8440-8446

Masson, JY, Tarsounas, MC, Stasiak, AZ, Stasiak, A, Shah, R, McIlwraith, MJ, Benson, FE, West, SC (2001b) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev* **15**: 3296-3307

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

Miller, KA, Sawicka, D, Barsky, D, Albala, JS (2004) Domain mapping of the Rad51 paralog protein complexes. *Nucleic Acids Res* **32**: 169-178

Osakabe, K, Abe, K, Yamanouchi, H, Takyuu, T, Yoshioka, T, Ito, Y, Kato, T, Tabata, S, Kurei, S, Yoshioka, Y, Machida, Y, Seki, M, Kobayashi, M, Shinozaki, K, Ichikawa, H, Toki, S (2005) *Arabidopsis* Rad51B is important for double-strand DNA breaks repair in somatic cells. *Plant Mol Biol* **57**: 819-833

Osakabe, K, Yoshioka, T, Ichikawa, H, Toki, S (2002) Molecular cloning and characterization of RAD51-like genes from *Arabidopsis thaliana*. *Plant Mol Biol* **50**: 71-81

Paprotka, T, Deuschle, K, Pilartz, M, Jeske, H (2015) Form follows function in geminiviral minichromosome architecture. *Virus Res* **196**: 44-55

Paprotka, T, Metzler, V, Jeske, H (2010) The first DNA 1-like alpha-satellites in association with New World begomoviruses in natural infections. *Virology* **404**: 148-157

Parrilla-Castellar, ER, Arlander, SJ, Karnitz, L (2004) Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair* **3**: 1009-1014

Patil, BL, Dutt, N, Briddon, RW, Bull, SE, Rothenstein, D, Borah, BK, Dasgupta, I, Stanley, J, Jeske, H (2007) Deletion and recombination events between the DNA-A and DNA-B

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

Pilartz, M, Jeske, H (2003) Mapping of abutilon mosaic geminivirus minichromosomes. *J Virol* **77**: 10808-10818

Pradillo, M, Varas, J, Oliver, C, Santos, JL (2014) On the role of *At*DMC1, *At*RAD51 and its paralogs during *Arabidopsis meiosis*. *Front Plant Sci* **5**: 23

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Puchta, H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J Exp Bot* **56**: 1-14

Richter, KS, Ende, L, Jeske, H (2015) Rad54 is not essential for any geminiviral replication mode in planta. *Plant Mol Biol* **87**: 193-202

Richter, KS, Kleinow, T, Jeske, H (2014) Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Virology* **452-453**: 287-296

Roth, N, Klimesch, J, Dukowic-Schulze, S, Pacher, M, Mannuss, A, Puchta, H (2012) The requirement for recombination factors differs considerably between different pathways of homologous double-strand break repair in somatic plant cells. *Plant J* **72**: 781-790

Samach, A, Melamed-Bessudo, C, Avivi-Ragolski, N, Pietrokovski, S, Levy, AA (2011) Identification of plant RAD52 homologs and characterization of the *Arabidopsis thaliana* RAD52-like genes. *Plant Cell* **23**: 4266-4279

San Filippo, J, Sung, P, Klein, H (2008) Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem* **77**: 229-257

Saunders, K, Lucy, A, Stanley, J (1991) DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res* **19**: 2325-2330

Serra, H, Da Ines, O, Degroote, F, Gallego, ME, White, CI (2013) Roles of XRCC2, RAD51B and RAD51D in RAD51-independent SSA recombination. *PLoS Genet* **9**: e1003971

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

Stainton, D, Kraberger, S, Walters, M, Wiltshire, EJ, Rosario, K, Halafihi, M, Lolohea, S, Katoa, I, Faitua, TH, Aholelei, W, Taufa, L, Thomas, JE, Collings, DA, Martin, DP, Varsani, A (2012) Evidence of inter-component recombination, intra-component recombination and reassortment in banana bunchy top virus. *J Gen Virol* **93**: 1103-1119

Stanley, J (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology* **206**: 707-712

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

Suwaki, N, Klare, K, Tarsounas, M (2011) RAD51 paralogs: roles in DNA damage signalling, recombinational repair and tumorigenesis. *Semin Cell Dev Biol* **22**: 898-905

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

Symington, LS (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* **66**: 630-670

Wang, S, Durrant, WE, Song, J, Spivey, NW, Dong, X (2010) *Arabidopsis* BRCA2 and RAD51 proteins are specifically involved in defense gene transcription during plant immune responses. *Proc Natl Acad Sci U S A* **107**: 22716-22721

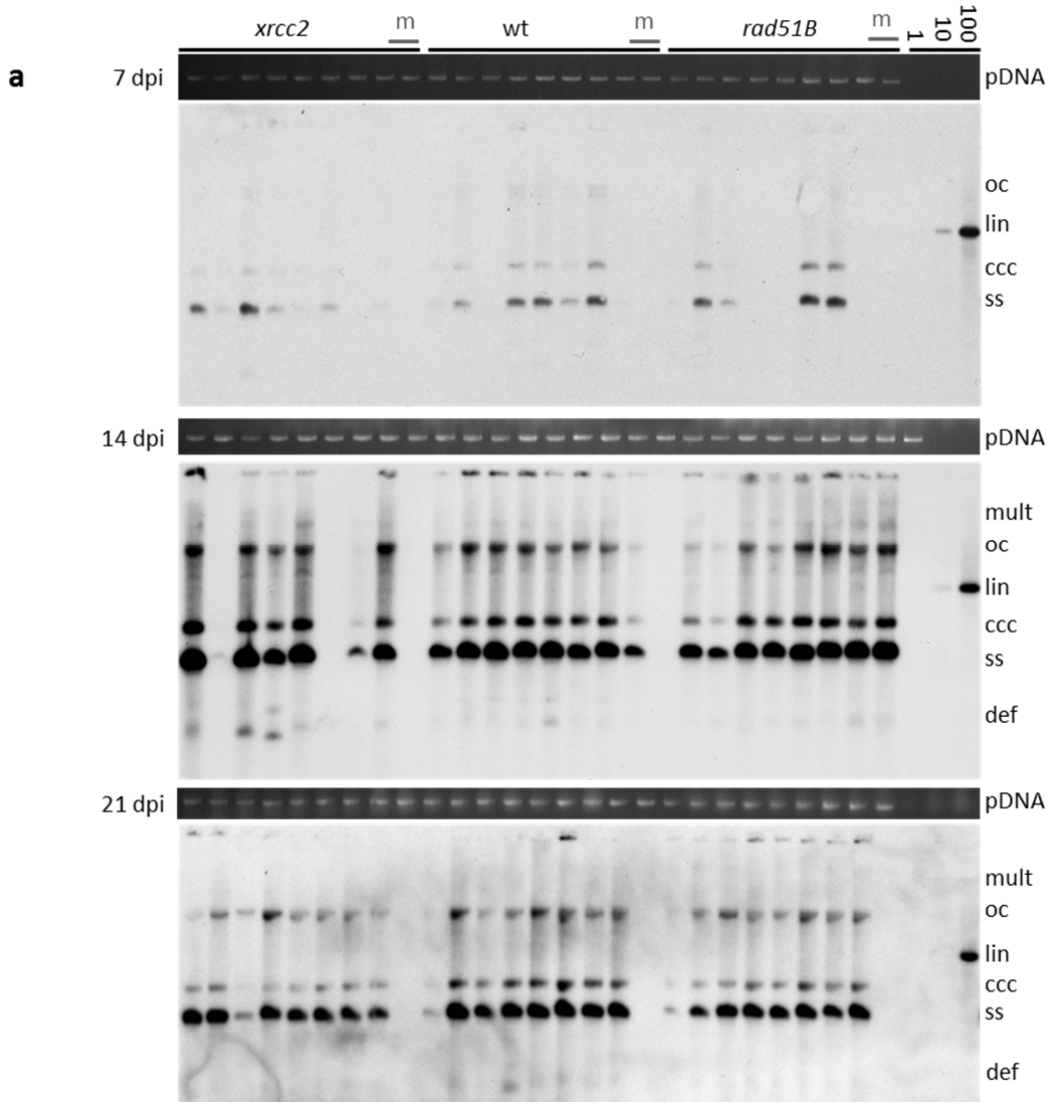
Wang, Y, Xiao, R, Wang, H, Cheng, Z, Li, W, Zhu, G, Ma, H (2014) The *Arabidopsis* RAD51 paralogs RAD51B, RAD51D and XRCC2 play partially redundant roles in somatic DNA repair and gene regulation. *New Phytol* **201**: 292-304

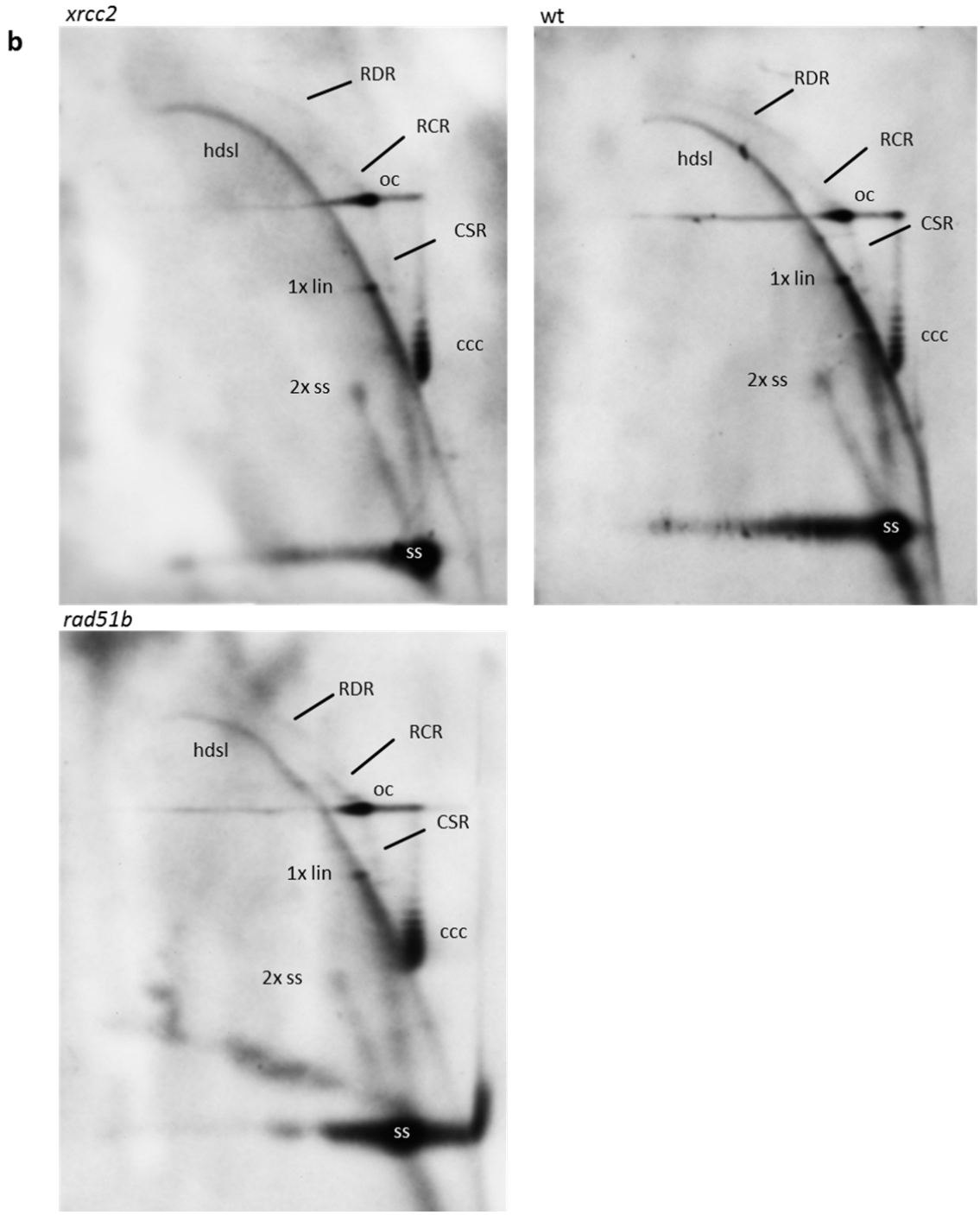
Williams, GJ, Lees-Miller, SP, Tainer, JA (2010) Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. *DNA Repair (Amst)* **9**: 1299-1306

Yokoyama, H, Sarai, N, Kagawa, W, Enomoto, R, Shibata, T, Kurumizaka, H, Yokoyama, S (2004) Preferential binding to branched DNA strands and strand-annealing activity of the human Rad51B, Rad51C, Rad51D and Xrcc2 protein complex. *Nucleic Acids Res* **32**: 2556-2565

Zhang, W, Olson, NH, Baker, TS, Faulkner, L, Agbandje-McKenna, M, Boulton, MI, Davies, JW, McKenna, R (2001) Structure of the Maize streak virus geminate particle. *Virology* **279**: 471-477

Supplementary data

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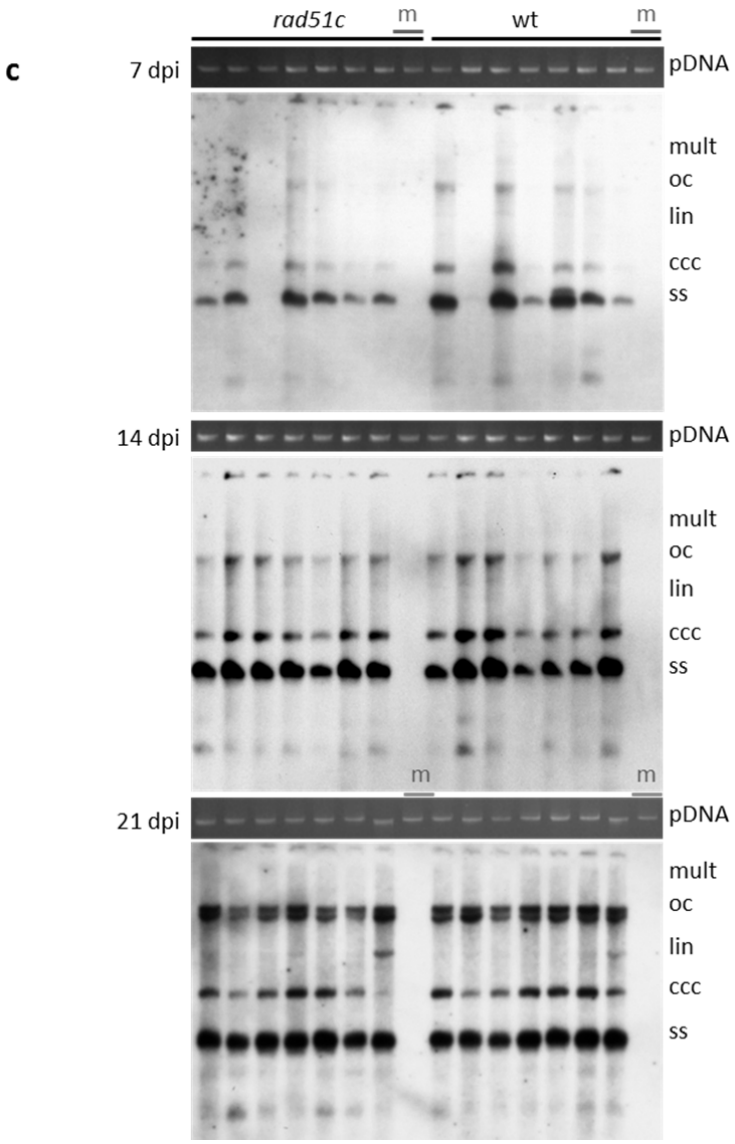
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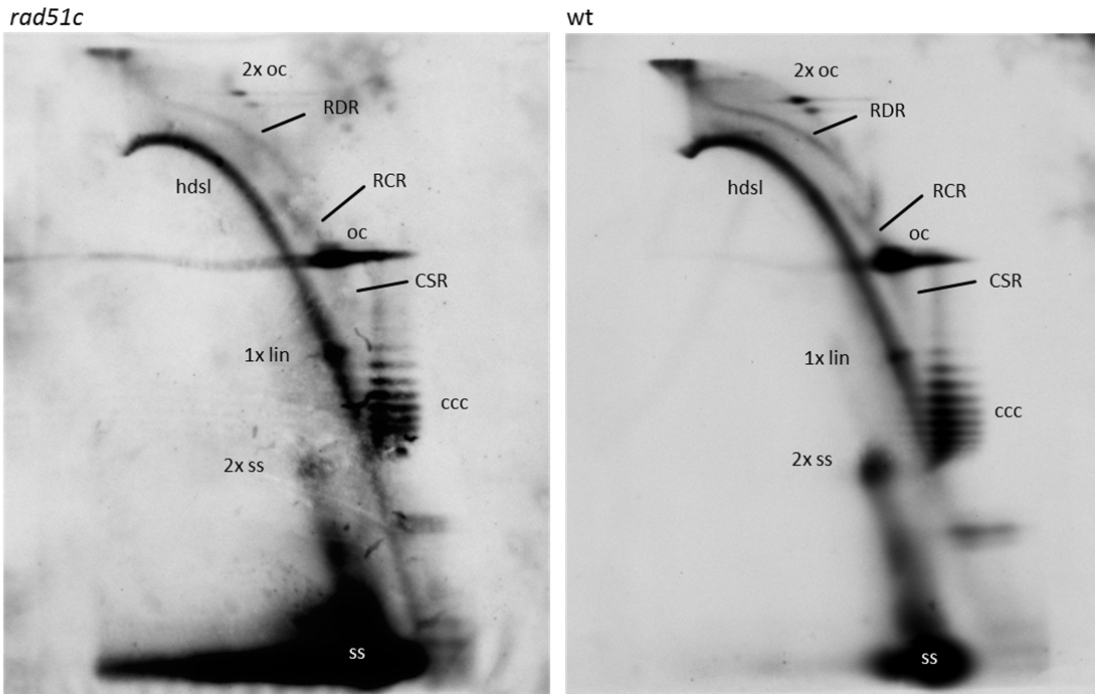
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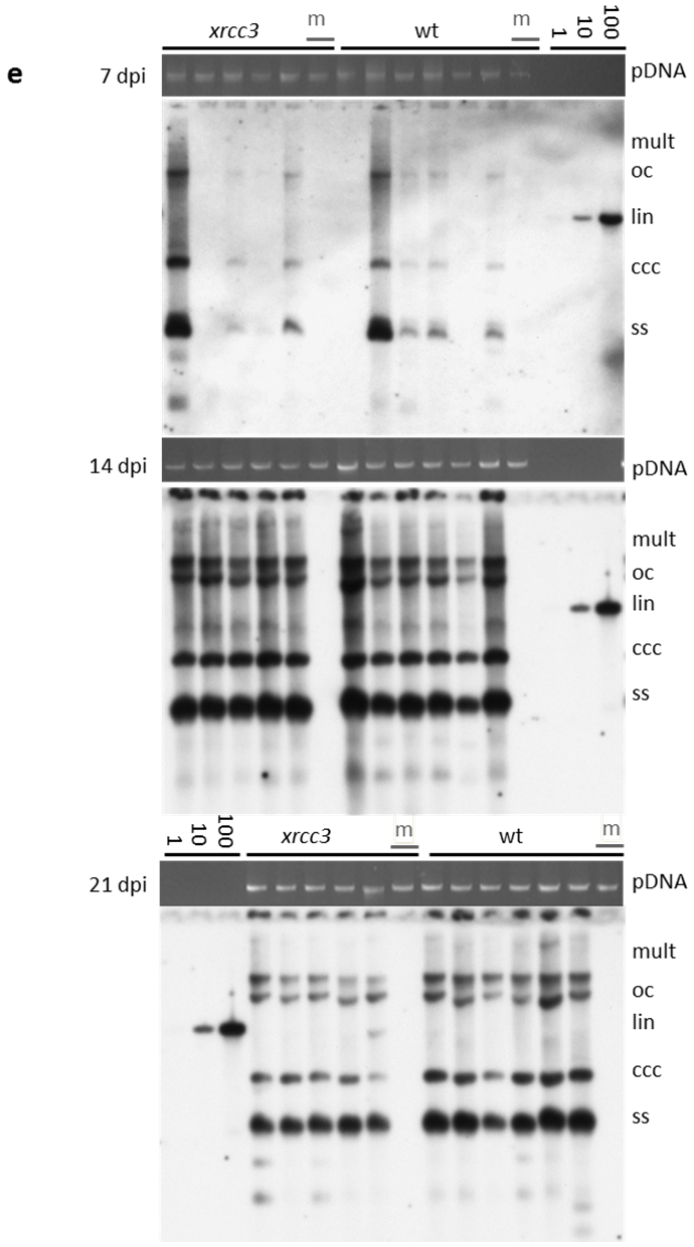
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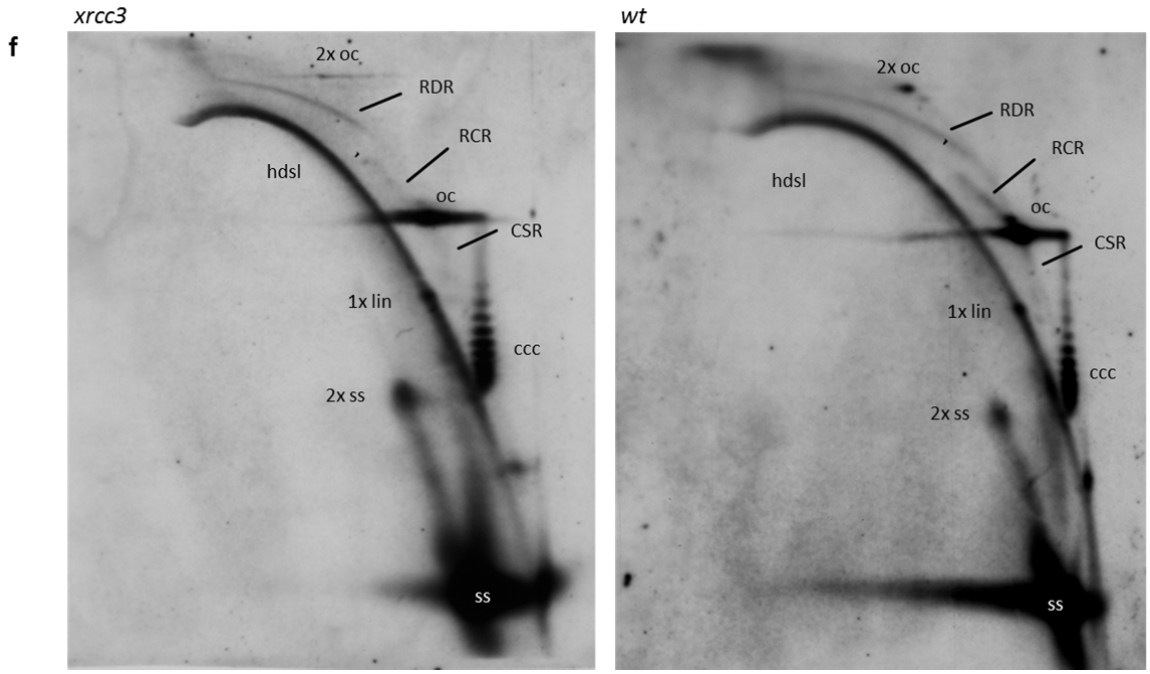
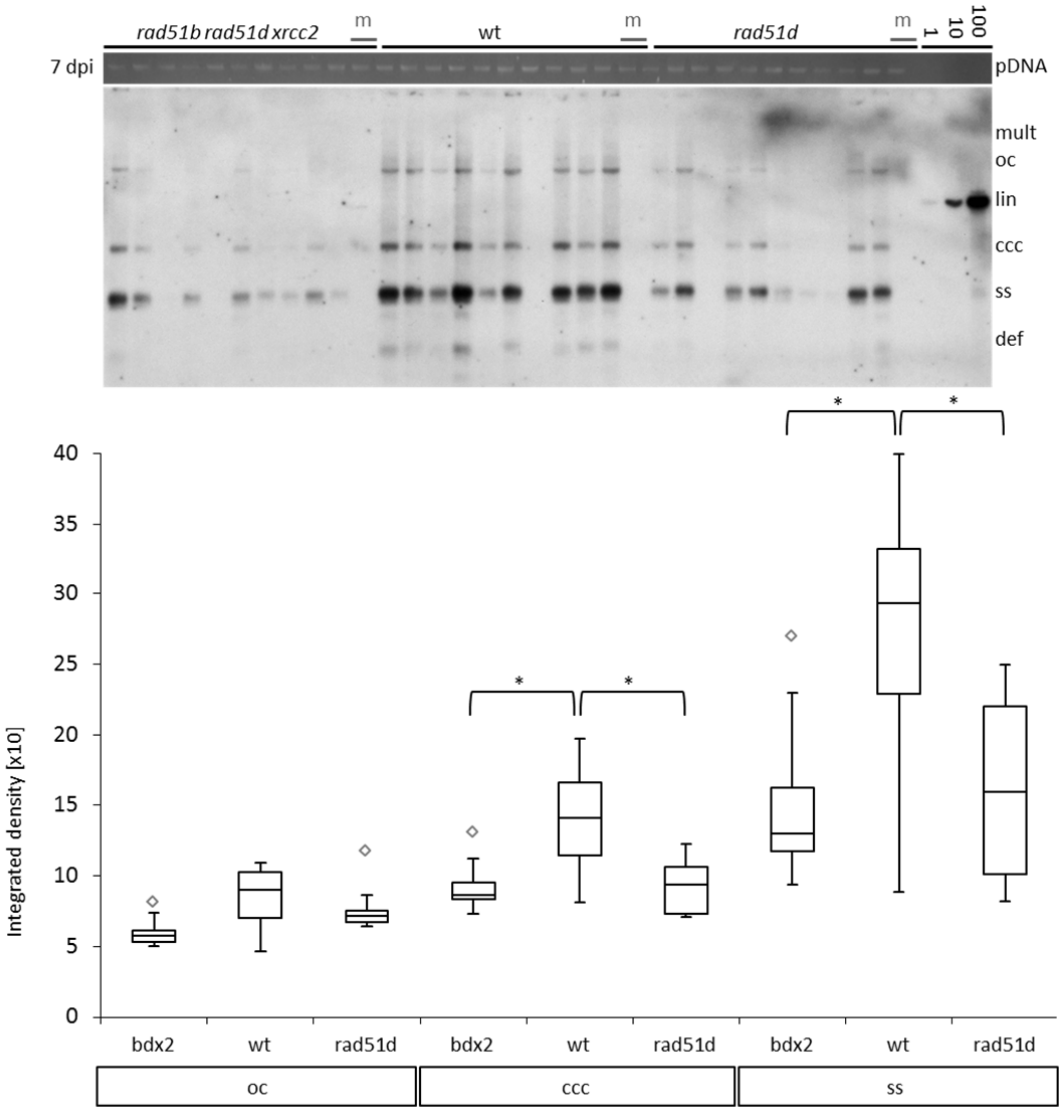


Figure S1: EuYMV DNA emergence at 7, 14 and 21 dpi and replicative intermediates at 14 dpi in *rad51b*, *xrcc2* and wt sibling plants of line *xrcc2* (a and b), *rad51c* and wt sibling plants (c and d) and *xrcc3* and wt sibling plants (e and f). Plants were inoculated and DNA was detected as described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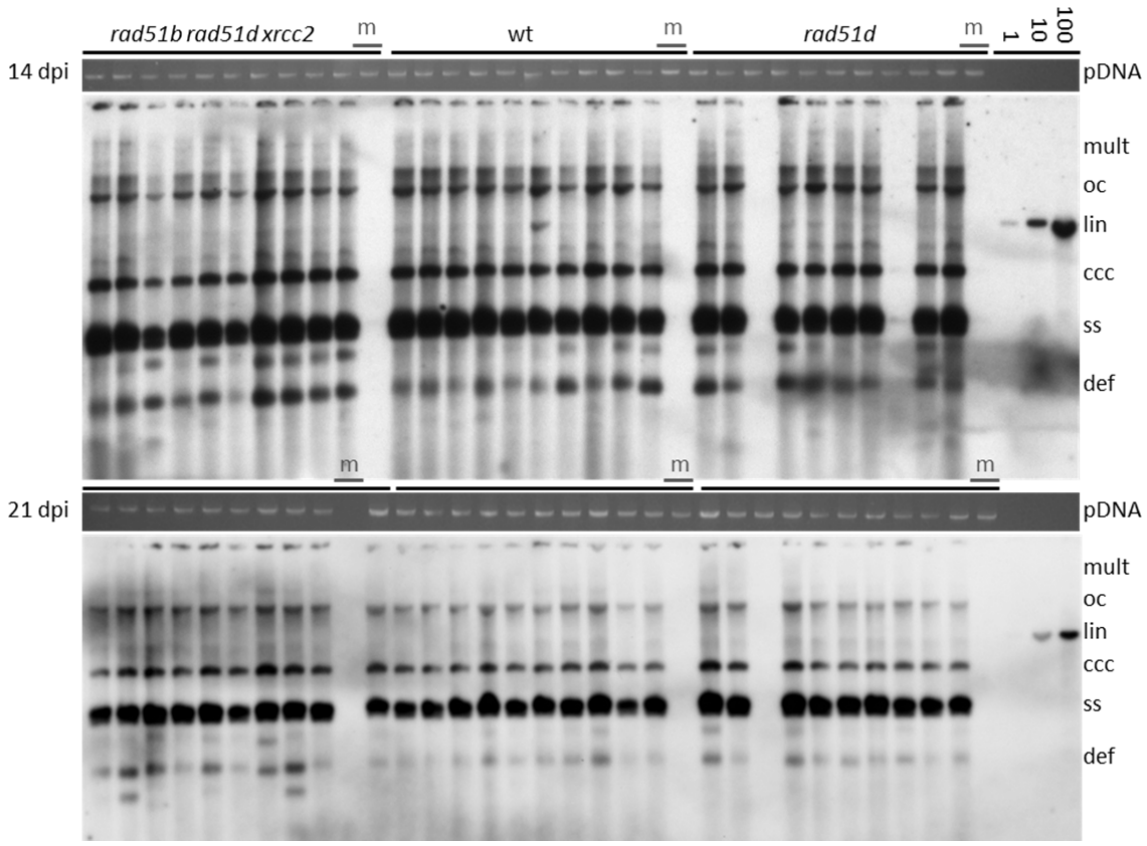
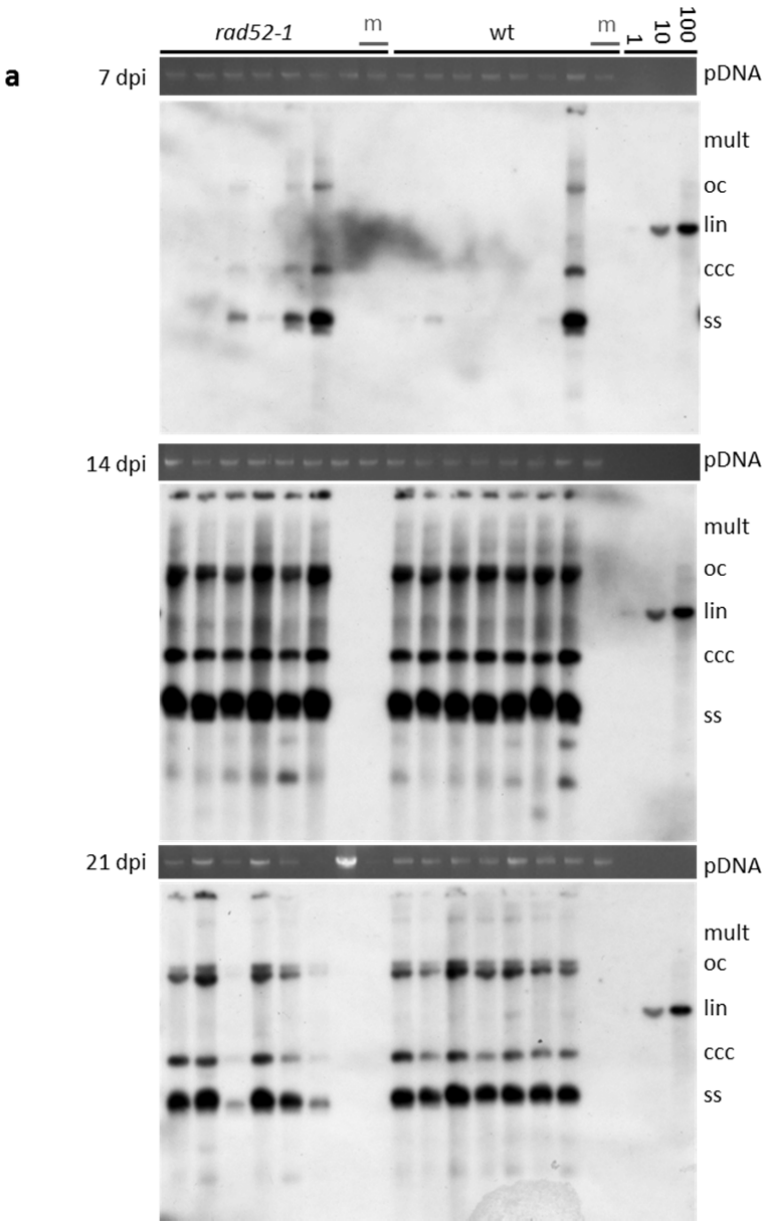


Figure S3: EuYMV DNA emergence in *rad51b rad51d xrcc2*, wt and *rad51d* plants at 7, 14 and 21 dpi, a biological replicate of the inoculation experiment shown in Fig. 6. Ten plants per genotype were inoculated and analyzed as described in Fig. 1. Statistical analysis was performed as described in Fig.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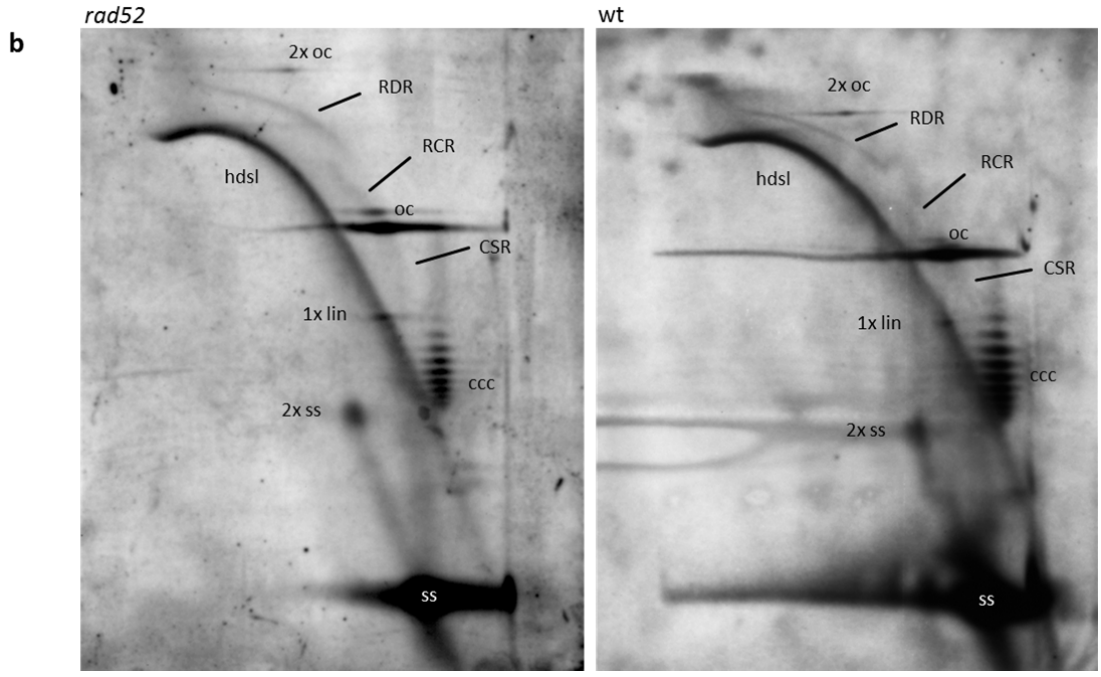


Figure S4: EuYMV DNA emergence at 7, 14 and 21 dpi (a) and replicative intermediates at 14 dpi (b) in *rad52-1* and wt sibling plants. Plants were inoculated and DNA was detected as described in Fig. 1.

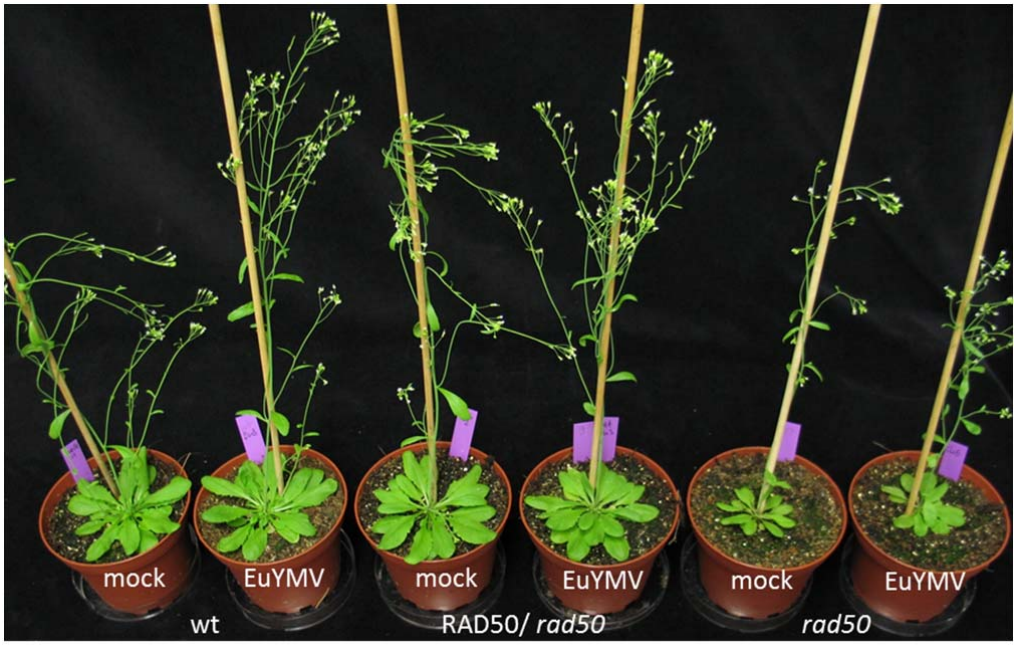


Figure S5: EuYMV-infected homozygous and hemizygous *rad50* and wt plants at 21 dpi. Homozygous *rad50* plants exhibited stunted rosettes, a reduced number of rosette leaves and slightly deformed leaf blades, which prevents a meaningful evaluation of virus infection on DNA and symptom level.

The contributions of translesion synthesis polymerases to geminivirus replication

Kathrin S. Richter[1], Monika Götz[2], Stephan Winter[2], and Holger Jeske[1#]

[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 capsulated 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

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;

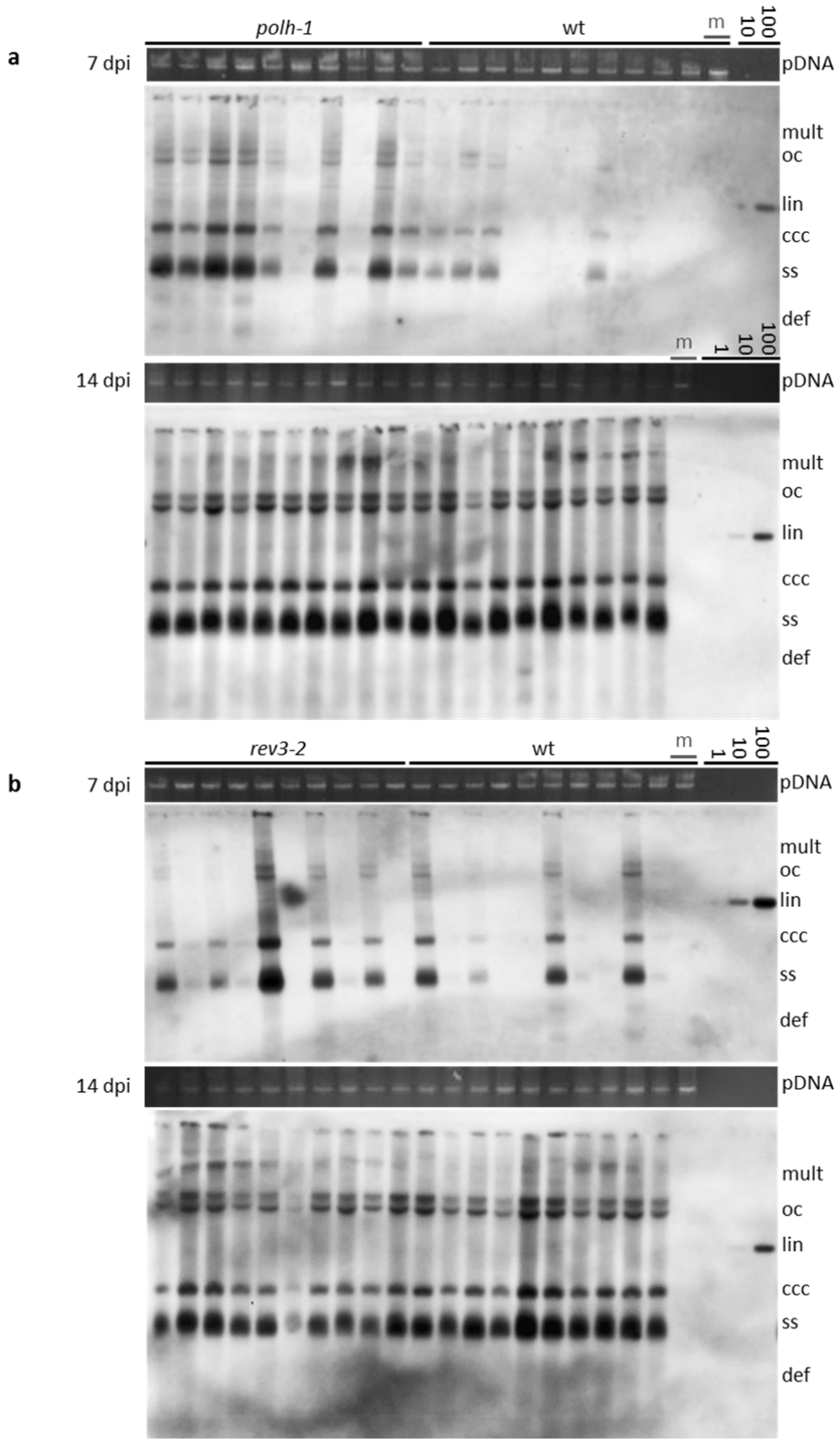
Washington *et al.*, 2002), and bypasses G lesions with bulky N(2)-adducts (Avkin *et al.*, 2004; Jia *et al.*, 2008; Minko *et al.*, 2008). The Y family Rev1 has nucleotidyl transferase activity, inserting only one or two nucleotides with subsequent extension probably accomplished by Polζ (Nelson *et 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 (ClLCrV) 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 ClLCrV infections (data not shown).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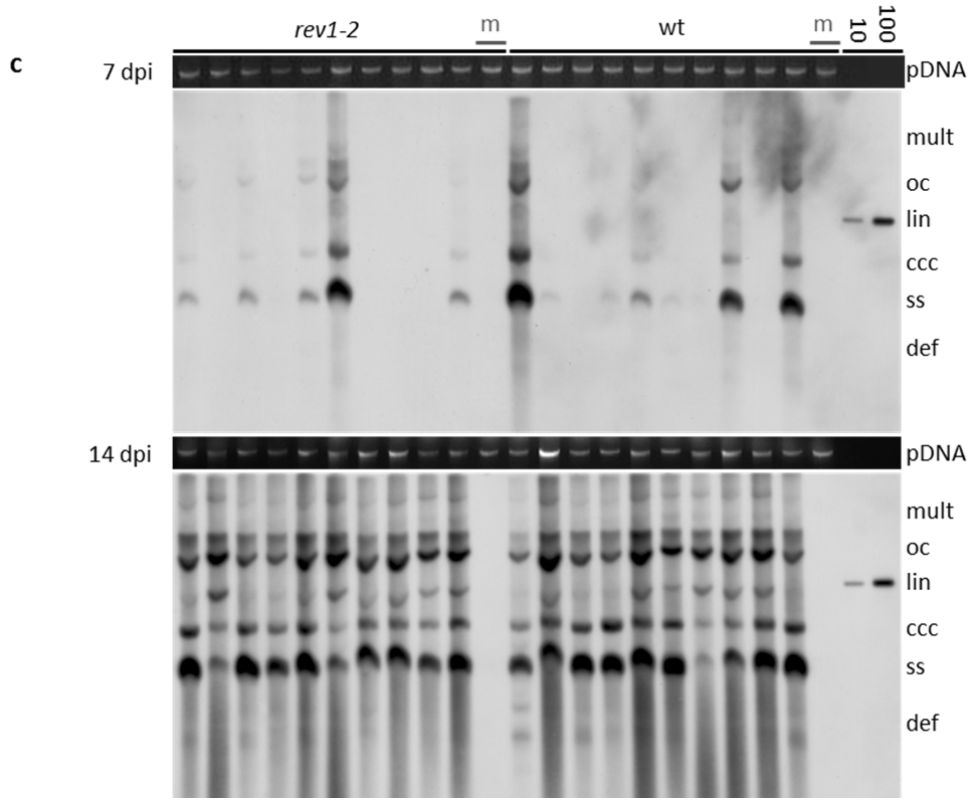


Figure 1: EuYMV DNA emergence at 7 and 14 dpi in *polh-1* (a), *rev3-2* (b) and *rev1-2* (c) *A. thaliana* plants in comparison to sibling wt plants after biolistic inoculation. Total nucleic acids (500 ng each) from ten plants per genotype were separated in agarose gels (1.4 %) in the presence of 5 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.

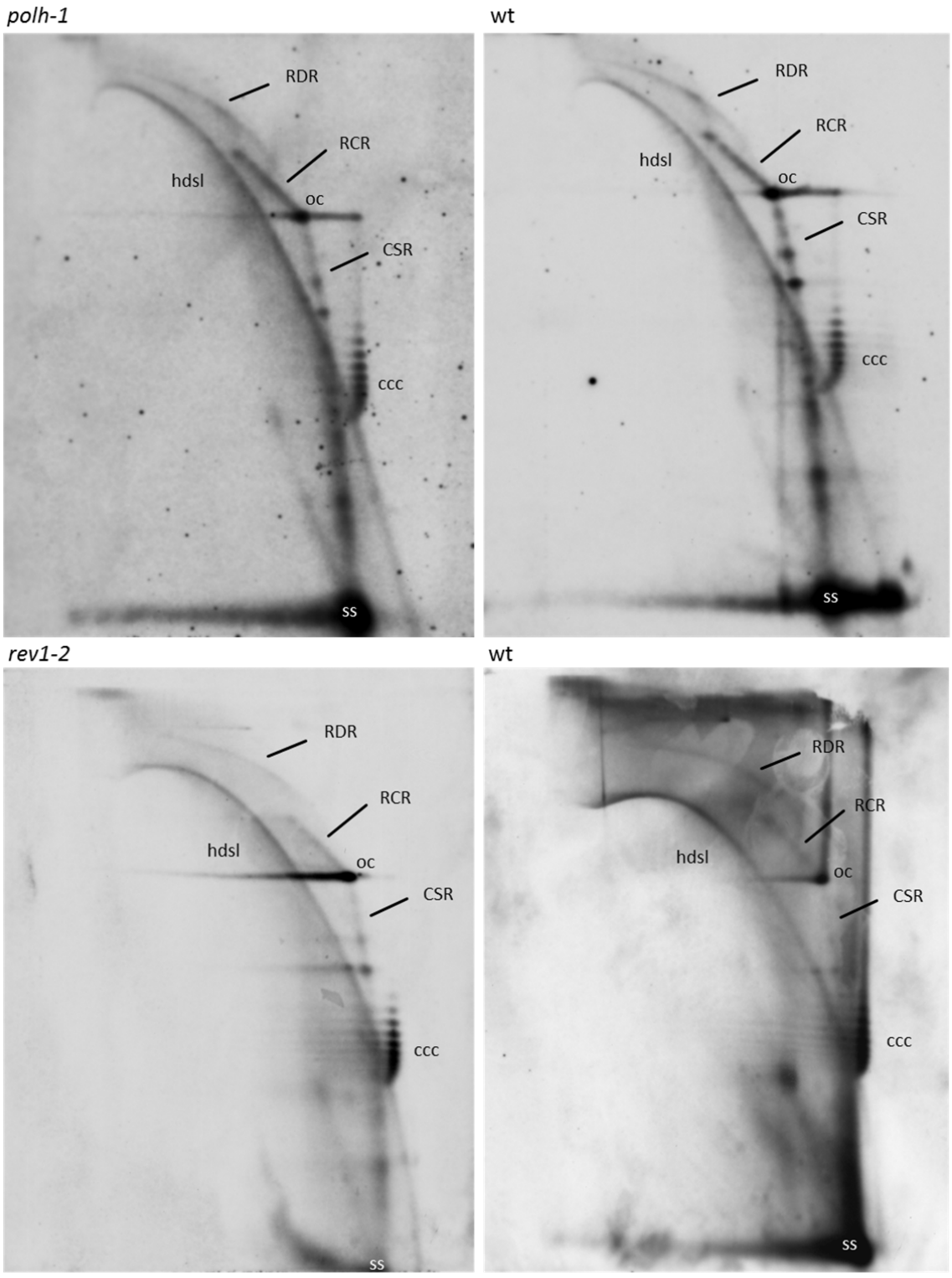
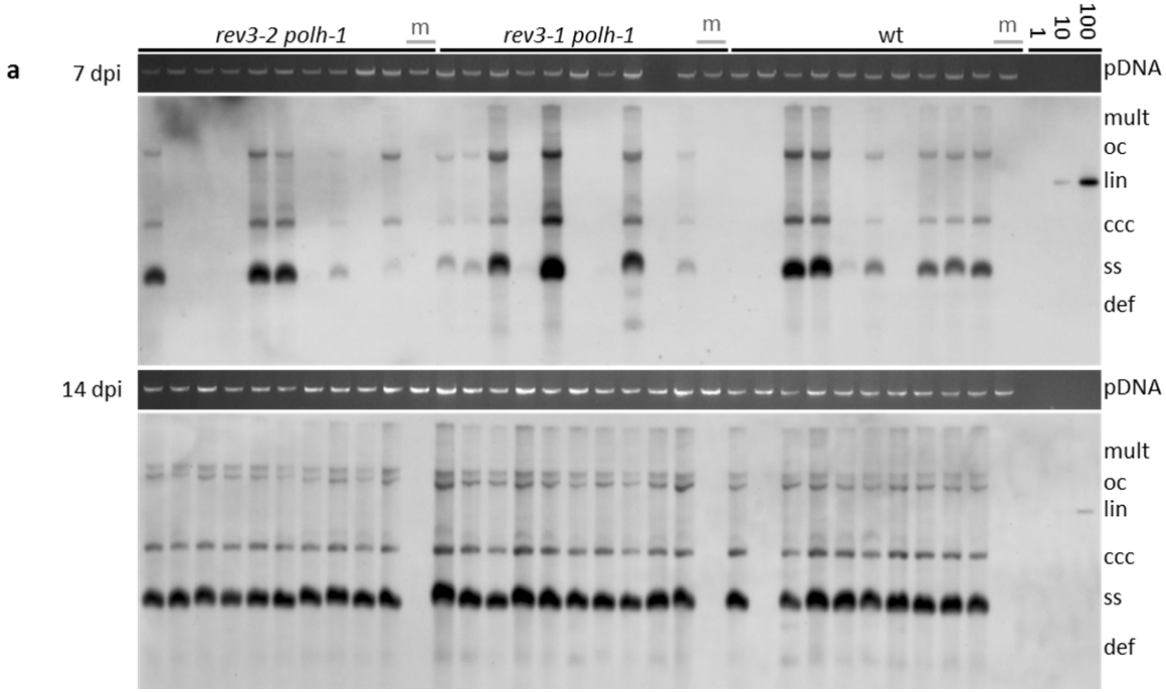


Figure 2: Replicative intermediates of EuYMV from individual *polh-1* (a) and *rev1-2* (b) plants and their respective wt siblings at 7 dpi. Separation by 2D gel electrophoresis was conducted in 0.5 % agarose gels with TBE buffer containing 0.03 % SDS in the first dimension followed by 1.4 % agarose 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.

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 ClLCrV (Fig. 3a for EuYMV, Fig S1c for ClLCrV).

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).



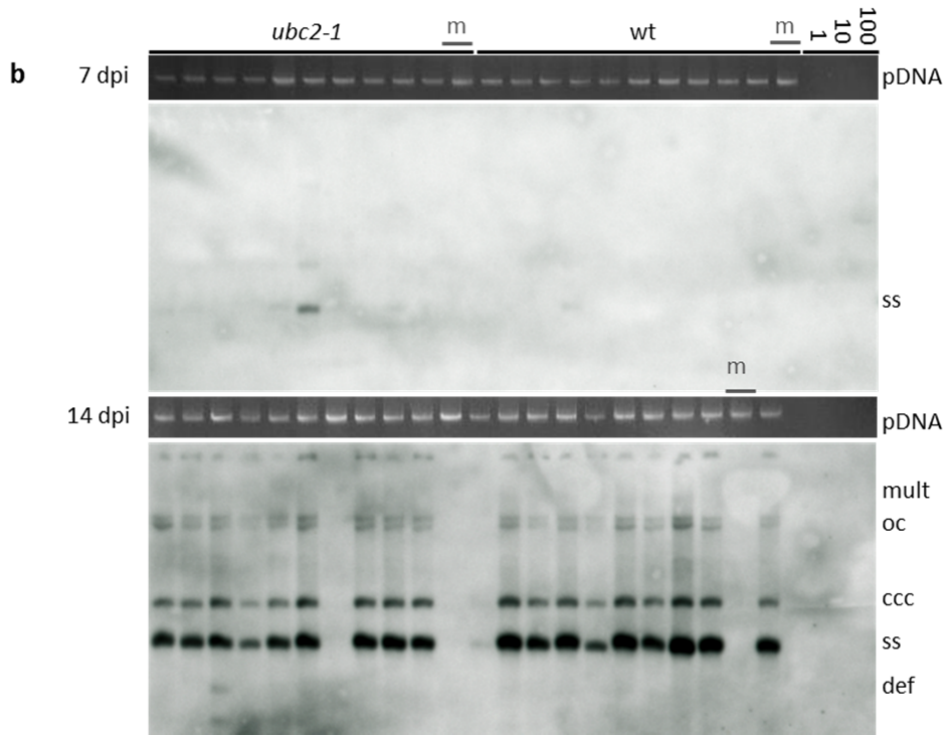


Figure 3: EuYMV DNA emergence at 7 and 14 dpi in *rev3-2 polh-1, rev3-1 polh-1* (a) and *ubc2-1* (b) in *A. thaliana* plants in comparison to wt plants as described in Fig. 1.

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 *At*POLK 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

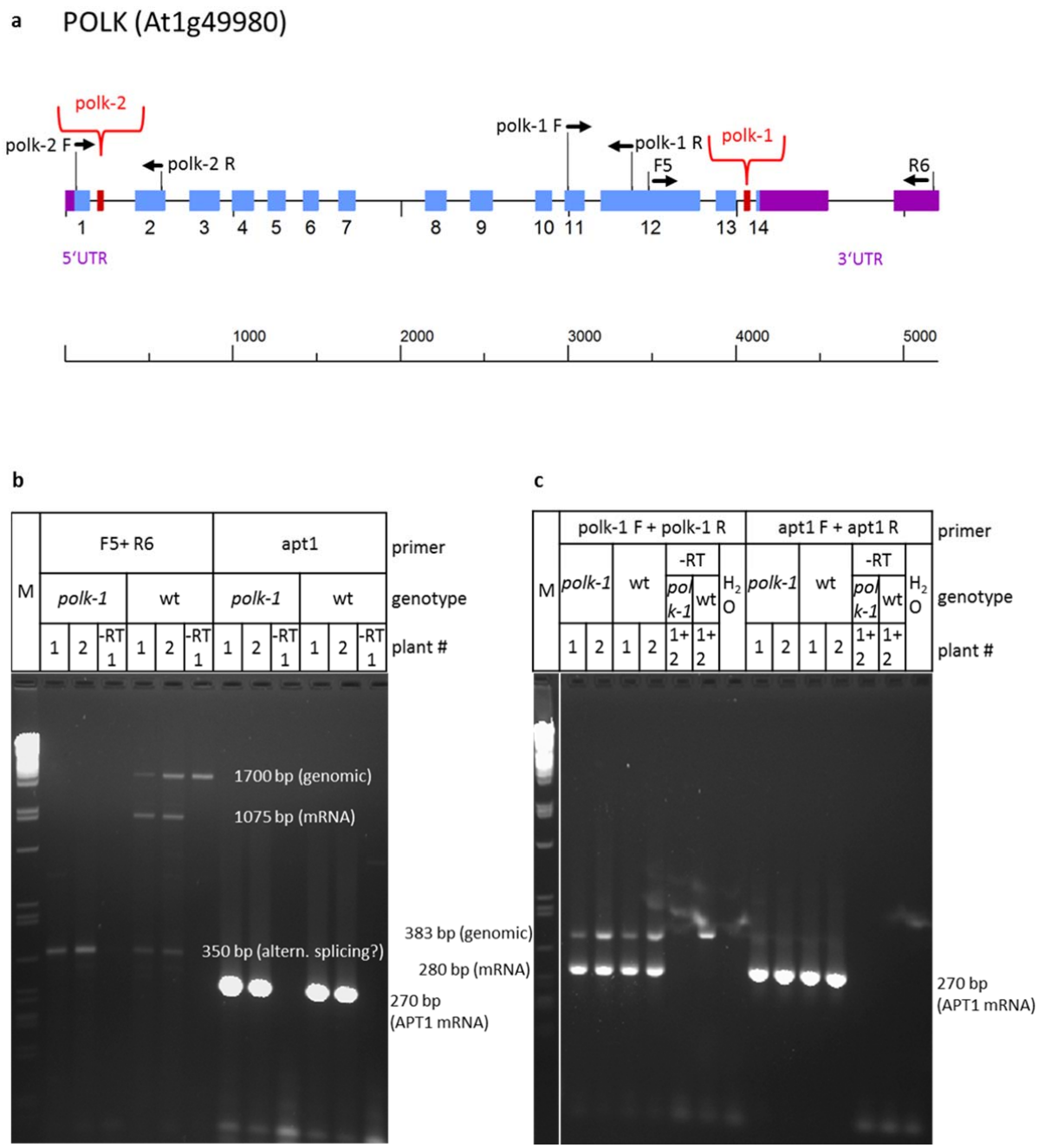


Figure 4: Gene organization of POLK (At1g49980) and RT-PCR analysis. (a) A gene model with exon/intron structure of *A. thaliana* POLK, the positions of the T-DNA insertions *polk-1* and *polk-2* and primer binding sites for RT-PCR are depicted. (b and c) POLK transcripts from two individual samples 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 alterative) and genomic DNA contaminations were depicted. (M: PstI digested λ phage DNA)

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

To determine whether truncated Polκ 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 *At*Polκ 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 *At*Polκ 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 *At*Polκ variant would exhibit an expected molecular mass of 20 kD. Altogether, these data indicate that Polκ variants might be expressed in wt as well as *polk-1* plants, and it cannot be completely excluded that those contain residual polymerase activity.

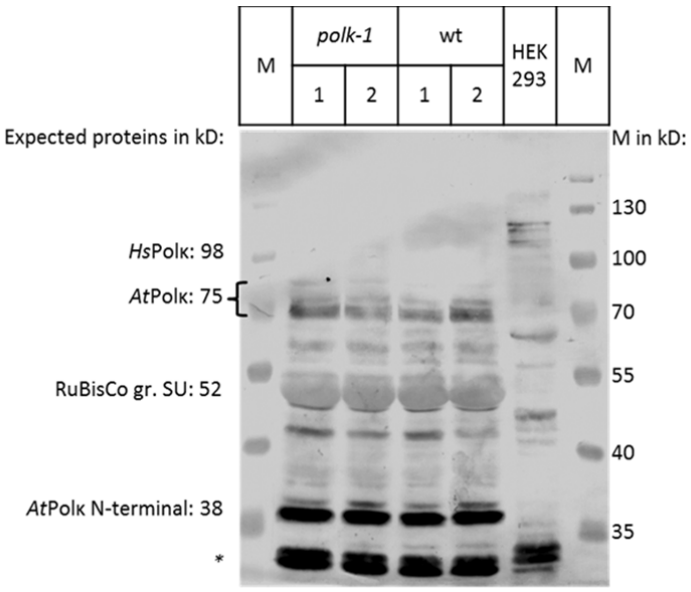


Figure 5: SDS-PAGE/Western blot to analyze expression of full-length Polκ or putative N-terminal Polκ fragments in *polk-1* and wt plants. Total protein extracts were separated by 10 % SDS-PAGE. Polκ was detected with a polyclonal rabbit anti-Polκ antiserum against the N-terminus of human Polκ, alkaline phosphatase-conjugated goat antirabbit 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 *At*Polκ and *Hs*Polκ 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.

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).

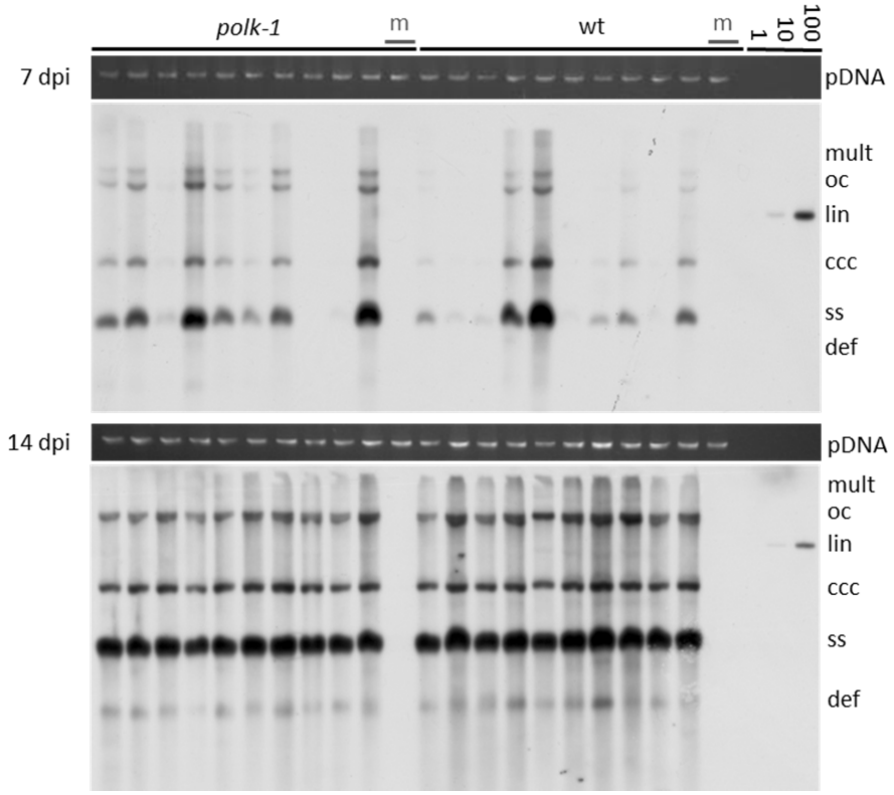


Figure 6: EuYMV DNA emergence at 7 and 14 dpi in *polk-1* and wt *A. thaliana* plants as described in Fig. 1.

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 1st and 2nd 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

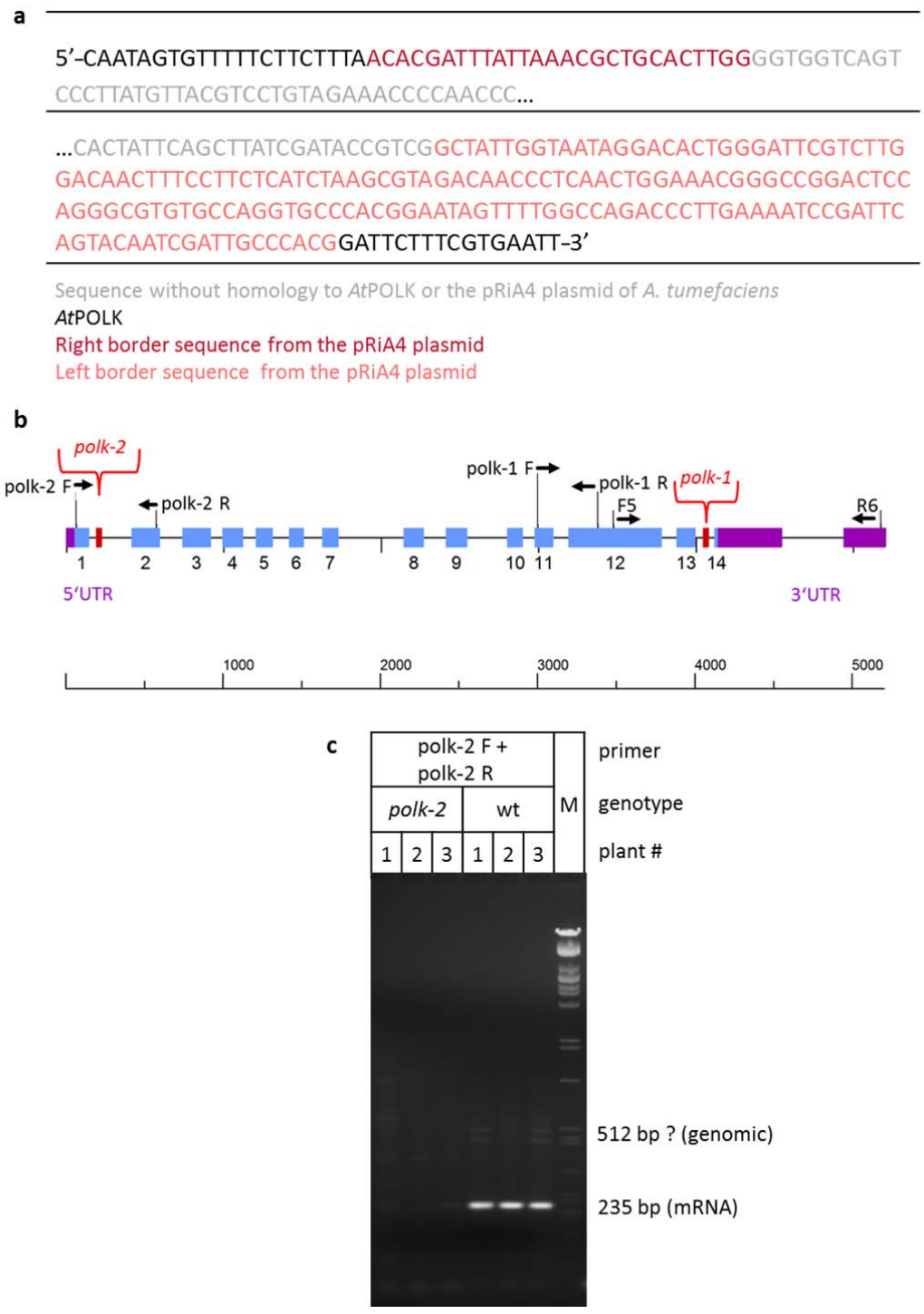


Figure 7: Analysis of *A. thaliana* line *polk-2*. (a) Sequence of the T-DNA/POLK junctions. The color code indicates *At*POLK, RB or LB T-DNA sequences of pRiA4. (b) Gene organization of POLK (At1g49980) with exon/intron structure, the positions of the T-DNA insertions *polk-1* and *polk-2* and primer 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 ClLCrV ssDNA were generated from viral particle preparation followed either by Cs_2SO_4 density gradient centrifugation or by *Sau*3AI digestion to dispose of viral dsDNA. This approach led to an infection rate of only 10-20 % with ClLCrV 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 ClLCrV DNA A and B were established, which revealed 100 % infection of *N. benthiamana* 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 ClLCrV (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.

Table 1: Infection rates of alternative inoculation tests.

Plant species	Inoculation procedure	EuYMV	ClLCrV
A. thaliana	Biolistics[a]	0/10	1/10
	Agroinoculation	8/20	n.t.
	B. tabaci transmission	7/7	3/7
N. benthamiana	Biolistics[b]	0/10	2/10
	Agroinoculation	4/4	4/4
	B. tabaci transmission	n.t.	n.t

Numbers represent infected individuals per inoculated individuals.

[a:] After virus particle preparation and Cs_2SO_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.

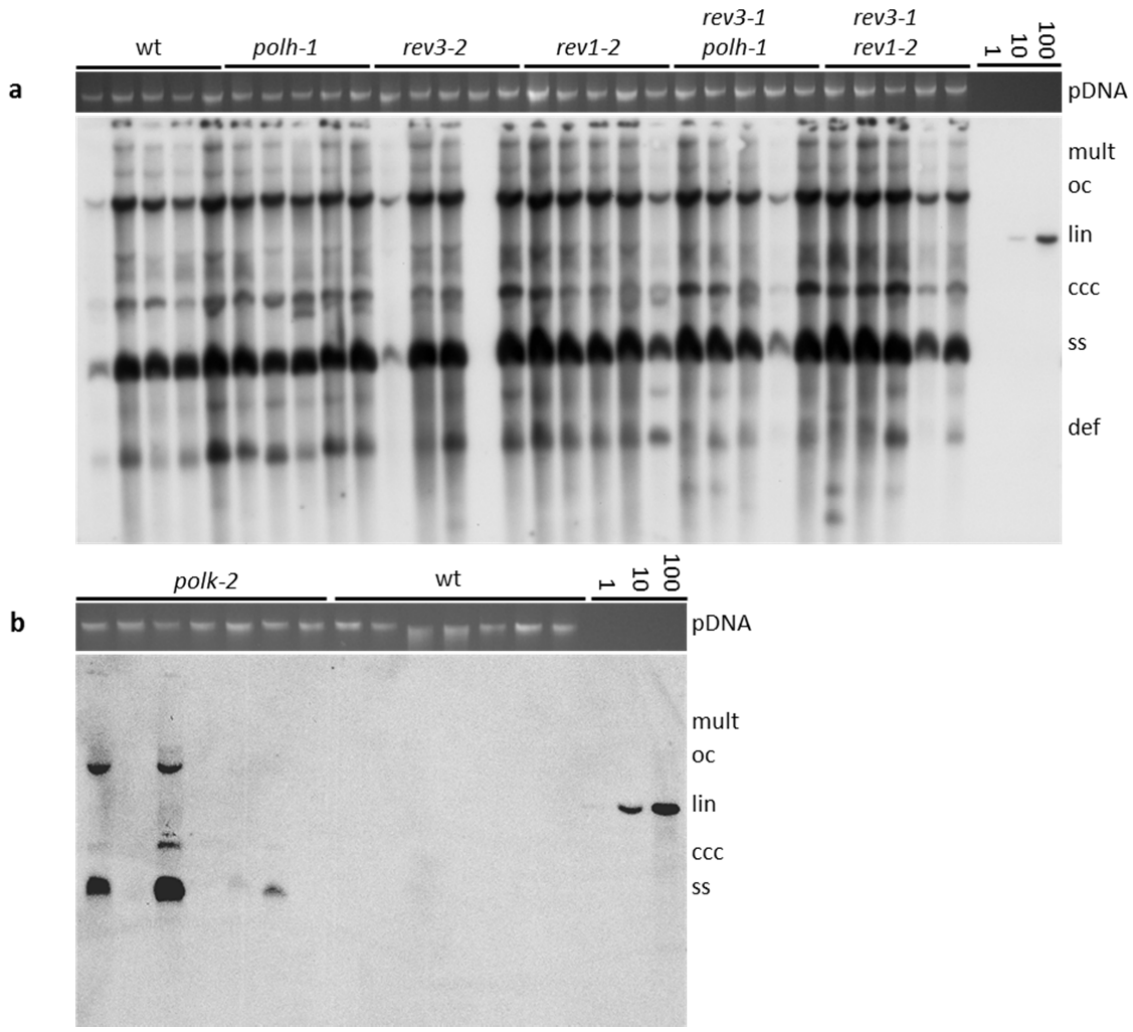


Figure 8: : *B. tabaci* transmission of EuYMV DNA: emergence at 14 dpi after in *A. thaliana* (a) *polh-1*, *rev3-2*, *rev1-2*, *rev3-1 polh-1*, *rev3-1 rev1-1* and wt siblings of line *rev3-2* and (b) in *polk-2* in comparison to its wt siblings as described in Fig. 1.

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

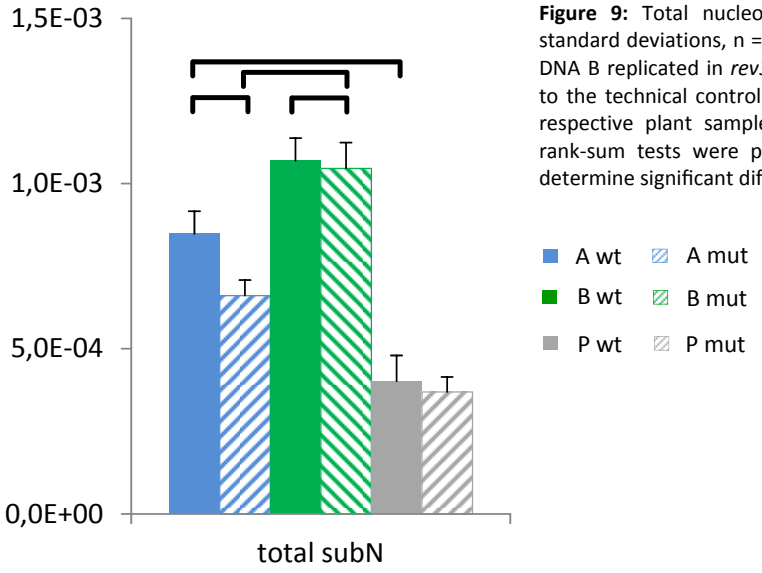


Figure 9: Total nucleotide substitution rates (mean values, standard deviations, n = 4 data batches each) for viral DNA A or DNA B replicated in *rev3-2 polh-1* (mut) or wt plants compared to the technical control of plasmid DNA (p) added each to the respective plant samples. Student's t-tests or Mann-Whitney 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, Polζ 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 Polζ/Polη or Polζ/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.

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

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 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 (ClLCrV) 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.

Knock-out line	Line no.	Gene no.	Primer sequences
polh-1	Salk_129731	At5G44740	LP: 5'GAGGACTGCCTAGTGCAGTTG
			RP: 5'GTCAGTTTGCAAGCTAGTGCC
rev3-2	Salk_029237	At1G67500	LP: 5'GATTGCCTAAGTGCTGGACTG
			RP: 5'TATACGTGCAAAGGCCATTTG
rev1-2	Salk_005721C	At5g44750	LP: 5'CTCTTCAAAAGGATTTTGGGG
			RP: 5'CGTTCAGTCTCAAGGACCAAC
polk-1	Salk_081715	At1g49980	LP: 5'GGTAAAGCGAGATCGGAATTC
			RP: 5'AATGCGTCTTGTGTGGGATAG
polk-2	Flag_566E01	"	LP: 5'CCAAAATAAGAAAGTCGTCGAAG
			RP: 5'TTTGTCAACTCCCTCCATACC
ubc2-1	Salk_060994	At2g02760	LP: 5'AGTCCAGCTTTGTTCGACAAC
			RP: 5'GGCAGATTCAAATGGTTTGAG
	Insertion lines	Primer name	Sequences
	Salk	LBb1.3	5'ATTTTGCCGATTTCGGAAC
	Flag	Tag5	5'CTACAAATTGCCTTTTCTTATCGAC

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 ClLCrV 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 ClLCrV particles were prepared from infected *N. benthamiana* plants and purified by Cs_2SO_4 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 H_20 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 ClLCrV 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) transfomants 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.

Knock-out line	Line no.	Primer name	Primer sequences
polk-1	Salk_081715	polk-1 F	5'GTTAGCCTGCAGAGGTACAC
		polk-1 R	5'AGGATACATTCCCGCTCTCG
		F5	5'TGTATGAAAAAAGAAGAAAGGCTGCAAA
		R6	5'ATTCTTGTCAGAAATCATATGGTGTAG
polk-2	Flag_566E01	polk-2 F	5' TAATGGCGAATCATCATCCA
		Polk-2 R	5' ATCAAGGGATGAAAGCTTTGC

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 % gycerol, 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 antiserum (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/choride–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) work flow and t-tests or Mann-Whitney rank-sum tests were carried out with the SigmaStat program (Systat Software, San Jose, CA, USA).

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References

Abouzid, AM, Frischmuth, T, Jeske, H (1988) A putative replicative form of the Abutilon mosaic virus (gemini group) in a chromatin-like structure. *Mol Gen Genet* **212**: 252-258

Anderson, HJ, Vonarx, EJ, Pastushok, L, Nakagawa, M, Katafuchi, A, Gruz, P, Di Rubbo, A, Grice, DM, Osmond, MJ, Sakamoto, AN, Nohmi, T, Xiao, W, Kunz, BA (2008) *Arabidopsis thaliana* Y-family DNA polymerase eta catalyses translesion synthesis and interacts functionally with PCNA2. *Plant J* **55**: 895-908

Arana, ME, Kunkel, TA (2010) Mutator phenotypes due to DNA replication infidelity. *Semin Cancer Biol* **20**: 304-311

Arguello-Astorga, G, Lopez-Ochoa, L, Kong, LJ, Orozco, BM, Settlage, SB, Hanley-Bowdoin, L (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma-related protein. *J Virol* **78**: 4817-4826

Avkin, S, Goldsmith, M, Velasco-Miguel, S, Geacintov, N, Friedberg, EC, Livneh, Z (2004) Quantitative analysis of translesion DNA synthesis across a benzo[a]pyrene-guanine adduct in mammalian cells: the role of DNA polymerase kappa. *J Biol Chem* **279**: 53298-53305

Bach, J, Jeske, H (2014) Defective DNAs of beet curly top virus from long-term survivor sugar beet plants. *Virus Res* **183**: 89-94

Böttcher, B, Unseld, S, Ceulemans, H, Russell, RB, Jeske, H (2004) Geminate structures of African cassava mosaic virus. *J Virol* **78**: 6758-6765

Bouchez, D, Camilleri, C, Caboche, M (1993) A binary vector based on Basta resistance for *in planta* transformation of *Arabidopsis thaliana*. *C R Acad Sci Paris, Life sciences* **316**: 1188–1193

Boykin, IM, de Barro, PJ (2014) A practical guide to identifying members of the *Bemisia tabaci* species complex: and other morphologically identical species. *Front Ecol Evol* **2**: 1-5

Chiel, E, Gottlieb, Y, Zchori-Fein, E, Mozes-Daube, N, Katzir, N, Inbar, M, Ghanim, M (2007) Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bull Entomol Res* **97**: 407-413

Choi, JY, Lim, S, Kim, EJ, Jo, A, Guengerich, FP (2010) Translesion synthesis across abasic lesions by human B-family and Y-family DNA polymerases alpha, delta, eta, iota, kappa, and REV1. *J Mol Biol* **404**: 34-44

Choudhury, NR, Malik, PS, Singh, DK, Islam, MN, Kaliappan, K, Mukherjee, SK (2006) The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. *Nucleic Acids Res* **34**: 6362-6377

Clerot, D, Bernardi, F (2006) DNA helicase activity is associated with the replication initiator protein rep of tomato yellow leaf curl geminivirus. *J Virol* **80**: 11322-11330

Curtis, MJ, Hays, JB (2007) Tolerance of dividing cells to replication stress in UVB-irradiated *Arabidopsis* roots: requirements for DNA translesion polymerases eta and zeta. *DNA Repair* **6**: 1341-1358

Duffy, S, Holmes, EC (2008) Phylogenetic evidence for rapid rates of molecular evolution in the single-stranded DNA begomovirus tomato yellow leaf curl virus. *J Virol* **82**: 957-965

Duffy, S, Holmes, EC (2009) Validation of high rates of nucleotide substitution in geminiviruses: phylogenetic evidence from East African cassava mosaic viruses. *J Gen Virol* **90**: 1539-1547

Fondong, VN (2013) Geminivirus protein structure and function. *Mol Plant Pathol* **14**: 635-649

Frischmuth, T, Engel, M, Jeske, H (1997) Beet curly top virus DI DNA-mediated resistance is linked to its size. *Mol Breeding* **3**: 213-217

Frohlich, DR, Torres-Jerez, II, Bedford, ID, Markham, PG, Brown, JK (1999) A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. *Mol Ecol* **8**: 1683-1691

Garcia-Ortiz, MV, Ariza, RR, Hoffman, PD, Hays, JB, Roldan-Arjona, T (2004) *Arabidopsis thaliana At*POLK encodes a DinB-like DNA polymerase that extends mispaired primer termini and is highly expressed in a variety of tissues. *Plant J* **39**: 84-97

Garcia-Ortiz, MV, Roldan-Arjona, T, Ariza, RR (2007) The noncatalytic C-terminus of *At*POLK Y-family DNA polymerase affects synthesis fidelity, mismatch extension and translesion replication. *FEBS J* **274**: 3340-3350

Ge, L, Zhang, J, Zhou, X, Li, H (2007) Genetic structure and population variability of tomato yellow leaf curl China virus. *J Virol* **81**: 5902-5907

Goodman, MF, Woodgate, R (2013) Translesion DNA polymerases. *Cold Spring Harb Perspect Biol* **5**: a010363

Gray, S, Cilia, M, Ghanim, M (2014) Circulative, "nonpropagative" virus transmission: an orchestra of virus-, insect-, and plant-derived instruments. *Adv Virus Res* **89**: 141-199

Gutierrez, C, Ramirez-Parra, E, Mar Castellano, M, Sanz-Burgos, AP, Luque, A, Missich, R (2004) Geminivirus DNA replication and cell cycle interactions. *Vet Microbiol* **98**: 111-119

Haible, D, Kober, S, Jeske, H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J Virol Methods* **135**: 9-16

Hanley-Bowdoin, L, Bejarano, ER, Robertson, D, Mansoor, S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat Rev Microbiol* **11**: 777-788

Haracska, L, Johnson, RE, Unk, I, Phillips, B, Hurwitz, J, Prakash, L, Prakash, S (2001) Physical and functional interactions of human DNA polymerase eta with PCNA. *Mol Cell Biol* **21**: 7199-7206

Haracska, L, Prakash, L, Prakash, S (2002a) Role of human DNA polymerase kappa as an extender in translesion synthesis. *Proc Natl Acad Sci U S A* **99**: 16000-16005

Haracska, L, Prakash, S, Prakash, L (2003) Yeast DNA polymerase zeta is an efficient extender of primer ends opposite from 7,8-dihydro-8-Oxoguanine and O6-methylguanine. *Mol Cell Biol* **23**: 1453-1459

Haracska, L, Unk, I, Johnson, RE, Phillips, BB, Hurwitz, J, Prakash, L, Prakash, S (2002b) Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA. *Mol Cell Biol* **22**: 784-791

Hellens, RP, Edwards, EA, Leyland, NR, Bean, S, Mullineaux, PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* **42**: 819-832

Hipp, K, Rau, P, Schafer, B, Gronenborn, B, Jeske, H (2014) The RXL motif of the African cassava mosaic virus Rep protein is necessary for rereplication of yeast DNA and viral infection in plants. *Virology* **462-463**: 189-198

Horn, J, Lauster, S, Krenz, B, Kraus, J, Frischmuth, T, Jeske, H (2011) Ambivalent effects of defective DNA in beet curly top virus-infected transgenic sugarbeet plants. *Virus Res* **158**: 169-178

Jeske, H, (2009). Geminiviruses, in: *Torque Teno Virus: The Still Elusive Human Pathogens*, pp. 185-226. Edited by zur Hausen, H., de Villiers, E.-M. Springer, Berlin.

Jeske, H, Lütgemeier, M, Preiss, W (2001) Distinct DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic geminivirus. *EMBO J* **20**: 6158-6167

Jia, L, Geacintov, NE, Broyde, S (2008) The N-clasp of human DNA polymerase kappa promotes blockage or error-free bypass of adenine- or guanine-benzo[a]pyrenyl lesions. *Nucleic Acids Res* **36**: 6571-6584

Johnson, RE, Prakash, S, Prakash, L (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. *Science* **283**: 1001-1004

Johnson, RE, Washington, MT, Haracska, L, Prakash, S, Prakash, L (2000) Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions. *Nature* **406**: 1015-1019

Jouanin, L, Bouchez, D, Drong, RF, Tepfer, D, Slightom, JL (1989) Analysis of TR-DNA/plant junctions in the genome of a *Convolvulus arvensis* clone transformed by *Agrobacterium rhizogenes* strain A4. *Plant Mol Biol* **12**: 75-85

Kittelmann, K, Jeske, H (2008) Disassembly of African cassava mosaic virus. *J Gen Virol* **89**: 2029-2036

Kittelmann, K, Rau, P, Gronenborn, B, Jeske, H (2009) Plant geminivirus rep protein induces rereplication in fission yeast. *J Virol* **83**: 6769-6778

Kollenberg, M, Winter, S, Gotz, M (2014) Quantification and localization of Watermelon chlorotic stunt virus and Tomato yellow leaf curl virus (Geminiviridae) in populations of *Bemisia tabaci* (Hemiptera, Aleyrodidae) with differential virus transmission characteristics. *PLoS One* **9**: e111968

Kong, LJ, Orozco, BM, Roe, JL, Nagar, S, Ou, S, Feiler, HS, Durfee, T, Miller, AB, Gruissem, W, Robertson, D, Hanley-Bowdoin, L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J* **19**: 3485-3495

Kozmin, SG, Pavlov, YI, Kunkel, TA, Sage, E (2003) Roles of *Saccharomyces cerevisiae* DNA polymerases Poleta and Polzeta in response to irradiation by simulated sunlight. *Nucleic Acids Res* **31**: 4541-4552

Laemmli, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685

Laufs, J, Traut, W, Heyraud, F, Matzeit, V, Rogers, SG, Schell, J, Gronenborn, B (1995) *In vitro* cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proc Natl Acad Sci U S A* **92**: 3879-3883

Lee, KY, Myung, KJ (2008) PCNA modifications for regulation of post-replication repair pathways. *Mol Cells* **26**: 5-11

Levy, A, Tzfira, T (2010) Bean dwarf mosaic virus: a model system for the study of viral movement. *Mol Plant Pathol* **11**: 451-461

Luque, A, Sanz-Burgos, AP, Ramirez-Parra, E, Castellano, MM, Gutierrez, C (2002) Interaction of geminivirus Rep protein with replication factor C and its potential role during geminivirus DNA replication. *Virology* **302**: 83-94

Matsuda, T, Bebenek, K, Masutani, C, Hanaoka, F, Kunkel, TA (2000) Low fidelity DNA synthesis by human DNA polymerase-eta. *Nature* **404**: 1011-1013

Matsuda, T, Bebenek, K, Masutani, C, Rogozin, IB, Hanaoka, F, Kunkel, TA (2001) Error rate and specificity of human and murine DNA polymerase eta. *J Mol Biol* **312**: 335-346

McCulloch, SD, Kunkel, TA (2008) The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res* **18**: 148-161

McDonald, JP, Rapic-Otrin, V, Epstein, JA, Broughton, BC, Wang, X, Lehmann, AR, Wolgemuth, DJ, Woodgate, R (1999) Novel human and mouse homologs of Saccharomyces cerevisiae DNA polymerase eta. *Genomics* **60**: 20-30

Minko, IG, Yamanaka, K, Kozekov, ID, Kozekova, A, Indiani, C, O'Donnell, ME, Jiang, Q, Goodman, MF, Rizzo, CJ, Lloyd, RS (2008) Replication bypass of the acrolein-mediated deoxyguanine DNA-peptide cross-links by DNA polymerases of the DinB family. *Chem Res Toxicol* **21**: 1983-1990

Monjane, AL, Pande, D, Lakay, F, Shepherd, DN, van der Walt, E, Lefeuvre, P, Lett, JM, Varsani, A, Rybicki, EP, Martin, DP (2012) Adaptive evolution by recombination is not associated with increased mutation rates in Maize streak virus. *BMC Evol Biol* **12**: 252

Nagar, S, Hanley-Bowdoin, L, Robertson, D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**: 2995-3007

Nagar, S, Pedersen, TJ, Carrick, KM, Hanley-Bowdoin, L, Robertson, D (1995) A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. *Plant Cell* **7**: 705-719

Nakagawa, M, Takahashi, S, Tanaka, A, Narumi, I, Sakamoto, AN (2011) Role of *At*Polzeta, *At*Rev1, and *At*Poleta in UV light-induced mutagenesis in *Arabidopsis*. *Plant Physiol* **155**: 414-420

Nelson, JR, Lawrence, CW, Hinkle, DC (1996) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature* **382**: 729-731

Ohashi, E, Bebenek, K, Matsuda, T, Feaver, WJ, Gerlach, VL, Friedberg, EC, Ohmori, H, Kunkel, TA (2000) Fidelity and processivity of DNA synthesis by DNA polymerase kappa, the product of the human DINB1 gene. *J Biol Chem* **275**: 39678-39684

Paprotka, T, Deuschle, K, Pilartz, M, Jeske, H (2015) Form follows function in geminiviral minichromosome architecture. *Virus Res* **196**: 44-55

Patil, BL, Dasgupta, I (2006) Defective interfering DNAs of plant viruses. *Crit Rev Plant Sci* **24**: 1–18

Patra, A, Zhang, Q, Lei, L, Su, Y, Egli, M, Guengerich, FP (2015) Structural and Kinetic Analysis of Nucleoside Triphosphate Incorporation Opposite an Abasic Site by Human Translesion DNA Polymerase eta. *J Biol Chem*

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

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Richter, KS, Ende, L, Jeske, H (2015) Rad54 is not essential for any geminiviral replication mode in planta. *Plant Mol Biol* **87**: 193-202

Richter, KS, Kleinow, T, Jeske, H (2014) Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Virology* **452-453**: 287-296

Rojas, MR, Hagen, C, Lucas, WJ, Gilbertson, RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annu Rev Phytopathol* **43**: 361-394

Sabbioneda, S, Minesinger, BK, Giannattasio, M, Plevani, P, Muzi-Falconi, M, Jinks-Robertson, S (2005) The 9-1-1 checkpoint clamp physically interacts with Pol zeta and is partially required for spontaneous Pol zeta-dependent mutagenesis in *Saccharomyces cerevisiae*. *J Biol Chem* **280**: 38657-38665

Sakamoto, A, Lan, VT, Hase, Y, Shikazono, N, Matsunaga, T, Tanaka, A (2003) Disruption of the *At*REV3 gene causes hypersensitivity to ultraviolet B light and gamma-rays in *Arabidopsis*: implication of the presence of a translesion synthesis mechanism in plants. *Plant Cell* **15**: 2042-2057

Santiago, MJ, Alejandre-Duran, E, Munoz-Serrano, A, Ruiz-Rubio, M (2008a) Two translesion synthesis DNA polymerase genes, *At*POLH and *At*REV1, are involved in development and UV light resistance in *Arabidopsis*. *J Plant Physiol* **165**: 1582-1591

Santiago, MJ, Alejandre-Duran, E, Ruiz-Rubio, M (2006) Analysis of UV-induced mutation spectra in *Escherichia coli* by DNA polymerase eta from *Arabidopsis thaliana*. *Mutat Res* **601**: 51-60

Santiago, MJ, Ruiz-Rubio, M, Dio, LD, Gonzalez-Reyes, JA, Alejandre-Duran, E (2008b) Ubiquitous expression of two translesion synthesis DNA polymerase genes in *Arabidopsis*. *Planta* **227**: 1269-1277

Sekimoto, T, Oda, T, Kurashima, K, Hanaoka, F, Yamashita, T (2015) Both high-fidelity replicative and low-fidelity Y-family polymerases are involved in DNA rereplication. *Mol Cell Biol* **35**: 699-715

Shen, WJ, Forde, BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res* **17**: 8385

Stanley, J (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology* **206**: 707-712

Takahashi, S, Sakamoto, A, Sato, S, Kato, T, Tabata, S, Tanaka, A (2005) Roles of *Arabidopsis* *At*REV1 and *At*REV7 in translesion synthesis. *Plant Physiol* **138**: 870-881

Takahashi, S, Sakamoto, AN, Tanaka, A, Shimizu, K (2007) *At*REV1, a Y-family DNA polymerase in *Arabidopsis*, has deoxynucleotidyl transferase activity *in vitro*. *Plant Physiol* **145**: 1052-1060

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

van der Walt, E, Martin, DP, Varsani, A, Polston, JE, Rybicki, EP (2008) Experimental observations of rapid Maize streak virus evolution reveal a strand-specific nucleotide substitution bias. *Virol J* **5**

Von Arnim, A, Frischmuth, T, Stanley, J (1993) Detection and possible functions of African cassava mosaic virus DNA B gene products. *Virology* **192**: 264-272

Washington, MT, Johnson, RE, Prakash, L, Prakash, S (2001) Accuracy of lesion bypass by yeast and human DNA polymerase eta. *Proc Natl Acad Sci U S A* **98**: 8355-8360

Washington, MT, Johnson, RE, Prakash, L, Prakash, S (2002) Human DINB1-encoded DNA polymerase kappa is a promiscuous extender of mispaired primer termini. *Proc Natl Acad Sci U S A* **99**: 1910-1914

Waters, LS, Minesinger, BK, Wiltrout, ME, D'Souza, S, Woodruff, RV, Walker, GC (2009) Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. *Microbiol Mol Biol Rev* **73**: 134-154

Wege, C, **(2007)**. Movement and localization of Tomato yellow leaf curl viruses in the infected plant, in: *Tomato yellow leaf curl virus disease*, pp. 185-206. Edited by Czosnek, H. Springer, Dordrecht, Netherlands.

Wei, J, Zhao, JJ, Zhang, T, Li, FF, Ghanim, M, Zhou, XP, Ye, GY, Liu, SS, Wang, XW (2014) Specific cells in the primary salivary glands of the whitefly *Bemisia tabaci* control retention and transmission of begomoviruses. *J Virol* **88**: 13460-13468

Wyant, PS, Gotthardt, D, Schafer, B, Krenz, B, Jeske, H (2011) The genomes of four novel begomoviruses and a new *Sida micrantha* mosaic virus strain from Bolivian weeds. *Arch Virol* **156**: 347-352

Xu, L, Menard, R, Berr, A, Fuchs, J, Cognat, V, Meyer, D, Shen, WH (2009) The E2 ubiquitin-conjugating enzymes, *At*UBC1 and *At*UBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in *Arabidopsis thaliana*. *Plant J* **57**: 279-288

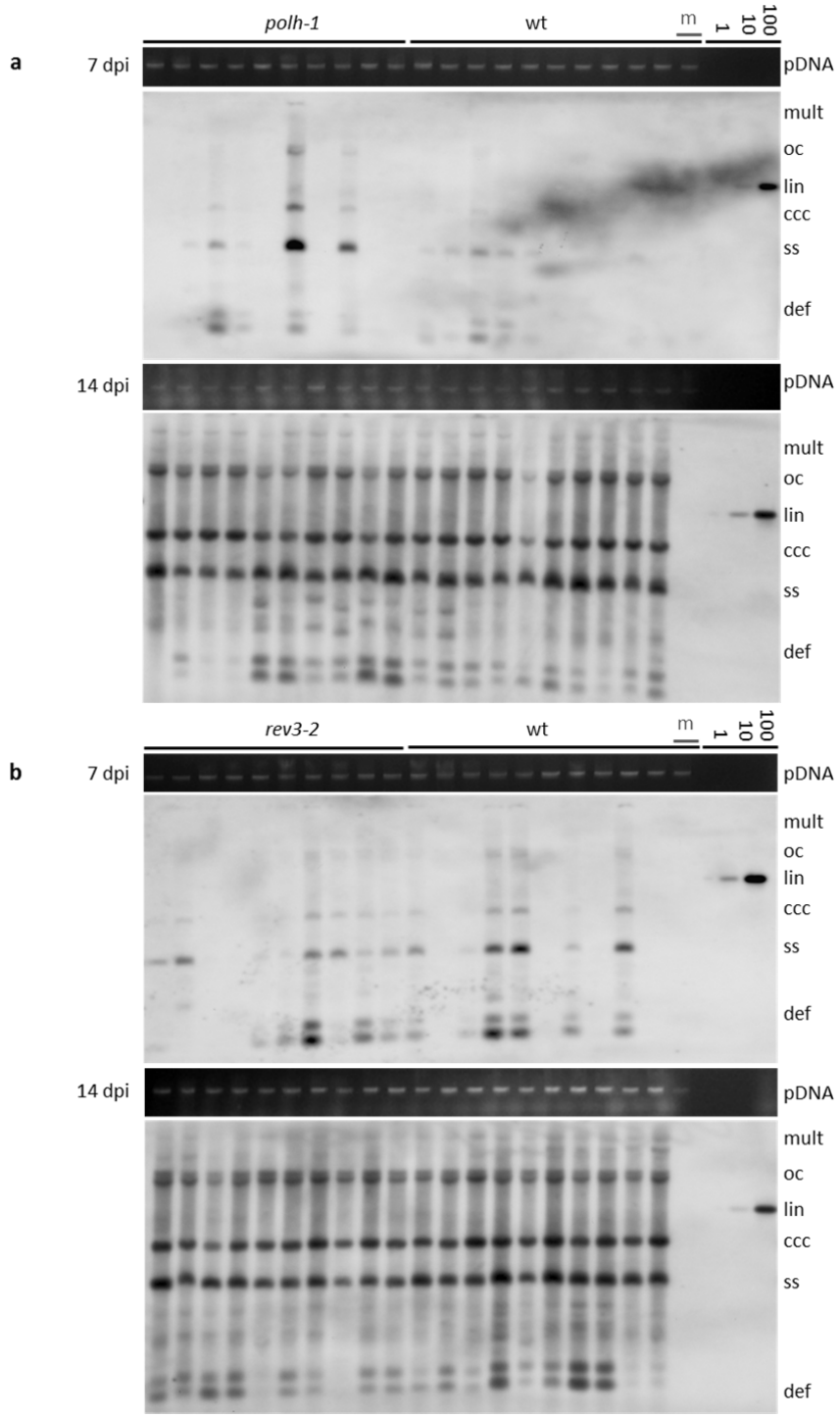
Zhang, W, Olson, NH, Baker, TS, Faulkner, L, Agbandje-McKenna, M, Boulton, MI, Davies, JW, McKenna, R (2001) Structure of the Maize streak virus geminate particle. *Virology* **279**: 471-477

Zhang, Y, Wu, X, Rechkoblit, O, Geacintov, NE, Taylor, JS, Wang, Z (2002) Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. *Nucleic Acids Res* **30**: 1630-1638

Zhong, X, Garg, P, Stith, CM, Nick McElhinny, SA, Kissling, GE, Burgers, PM, Kunkel, TA (2006) The fidelity of DNA synthesis by yeast DNA polymerase zeta alone and with accessory proteins. *Nucleic Acids Res* **34**: 4731-4742

Zwirn, P, Stary, S, Luschnig, C, Bachmair, A (1997) *Arabidopsis thaliana* RAD6 homolog *At*UBC2 complements UV sensitivity, but not N-end rule degradation deficiency, of *Saccharomyces cerevisiae* rad6 mutants. *Curr Genet* **32**: 309-314

Supplementary data



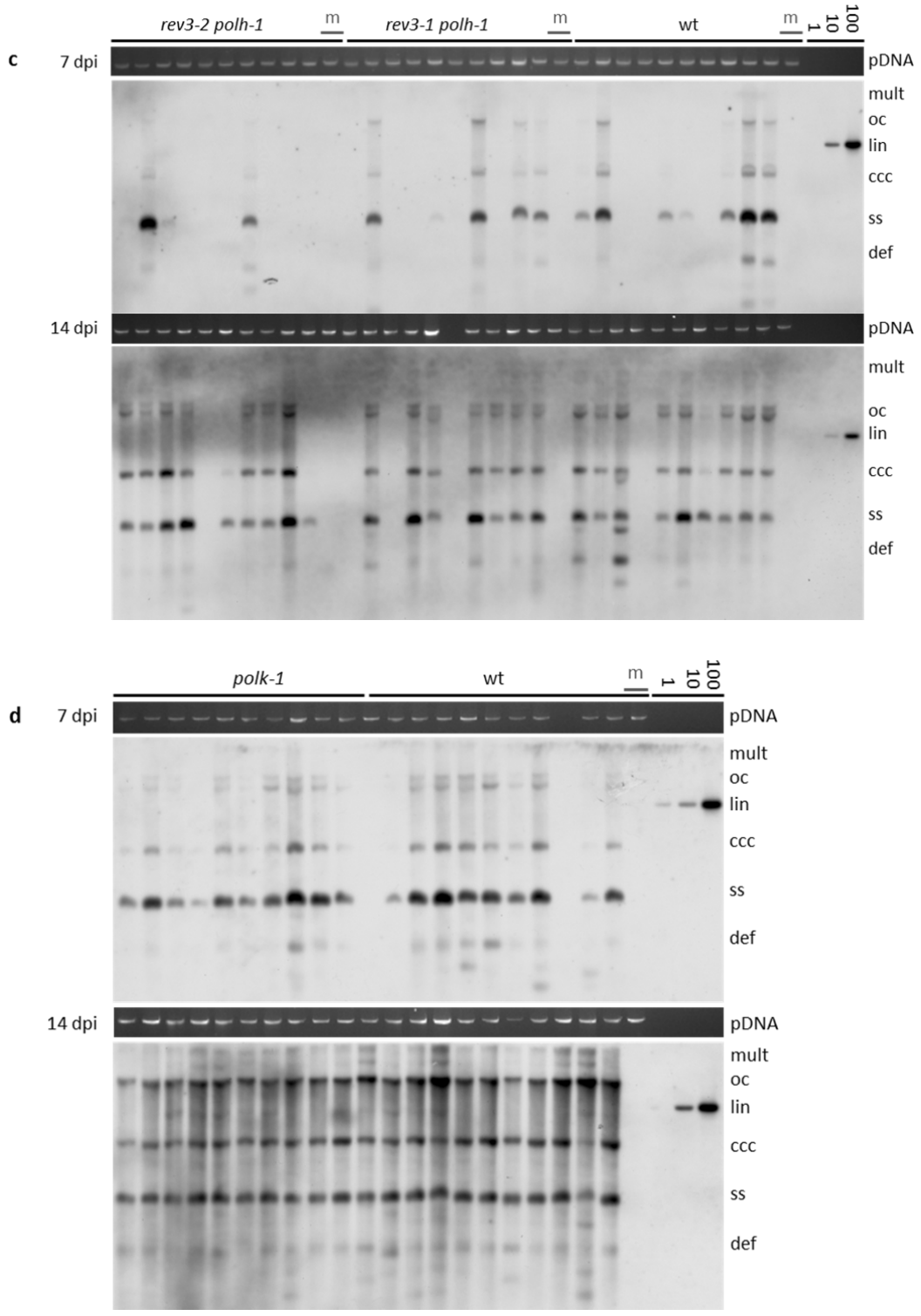


Figure S1: ClLCrV DNA emergence at 7 and 14 dpi in *polh-1* (a), *rev3-2* (b), *rev3-2 polh-1*, *rev3-1 polh-1* (c), *polk-1* (d) and respective wt sibling plants. Virus DNA was hybridized with DIG-labeled full-length ClLCrV DNA A probes and detected as described in Fig. 1.

Table S1: Summary of individual inoculation experiments.

Inoculation procedure	Simultaneously tested plant lines	EuYMV	ClLCrV
Biolistics	*polh-1* + wt siblings	3 x 10	3 x 10
	rev3-2 + wt siblings	3 x 10	3 x 10
	rev1-2 + wt siblings of *rev3-2*	1 x 10	n.t.
	polk-1 + wt sibings	1 x 10	1 x 10
	rev3-2 polh-1 + wt siblings+ *rev3-1 polh-1*	2 x 10	2 x 10
Insect transmission	*polh-1* + *rev3-2* + *rev1-2*+ *rev3-1 polh-1* + *rev3-1* *rev1-1* + wt siblings of *rev3-2*	1 x 5	n.t.
	polk-2+ wt siblings	1 x 7	n.t.

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

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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

Abouzid, AM, Frischmuth, T, Jeske, H (1988) A putative replicative form of the Abutilon mosaic virus (gemini group) in a chromatin-like structure. *Mol Gen Genet* **212**: 252-258

Amiard, S, Charbonnel, C, Allain, E, Depeiges, A, White, CI, Gallego, ME (2010) Distinct roles of the ATR kinase and the Mre11-Rad50-Nbs1 complex in the maintenance of chromosomal stability in *Arabidopsis*. *Plant Cell* **22**: 3020-3033

Andersen, PL, Xu, F, Xiao, W (2008) Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. *Cell Res* **18**: 162-173

Arana, ME, Kunkel, TA (2010) Mutator phenotypes due to DNA replication infidelity. *Semin Cancer Biol* **20**: 304-311

Arguello-Astorga, G, Lopez-Ochoa, L, Kong, LJ, Orozco, BM, Settlage, SB, Hanley-Bowdoin, L (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma-related protein. *J Virol* **78**: 4817-4826

Arguello-Astorga, GR, Guevara-Gonzalez, RG, Herrera-Estrella, LR, Rivera-Bustamante, RF (1994) Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. *Virology* **203**: 90-100

Arguello-Astorga, GR, Ruiz-Medrano, R (2001) An iteron-related domain is associated to Motif 1 in the replication proteins of geminiviruses: identification of potential interacting amino acid-base pairs by a comparative approach. *Arch Virol* **146**: 1465-1485

Ascencio-Ibanez, JT, Sozzani, R, Lee, TJ, Chu, TM, Wolfinger, RD, Cella, R, Hanley-Bowdoin, L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol* **148**: 436-454

Bach, J, Jeske, H (2014) Defective DNAs of beet curly top virus from long-term survivor sugar beet plants. *Virus Res* **183**: 89-94

Bleuyard, JY, Gallego, ME, Savigny, F, White, CI (2005) Differing requirements for the *Arabidopsis* Rad51 paralogs in meiosis and DNA repair. *Plant J* **41**: 533-545

Böttcher, B, Unseld, S, Ceulemans, H, Russell, RB, Jeske, H (2004) Geminate structures of African cassava mosaic virus. *J Virol* **78**: 6758-6765

Boyko, A, Kovalchuk, I (2010) Transgenerational response to stress in *Arabidopsis thaliana*. *Plant Signal Behav* **5**: 995-998

Brewer, BJ (1988) When polymerases collide - replication and the transcriptional organization of the *Escherichia coli* chromosome. *Cell* **53**: 679-686

Briddon, RW, Pinner, MS, Stanley, J, Markham, PG (1990) Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177**: 85-94

Caldecott, KW (2014) DNA single-strand break repair. *Exp Cell Res* **329**: 2-8

Carvalho, CM, Fontenelle, MR, Florentino, LH, Santos, AA, Zerbini, FM, Fontes, EP (2008) A novel nucleocytoplasmic traffic GTPase identified as a functional target of the bipartite geminivirus nuclear shuttle protein. *Plant J* **55**: 869-880

Castillo, AG, Collinet, D, Deret, S, Kashoggi, A, Bejarano, ER (2003) Dual interaction of plant PCNA with geminivirus replication accessory protein (Ren) and viral replication protein (Rep). *Virology* **312**: 381-394

Ceballos, SJ, Heyer, WD (2011) Functions of the Snf2/Swi2 family Rad54 motor protein in homologous recombination. *Biochim Biophys Acta* **1809**: 509-523

Chapman, JR, Taylor, MR, Boulton, SJ (2012) Playing the end game: DNA double-strand break repair pathway choice. *Mol Cell* **47**: 497-510

Charbonnel, C, Allain, E, Gallego, ME, White, CI (2011) Kinetic analysis of DNA double-strand break repair pathways in *Arabidopsis*. *DNA Repair* **10**: 611-619

Charbonnel, C, Gallego, ME, White, CI (2010) Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in *Arabidopsis* plants. *Plant J* **64**: 280-290

Chellappan, P, Vanitharani, R, Fauquet, CM (2005) MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc Natl Acad Sci U S A* **102**: 10381-10386

Choudhury, NR, Malik, PS, Singh, DK, Islam, MN, Kaliappan, K, Mukherjee, SK (2006) The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. *Nucleic Acids Res* **34**: 6362-6377

Clerot, D, Bernardi, F (2006) DNA helicase activity is associated with the replication initiator protein rep of tomato yellow leaf curl geminivirus. *J Virol* **80**: 11322-11330

Culligan, KM, Robertson, CE, Foreman, J, Doerner, P, Britt, AB (2006) ATR and ATM play both distinct and additive roles in response to ionizing radiation. *Plant J* **48**: 947-961

Donson, J, Accotto, GP, Boulton, MI, Mullineaux, PM, Davies, JW (1987) The nucleotide sequence of a geminivirus from *Digitaria sanguinalis*. *Virology* **161**: 160-169

Donson, J, Morris-Krsinich, BA, Mullineaux, PM, Boulton, MI, Davies, JW (1984) A putative primer for second-strand DNA synthesis of maize streak virus is virion-associated. *Embo J* **3**: 3069-3073

Doutriaux, MP, Couteau, F, Bergounioux, C, White, C (1998) Isolation and characterisation of the RAD51 and DMC1 homologs from *Arabidopsis thaliana*. *Mol Gen Genet* **257**: 283-291

Duffy, S, Holmes, EC (2008) Phylogenetic evidence for rapid rates of molecular evolution in the single-stranded DNA begomovirus tomato yellow leaf curl virus. *J Virol* **82**: 957-965

Duffy, S, Holmes, EC (2009) Validation of high rates of nucleotide substitution in geminiviruses: phylogenetic evidence from East African cassava mosaic viruses. *J Gen Virol* **90**: 1539-1547

Erdmann, JB, Shepherd, DN, Martin, DP, Varsani, A, Rybicki, EP, Jeske, H (2010) Replicative intermediates of maize streak virus found during leaf development. *J Gen Virol* **91**: 1077-1081

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

Fondong, VN (2013) Geminivirus protein structure and function. *Mol Plant Pathol* **14**: 635-649

Friesner, J, Britt, AB (2003) Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration. *Plant J* **34**: 427-440

Frischmuth, S, Wege, C, Hulser, D, Jeske, H (2007) The movement protein BC1 promotes redirection of the nuclear shuttle protein BV1 of Abutilon mosaic geminivirus to the plasma membrane in fission yeast. *Protoplasma* **230**: 117-123

Frischmuth, T, Engel, M, Jeske, H (1997) Beet curly top virus DI DNA-mediated resistance is linked to its size. *Mol Breeding* **3**: 213-217

Frit, P, Barboule, N, Yuan, Y, Gomez, D, Calsou, P (2014) Alternative end-joining pathway(s): bricolage at DNA breaks. *DNA Repair* **17**: 81-97

Garcia-Andres, S, Monci, F, Navas-Castillo, J, Moriones, E (2006) Begomovirus genetic diversity in the native plant reservoir *Solanum nigrum*: Evidence for the presence of a new virus species of recombinant nature. *Virology* **350**: 433-442

Garcia-Ortiz, MV, Ariza, RR, Hoffman, PD, Hays, JB, Roldan-Arjona, T (2004) *Arabidopsis thaliana At*POLK encodes a DinB-like DNA polymerase that extends mispaired primer termini and is highly expressed in a variety of tissues. *Plant J* **39**: 84-97

Garcia-Ortiz, MV, Roldan-Arjona, T, Ariza, RR (2007) The noncatalytic C-terminus of AtPOLK Y-family DNA polymerase affects synthesis fidelity, mismatch extension and translesion replication. *FEBS J* **274**: 3340-3350

Ge, L, Zhang, J, Zhou, X, Li, H (2007) Genetic structure and population variability of tomato yellow leaf curl China virus. *J Virol* **81**: 5902-5907

Geuting, V, Kobbe, D, Hartung, F, Durr, J, Focke, M, Puchta, H (2009) Two distinct MUS81-EME1 complexes from *Arabidopsis* process Holliday junctions. *Plant Physiol* **150**: 1062-1071

Glazov, E, Phillips, K, Budziszewski, GJ, Schob, H, Meins, F, Jr., Levin, JZ (2003) A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*. *Plant J* **35**: 342-349

Goodman, MF, Woodgate, R (2013) Translesion DNA polymerases. *Cold Spring Harb Perspect Biol* **5**: a010363

Gray, S, Cilia, M, Ghanim, M (2014) Circulative, "nonpropagative" virus transmission: an orchestra of virus-, insect-, and plant-derived instruments. *Adv Virus Res* **89**: 141-199

Gregorio-Jorge, J, Bernal-Alcocer, A, Banuelos-Hernandez, B, Alpuche-Solis, AG, Hernandez-Zepeda, C, Moreno-Valenzuela, O, Frias-Trevino, G, Arguello-Astorga, GR (2010) Analysis of a new strain of Euphorbia mosaic virus with distinct replication specificity unveils a lineage of begomoviruses with short Rep sequences in the DNA-B intergenic region. *Virol J* **7**: 275

Gutierrez, C, Ramirez-Parra, E, Mar Castellano, M, Sanz-Burgos, AP, Luque, A, Missich, R (2004) Geminivirus DNA replication and cell cycle interactions. *Vet Microbiol* **98**: 111-119

Hanley-Bowdoin, L, Bejarano, ER, Robertson, D, Mansoor, S (2013) Geminiviruses: masters at redirecting and reprogramming plant processes. *Nat Rev Microbiol* **11**: 777-788

Hanley-Bowdoin, L, Settlage, SB, Robertson, D (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. *Mol Plant Pathol* **5**: 149-156

Haracska, L, Johnson, RE, Unk, I, Phillips, B, Hurwitz, J, Prakash, L, Prakash, S (2001) Physical and functional interactions of human DNA polymerase eta with PCNA. *Mol Cell Biol* **21**: 7199-7206

Haracska, L, Unk, I, Johnson, RE, Phillips, BB, Hurwitz, J, Prakash, L, Prakash, S (2002) Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA. *Mol Cell Biol* **22**: 784-791

Hartung, F, Plchova, H, Puchta, H (2000) Molecular characterisation of RecQ homologues in *Arabidopsis thaliana*. *Nucleic Acids Res* **28**: 4275-4282

Hartung, F, Suer, S, Bergmann, T, Puchta, H (2006) The role of *At*MUS81 in DNA repair and its genetic interaction with the helicase *At*RecQ4A. *Nucleic Acids Res* **34**: 4438-4448

Hehnle, S, Wege, C, Jeske, H (2004) Interaction of DNA with the movement proteins of geminiviruses revisited. *J Virol* **78**: 7698-7706

Heitzeberg, F, Chen, IP, Hartung, F, Orel, N, Angelis, KJ, Puchta, H (2004) The Rad17 homologue of *Arabidopsis* is involved in the regulation of DNA damage repair and homologous recombination. *Plant J* **38**: 954-968

Heyraud-Nitschke, F, Schumacher, S, Laufs, J, Schaefer, S, Schell, J, Gronenborn, B (1995) Determination of the origin cleavage and joining domain of geminivirus Rep proteins. *Nucleic Acids Res* **23**: 910-916

Hipp, K, Rau, P, Schafer, B, Gronenborn, B, Jeske, H (2014) The RXL motif of the African cassava mosaic virus Rep protein is necessary for rereplication of yeast DNA and viral infection in plants. *Virology* **462-463**: 189-198

Höhnle, M, Höfer, P, Bedford, ID, Briddon, RW, Markham, PG, Frischmuth, T (2001) Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible Abutilon mosaic virus isolate. *Virology* **290**: 164-171

Horn, J, Lauster, S, Krenz, B, Kraus, J, Frischmuth, T, Jeske, H (2011) Ambivalent effects of defective DNA in beet curly top virus-infected transgenic sugarbeet plants. *Virus Res* **158**: 169-178

Iyama, T, Wilson, DM, 3rd (2013) DNA repair mechanisms in dividing and non-dividing cells. *DNA Repair* **12**: 620-636

Jasin, M, Rothstein, R (2013) Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol* **5**: a012740

Jeske, H, **(2009)**. Geminiviruses, in: *Torque Teno Virus: The Still Elusive Human Pathogens*, pp. 185-226. Edited by zur Hausen, H., de Villiers, E.-M. Springer, Berlin.

Jeske, H, Lütgemeier, M, Preiss, W (2001) Distinct DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic geminivirus. *EMBO J* **20**: 6158-6167

Jovel, J, Preiss, W, Jeske, H (2007) Characterization of DNA intermediates of an arising geminivirus. *Virus Res* **130**: 63-70

Kagawa, W, Kurumizaka, H (2010) From meiosis to postmeiotic events: uncovering the molecular roles of the meiosis-specific recombinase Dmc1. *FEBS J* **277**: 590-598

Kaliappan, K, Choudhury, NR, Suyal, G, Mukherjee, SK (2012) A novel role for RAD54: this host protein modulates geminiviral DNA replication. *FASEB J* **26**: 1142-1160

Ketting, RF, Haverkamp, TH, van Luenen, HG, Plasterk, RH (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**: 133-141

Kittelmann, K, Rau, P, Gronenborn, B, Jeske, H (2009) Plant geminivirus rep protein induces rereplication in fission yeast. *J Virol* **83**: 6769-6778

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

Knoll, A, Fauser, F, Puchta, H (2014) DNA recombination in somatic plant cells: mechanisms and evolutionary consequences. *Chromosome Res* **22**: 191-201

Knoll, A, Puchta, H (2011) The role of DNA helicases and their interaction partners in genome stability and meiotic recombination in plants. *J Exp Bot* **62**: 1565-1579

Kobbe, D, Blanck, S, Demand, K, Focke, M, Puchta, H (2008) *At*RECQ2, a RecQ helicase homologue from *Arabidopsis thaliana*, is able to disrupt various recombinogenic DNA structures *in vitro*. *Plant J* **55**: 397-405

Kong, LJ, Orozco, BM, Roe, JL, Nagar, S, Ou, S, Feiler, HS, Durfee, T, Miller, AB, Gruissem, W, Robertson, D, Hanley-Bowdoin, L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J* **19**: 3485-3495

Kovalchuk, I, Kovalchuk, O, Kalck, V, Boyko, V, Filkowski, J, Heinlein, M, Hohn, B (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. *Nature* **423**: 760-762

Kozmin, SG, Pavlov, YI, Kunkel, TA, Sage, E (2003) Roles of *Saccharomyces cerevisiae* DNA polymerases Poleta and Polzeta in response to irradiation by simulated sunlight. *Nucleic Acids Res* **31**: 4541-4552

Kraberger, S, Harkins, GW, Kumari, SG, Thomas, JE, Schwinghamer, MW, Sharman, M, Collings, DA, Briddon, RW, Martin, DP, Varsani, A (2013) Evidence that dicot-infecting mastreviruses are particularly prone to inter-species recombination and have likely been circulating in Australia for longer than in Africa and the Middle East. *Virology* **444**: 282-291

Kraberger, S, Kumari, SG, Hamed, AA, Gronenborn, B, Thomas, JE, Sharman, M, Harkins, GW, Muhire, BM, Martin, DP, Varsani, A (2015) Molecular diversity of Chickpea chlorotic dwarf virus in Sudan: high rates of intra-species recombination - a driving force in the emergence of new strains. *Infect Genet Evol* **29**: 203-215

Krenz, B, Deuschle, K, Deigner, T, Unseld, S, Kepp, G, Wege, C, Kleinow, T, Jeske, H (2015) Early function of the Abutilon mosaic virus AC2 gene as a replication brake. *J Virol* **89**: 3683-3699

Krenz, B, Jeske, H, Kleinow, T (2012) The induction of stromule formation by a plant DNA-virus in epidermal leaf tissues suggests a novel intra- and intercellular macromolecular trafficking route. *Front Plant Sci* **3**: 291

Krenz, B, Windeisen, V, Wege, C, Jeske, H, Kleinow, T (2010) A plastid-targeted heat shock cognate 70kDa protein interacts with the Abutilon mosaic virus movement protein. *Virology* **401**: 6-17

Lamarche, BJ, Orazio, NI, Weitzman, MD (2010) The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett* **584**: 3682-3695

Laufs, J, Traut, W, Heyraud, F, Matzeit, V, Rogers, SG, Schell, J, Gronenborn, B (1995) *In vitro* cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proc Natl Acad Sci U S A* **92**: 3879-3883

Lefeuvre, P, Moriones, E (2015) Recombination as a motor of host switches and virus emergence: geminiviruses as case studies. *Curr Opin Virol* **10C**: 14-19

Li, B, Conway, N, Navarro, S, Comai, L (2005) A conserved and species-specific functional interaction between the Werner syndrome-like exonuclease atWEX and the Ku heterodimer in *Arabidopsis*. *Nucleic Acids Res* **33**: 6861-6867

Li, X, Heyer, WD (2008) Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* **18**: 99-113

Liu, L, Chung, HY, Lacatus, G, Baliji, S, Ruan, J, Sunter, G (2014) Altered expression of *Arabidopsis* genes in response to a multifunctional geminivirus pathogenicity protein. *BMC Plant Biol* **14**: 302

Lucht, JM, Mauch-Mani, B, Steiner, HY, Metraux, JP, Ryals, J, Hohn, B (2002) Pathogen stress increases somatic recombination frequency in *Arabidopsis*. *Nature Genet* **30**: 311-314

Luque, A, Sanz-Burgos, AP, Ramirez-Parra, E, Castellano, MM, Gutierrez, C (2002) Interaction of geminivirus Rep protein with replication factor C and its potential role during geminivirus DNA replication. *Virology* **302**: 83-94

Mannuss, A, Trapp, O, Puchta, H (2012) Gene regulation in response to DNA damage. *Biochim Biophys Acta* **1819**: 154-165

Maréchal, A, Zou, L (2013) DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol* **5**

Martin, DP, Lefeuvre, P, Varsani, A, Hoareau, M, Semegni, JY, Dijoux, B, Vincent, C, Reynaud, B, Lett, JM (2011) Complex recombination patterns arising during geminivirus coinfections preserve and demarcate biologically important intra-genome interaction networks. *PLoS Pathog* **7**: e1002203

Masson, JY, Stasiak, AZ, Stasiak, A, Benson, FE, West, SC (2001a) Complex formation by the human RAD51C and XRCC3 recombination repair proteins. *Proc Natl Acad Sci U S A* **98**: 8440-8446

Masson, JY, Tarsounas, MC, Stasiak, AZ, Stasiak, A, Shah, R, McIlwraith, MJ, Benson, FE, West, SC (2001b) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev* **15**: 3296-3307

Matsuda, T, Bebenek, K, Masutani, C, Hanaoka, F, Kunkel, TA (2000) Low fidelity DNA synthesis by human DNA polymerase-eta. *Nature* **404**: 1011-1013

Matsuda, T, Bebenek, K, Masutani, C, Rogozin, IB, Hanaoka, F, Kunkel, TA (2001) Error rate and specificity of human and murine DNA polymerase eta. *J Mol Biol* **312**: 335-346

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

McCulloch, SD, Kunkel, TA (2008) The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res* **18**: 148-161

Miller, KA, Sawicka, D, Barsky, D, Albala, JS (2004) Domain mapping of the Rad51 paralog protein complexes. *Nucleic Acids Res* **32**: 169-178

Moffat, AS (1999) Plant pathology - Geminiviruses emerge as serious crop threat. *Science* **286**: 1835-1835

Molinier, J, Oakeley, EJ, Niederhauser, O, Kovalchuk, I, Hohn, B (2005) Dynamic response of plant genome to ultraviolet radiation and other genotoxic stresses. *Mutat Res* **571**: 235-247

Molinier, J, Ries, G, Zipfel, C, Hohn, B (2006) Transgeneration memory of stress in plants. *Nature* **442**: 1046-1049

Monjane, AL, Pande, D, Lakay, F, Shepherd, DN, van der Walt, E, Lefeuvre, P, Lett, JM, Varsani, A, Rybicki, EP, Martin, DP (2012) Adaptive evolution by recombination is not associated with increased mutation rates in Maize streak virus. *BMC Evol Biol* **12**: 252

Morales, FJ (2007) Tropical Whitefly IPM Project. *Adv Virus Res* **69**: 249-311

Morra, MR, Petty, IT (2000) Tissue specificity of geminivirus infection is genetically determined. *Plant Cell* **12**: 2259-2270

Nagar, S, Hanley-Bowdoin, L, Robertson, D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**: 2995-3007

Nagar, S, Pedersen, TJ, Carrick, KM, Hanley-Bowdoin, L, Robertson, D (1995) A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. *Plant Cell* **7**: 705-719

Nakagawa, M, Takahashi, S, Tanaka, A, Narumi, I, Sakamoto, AN (2011) Role of *At*Polzeta, *At*Rev1, and *At*Poleta in UV light-induced mutagenesis in *Arabidopsis*. *Plant Physiol* **155**: 414-420

Navas-Castillo, J, Fiallo-Olive, E, Sanchez-Campos, S (2011) Emerging virus diseases transmitted by whiteflies. *Annu Rev Phytopathol* **49**: 219-248

Ohashi, E, Bebenek, K, Matsuda, T, Feaver, WJ, Gerlach, VL, Friedberg, EC, Ohmori, H, Kunkel, TA (2000) Fidelity and processivity of DNA synthesis by DNA polymerase kappa, the product of the human DINB1 gene. *J Biol Chem* **275**: 39678-39684

Osakabe, K, Abe, K, Yamanouchi, H, Takyuu, T, Yoshioka, T, Ito, Y, Kato, T, Tabata, S, Kurei, S, Yoshioka, Y, Machida, Y, Seki, M, Kobayashi, M, Shinozaki, K, Ichikawa, H, Toki, S (2005) *Arabidopsis* Rad51B is important for double-strand DNA breaks repair in somatic cells. *Plant Mol Biol* **57**: 819-833

Osakabe, K, Abe, K, Yoshioka, T, Osakabe, Y, Todoriki, S, Ichikawa, H, Hohn, B, Toki, S (2006) Isolation and characterization of the RAD54 gene from *Arabidopsis thaliana*. *Plant J* **48**: 827-842

Osakabe, K, Yoshioka, T, Ichikawa, H, Toki, S (2002) Molecular cloning and characterization of RAD51-like genes from *Arabidopsis thaliana*. *Plant Mol Biol* **50**: 71-81

Osman, K, Higgins, JD, Sanchez-Moran, E, Armstrong, SJ, Franklin, FC (2011) Pathways to meiotic recombination in *Arabidopsis thaliana*. *New Phytol* **190**: 523-544

Padidam, M, Sawyer, S, Fauquet, CM (1999) Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**: 218-225

Paprotka, T, Deuschle, K, Pilartz, M, Jeske, H (2015) Form follows function in geminiviral minichromosome architecture. *Virus Res* **196**: 44-55

Paprotka, T, Metzler, V, Jeske, H (2010) The first DNA 1-like alpha-satellites in association with New World begomoviruses in natural infections. *Virology* **404**: 148-157

Pascal, E, Sanderfoot, AA, Ward, BM, Medville, R, Turgeon, R, Lazarowitz, SG (1994) The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. *Plant Cell* **6**: 995-1006

Patil, BL, Dasgupta, I (2006) Defective interfering DNAs of plant viruses. *Crit Rev Plant Sci* **24**: 1–18

Patil, BL, Dutt, N, Briddon, RW, Bull, SE, Rothenstein, D, Borah, BK, Dasgupta, I, Stanley, J, Jeske, H (2007) Deletion and recombination events between the DNA-A and DNA-B components of Indian cassava-infecting geminiviruses generate defective molecules in *Nicotiana benthamiana*. *Virus Res* **124**: 59-67

Pecinka, A, Rosa, M, Schikora, A, Berlinger, M, Hirt, H, Luschnig, C, Scheid, OM (2009) Transgenerational stress memory is not a general response in *Arabidopsis*. *Plos One* **4**

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

Pilartz, M, Jeske, H (2003) Mapping of abutilon mosaic geminivirus minichromosomes. *J Virol* **77**: 10808-10818

Plchova, H, Hartung, F, Puchta, H (2003) Biochemical characterization of an exonuclease from *Arabidopsis thaliana* reveals similarities to the DNA exonuclease of the human Werner syndrome protein. *J Biol Chem* **278**: 44128-44138

Pradillo, M, Varas, J, Oliver, C, Santos, JL (2014) On the role of *At*DMC1, *At*RAD51 and its paralogs during *Arabidopsis* meiosis. *Front Plant Sci* **5**: 23

Preiss, W, Jeske, H (2003) Multitasking in replication is common among geminiviruses. *J Virol* **77**: 2972-2980

Puchta, H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J Exp Bot* **56**: 1-14

Rahavi, SM, Kovalchuk, I (2013) Changes in homologous recombination frequency in *Arabidopsis thaliana* plants exposed to stress depend on time of exposure during development and on duration of stress exposure. *Physiol Mol Biol Plants* **19**: 479-488

Rigden, JE, Dry, IB, Krake, LR, Rezaian, MA (1996) Plant virus DNA replication processes in *Agrobacterium*: insight into the origins of geminiviruses? *Proc Natl Acad Sci U S A* **93**: 10280-10284

Rocha, CS, Castillo-Urquiza, GP, Lima, AT, Silva, FN, Xavier, CA, Hora-Junior, BT, Beserra-Junior, JE, Malta, AW, Martin, DP, Varsani, A, Alfenas-Zerbini, P, Mizubuti, ES, Zerbini, FM (2013) Brazilian begomovirus populations are highly recombinant, rapidly evolving, and segregated based on geographical location. *J Virol* **87**: 5784-5799

Rojas, MR, Hagen, C, Lucas, WJ, Gilbertson, RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annu Rev Phytopathol* **43**: 361-394

Rojas, MR, Noueiry, AO, Lucas, WJ, Gilbertson, RL (1998) Bean Dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell* **95**: 105-113

Ruschhaupt, M, Martin, DP, Lakay, F, Bezuidenhout, M, Rybicki, EP, Jeske, H, Shepherd, DN (2013) Replication modes of Maize streak virus mutants lacking RepA or the RepA-pRBR interaction motif. *Virology* **442**: 173-179

Sabbioneda, S, Minesinger, BK, Giannattasio, M, Plevani, P, Muzi-Falconi, M, Jinks-Robertson, S (2005) The 9-1-1 checkpoint clamp physically interacts with Pol zeta and is partially required for spontaneous Pol zeta-dependent mutagenesis in *Saccharomyces cerevisiae*. *J Biol Chem* **280**: 38657-38665

Sakamoto, A, Lan, VT, Hase, Y, Shikazono, N, Matsunaga, T, Tanaka, A (2003) Disruption of the *At*REV3 gene causes hypersensitivity to ultraviolet B light and gamma-rays in *Arabidopsis*: implication of the presence of a translesion synthesis mechanism in plants. *Plant Cell* **15**: 2042-2057

Sale, JE (2013) Translesion DNA synthesis and mutagenesis in eukaryotes. *Cold Spring Harb Perspect Biol* **5**: a012708

Samach, A, Melamed-Bessudo, C, Avivi-Ragolski, N, Pietrokovski, S, Levy, AA (2011) Identification of plant RAD52 homologs and characterization of the *Arabidopsis thaliana* RAD52-like genes. *Plant Cell* **23**: 4266-4279

San Filippo, J, Sung, P, Klein, H (2008) Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem* **77**: 229-257

Sanderfoot, AA, Ingham, DJ, Lazarowitz, SG (1996) A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. *Plant Physiol* **110**: 23-33

Sanderfoot, AA, Lazarowitz, SG (1995) Cooperation in viral movement: The geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. *Plant Cell* **7**: 1185-1194

Santiago, MJ, Alejandre-Duran, E, Munoz-Serrano, A, Ruiz-Rubio, M (2008) Two translesion synthesis DNA polymerase genes, *At*POLH and *At*REV1, are involved in development and UV light resistance in *Arabidopsis*. *J Plant Physiol* **165**: 1582-1591

Saunders, K, Lucy, A, Stanley, J (1991) DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res* **19**: 2325-2330

Saunders, K, Lucy, A, Stanley, J (1992) RNA-primed complementary-sense DNA synthesis of the geminivirus African cassava mosaic virus. *Nucleic Acids Res* **20**: 6311-6315

Schuermann, D, Molinier, J, Fritsch, O, Hohn, B (2005) The dual nature of homologous recombination in plants. *Trends Genet* **21**: 172-181

Sekimoto, T, Oda, T, Kurashima, K, Hanaoka, F, Yamashita, T (2015) Both high-fidelity replicative and low-fidelity Y-family polymerases are involved in DNA rereplication. *Mol Cell Biol* **35**: 699-715

Selth, LA, Dogra, SC, Rasheed, MS, Healy, H, Randles, JW, Rezaian, MA (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. *Plant Cell* **17**: 311-325

Selth, LA, Randles, JW, Rezaian, MA (2002) *Agrobacterium tumefaciens* supports DNA replication of diverse geminivirus types. *FEBS Lett* **516**: 179-182

Serra, H, Da Ines, O, Degroote, F, Gallego, ME, White, CI (2013) Roles of XRCC2, RAD51B and RAD51D in RAD51-independent SSA recombination. *PLoS Genet* **9**: e1003971

Settlage, SB, See, RG, Hanley-Bowdoin, L (2005) Geminivirus C3 protein: replication enhancement and protein interactions. *J Virol* **79**: 9885-9895

Shepherd, DN, Martin, DP, Van Der Walt, E, Dent, K, Varsani, A, Rybicki, EP (2010) Maize streak virus: an old and complex 'emerging' pathogen. *Mol Plant Pathol* **11**: 1-12

Shinohara, A, Ogawa, H, Ogawa, T (1992) Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell* **69**: 457-470

Shrivastav, M, De Haro, LP, Nickoloff, JA (2008) Regulation of DNA double-strand break repair pathway choice. *Cell Res* **18**: 134-147

Snapka, RM, Permana, PA, Marquit, G, Shin, CG (1991) Two-dimensional agarose gel electrophoresis of simian virus 40 DNA replication intermediates. *Methods* **3**: 73-82

Soitamo, AJ, Jada, B, Lehto, K (2012) Expression of geminiviral AC2 RNA silencing suppressor changes sugar and jasmonate responsive gene expression in transgenic tobacco plants. *BMC Plant Biol* **12**: 204

Stainton, D, Kraberger, S, Walters, M, Wiltshire, EJ, Rosario, K, Halafihi, M, Lolohea, S, Katoa, I, Faitua, TH, Aholelei, W, Taufa, L, Thomas, JE, Collings, DA, Martin, DP, Varsani, A (2012) Evidence of inter-component recombination, intra-component recombination and reassortment in banana bunchy top virus. *J Gen Virol* **93**: 1103-1119

Stanley, J (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology* **206**: 707-712

Stenger, DC, Revington, GN, Stevenson, MC, Bisaro, DM (1991) Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proc Natl Acad Sci U S A* **88**: 8029-8033

Strzalka, W, Bartnicki, F, Pels, K, Jakubowska, A, Tsurimoto, T, Tanaka, K (2013) RAD5a ubiquitin ligase is involved in ubiquitination of *Arabidopsis thaliana* proliferating cell nuclear antigen. *J Exp Bot* **64**: 859-869

Sunitha, S, Shanmugapriya, G, Balamani, V, Veluthambi, K (2013) Mungbean yellow mosaic virus (MYMV) AC4 suppresses post-transcriptional gene silencing and an AC4 hairpin RNA gene reduces MYMV DNA accumulation in transgenic tobacco. *Virus Genes* **46**: 496-504

Sunter, G, Bisaro, DM (1991) Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. *Virology* **180**: 416-419

Sunter, G, Bisaro, DM (1992) Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. *Plant Cell* **4**: 1321-1331

Sunter, G, Bisaro, DM (1997) Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms. *Virology* **232**: 269-280

Suwaki, N, Klare, K, Tarsounas, M (2011) RAD51 paralogs: roles in DNA damage signalling, recombinational repair and tumorigenesis. *Semin Cell Dev Biol* **22**: 898-905

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

Takahashi, S, Sakamoto, A, Sato, S, Kato, T, Tabata, S, Tanaka, A (2005) Roles of *Arabidopsis At*REV1 and *At*REV7 in translesion synthesis. *Plant Physiol* **138**: 870-881

Tamura, K, Adachi, Y, Chiba, K, Oguchi, K, Takahashi, H (2002) Identification of Ku70 and Ku80 homologues in *Arabidopsis thaliana*: evidence for a role in the repair of DNA double-strand breaks. *Plant J* **29**: 771-781

Tiendrébéogo, F, Lefeuvre, P, Hoareau, M, Harimalala, MA, De Bruyn, A, Villemot, J, Traoré, VS, Konaté, G, Traoré, AS, Barro, N, Reynaud, B, Traoré, O, Lett, JM (2012) Evolution of

African cassava mosaic virus by recombination between bipartite and monopartite begomoviruses. *Virol J* **9**: 67

Tops, BB, Tabara, H, Sijen, T, Simmer, F, Mello, CC, Plasterk, RH, Ketting, RF (2005) RDE-2 interacts with MUT-7 to mediate RNA interference in *Caenorhabditis elegans*. *Nucleic Acids Res* **33**: 347-355

Trinks, D, Rajeswaran, R, Shivaprasad, PV, Akbergenov, R, Oakeley, EJ, Veluthambi, K, Hohn, T, Pooggin, MM (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J Virol* **79**: 2517-2527

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

Unk, I, Hajdu, I, Blastyak, A, Haracska, L (2010) Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance. *DNA Repair* **9**: 257-267

van der Walt, E, Martin, DP, Varsani, A, Polston, JE, Rybicki, EP (2008) Experimental observations of rapid Maize streak virus evolution reveal a strand-specific nucleotide substitution bias. *Virol J* **5**

van der Walt, E, Rybicki, EP, Varsani, A, Polston, JE, Billharz, R, Donaldson, L, Monjane, AL, Martin, DP (2009) Rapid host adaptation by extensive recombination. *J Gen Virol* **90**: 734-746

van Wezel, R, Dong, X, Blake, P, Stanley, J, Hong, Y (2002) Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in *Nicotiana benthamiana*. *Mol Plant Pathol* **3**: 461-471

Vanitharani, R, Chellappan, P, Pita, JS, Fauquet, CM (2004) Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J Virol* **78**: 9487-9498

Varma, A, Malathi, VG (2003) Emerging geminivirus problems: A serious threat to crop production. *Ann Appl Biol* **142**: 145-164

Varsani, A, Navas-Castillo, J, Moriones, E, Hernandez-Zepeda, C, Idris, A, Brown, JK, Murilo Zerbini, F, Martin, DP (2014) Establishment of three new genera in the family *Geminiviridae*: *Becurtovirus*, *Eragrovirus* and *Turncurtovirus*. *Arch Virol* **159**: 2193-2203

Varsani, A, Shepherd, DN, Monjane, AL, Owor, BE, Erdmann, JB, Rybicki, EP, Peterschmitt, M, Briddon, RW, Markham, PG, Oluwafemi, S, Windram, OP, Lefeuvre, P, Lett, JM, Martin, 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

Wang, H, Buckley, KJ, Yang, X, Buchmann, RC, Bisaro, DM (2005) Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. *J Virol* **79**: 7410-7418

Wang, H, Hao, L, Shung, CY, Sunter, G, Bisaro, DM (2003) Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* **15**: 3020-3032

Wang, Q, Goldstein, M, Alexander, P, Wakeman, TP, Sun, T, Feng, J, Lou, Z, Kastan, MB, Wang, XF (2014) Rad17 recruits the MRE11-RAD50-NBS1 complex to regulate the cellular response to DNA double-strand breaks. *EMBO J* **33**: 862-877

Wang, S, Wen, R, Shi, X, Lambrecht, A, Wang, H, Xiao, W (2011) RAD5a and REV3 function in two alternative pathways of DNA-damage tolerance in *Arabidopsis*. *DNA Repair* **10**: 620-628

Watanabe, K, Pacher, M, Dukowic, S, Schubert, V, Puchta, H, Schubert, I (2009) The STRUCTURAL MAINTENANCE OF CHROMOSOMES 5/6 complex promotes sister chromatid alignment and homologous recombination after DNA damage in *Arabidopsis thaliana*. *Plant Cell* **21**: 2688-2699

Waters, LS, Minesinger, BK, Wiltrout, ME, D'Souza, S, Woodruff, RV, Walker, GC (2009) Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. *Microbiol Mol Biol Rev* **73**: 134-154

Waterworth, WM, Altun, C, Armstrong, SJ, Roberts, N, Dean, PJ, Young, K, Weil, CF, Bray, CM, West, CE (2007) NBS1 is involved in DNA repair and plays a synergistic role with ATM in mediating meiotic homologous recombination in plants. *Plant J* **52**: 41-52

Waterworth, WM, Drury, GE, Bray, CM, West, CE (2011) Repairing breaks in the plant genome: the importance of keeping it together. *New Phytol* **192**: 805-822

Wege, C, **(2007)**. Movement and localization of Tomato yellow leaf curl viruses in the infected plant, in: *Tomato yellow leaf curl virus disease*, pp. 185–206. Edited by Czosnek, H. Springer, Dordrecht.

Wege, C, Saunders, K, Stanley, J, Jeske, H (2001) Comparative analysis of tissue tropism of bipartite geminiviruses. *J Phytopathol* **149**: 359-368

West, CE, Waterworth, WM, Story, GW, Sunderland, PA, Jiang, Q, Bray, CM (2002) Disruption of the *Arabidopsis At*Ku80 gene demonstrates an essential role for *At*Ku80 protein in efficient repair of DNA double-strand breaks *in vivo*. *Plant J* **31**: 517-528

Whitfield, AE, Falk, BW, Rotenberg, D (2015) Insect vector-mediated transmission of plant viruses. *Virology* **479-480C**: 278-289

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

Xie, Y, Zhao, L, Jiao, X, Jiang, T, Gong, H, Wang, B, Briddon, RW, Zhou, X (2013) A recombinant begomovirus resulting from exchange of the C4 gene. *J Gen Virol* **94**: 1896-1907

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

Yao, YL, Bilichak, A, Golubov, A, Kovalchuk, I (2011) Local infection with oilseed rape mosaic virus promotes genetic rearrangements in systemic *Arabidopsis* tissue. *Mutat Res* **709-10**: 7-14

Yoshiyama, KO, Kimura, S, Maki, H, Britt, AB, Umeda, M (2014) The role of SOG1, a plant-specific transcriptional regulator, in the DNA damage response. *Plant Signal Behav* **9**: e28889

Yoshiyama, KO, Kobayashi, J, Ogita, N, Ueda, M, Kimura, S, Maki, H, Umeda, M (2013) ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in *Arabidopsis*. *EMBO Rep* **14**: 817-822

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

Zhang, W, Olson, NH, Baker, TS, Faulkner, L, Agbandje-McKenna, M, Boulton, MI, Davies, JW, McKenna, R (2001b) Structure of the Maize streak virus geminate particle. *Virology* **279**: 471-477

Zhong, X, Garg, P, Stith, CM, Nick McElhinny, SA, Kissling, GE, Burgers, PM, Kunkel, TA (2006) The fidelity of DNA synthesis by yeast DNA polymerase zeta alone and with accessory proteins. *Nucleic Acids Res* **34**: 4731-4742

Zhou, Y, Rojas, MR, Park, MR, Seo, YS, Lucas, WJ, Gilbertson, RL (2011) Histone H3 interacts and colocalizes with the nuclear shuttle protein and the movement protein of a geminivirus. *J Virol* **85**: 11821-11832

Zwirn, P, Stary, S, Luschnig, C, Bachmair, A (1997) *Arabidopsis thaliana* RAD6 homolog *At*UBC2 complements UV sensitivity, but not N-end rule degradation deficiency, of *Saccharomyces cerevisiae* rad6 mutants. *Curr Genet* **32**: 309-314

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

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