Gene Silencing und das Abutilon Mosaik Virus

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Zusammenfassung

Geminiviren richten weltweit einen großen ökologischen und ökonomischen Schaden an. Es gibt ein gesteigertes Interesse, Pflanzenpathogenen entweder durch konservative Mittel oder durch biotechnologische Nutzung von neuentdeckten Phänomenen, wie dem *Post-transcriptional Gene Silencing* (PTGS) Einhalt zu gebieten. Dies ist vielversprechend, weil PTGS ein pflanzeneigenes Abwehrsystem gegen Pathogene darstellt, das ubiquitär im Pflanzenreich verbreitet ist.

Verschiedene PTGS-Auslöser zur Unterdrückung von Geminivirusinfektionen sollten hergestellt und charakterisiert werden. Dazu sollten Silencing-Konstrukte gegen eine *Abutilon Mosaic Virus* (AbMV)-Infektion entwickelt und eingesetzt werden, um letztendlich Wirtsfaktoren zu charakterisieren, die für eine geminivirale Infektion essentiell sind, zugleich aber die Wirtspflanze nicht in ihrer Entwicklung hemmen. Die DNA des AbMV wurde dazu als Gene Silencing- und Proteinexpressionsvektor umkonstruiert und mit Hilfe des Markergens Phytoendesature (PDS) und des Reportergens *Green Fluorescence Protein* GFP funktionell überprüft. In die infektiöse Hüllprotein-Deletionsmutante von AbMV wurde ein cDNA-Fragment von Phytoendesaturase (PDS) inseriert. Inokulierte *Nicotiana benthamiana*-Pflanzen zeigten im Verlauf der Infektion den erwarteten Albino-Phänotyp, der durch PDS-Silencing entsteht. Der offene Leserahmen (ORF) von GFP wurde in die gleiche AbMV-Mutante inseriert und führte zur Expression von GFP unter der Kontrolle des Hüllprotein-Promotors sowohl in Agrobakterium-infiltriertem Gewebe, als auch in systemisch infizierten Geweben, so dass AbMV als Gene Silencing- und Proteinexpressionsvektor genutzt werden kann.

Im Hefe-2-Hybrid-System konnte Dr. T. Kleinow nachweisen, dass die N-terminale Domäne des *movement protein* (MP) von AbMV mit dem C-terminalen Bereich des Chloroplasten-lokalisierten Hitzeschock-Proteins von 70 kDa (cpHsc70) aus *Arabidopsis thaliana* interagiert. Um zu überprüfen, wie sich ein cpHsc70-*knock down* Phänotyp auf eine AbMV-Infektion auswirkt, wurde das etablierte AbMV-basierte Silencing-Vektorsystem genutzt. Als Silencing-Phänotyp konnten entlang der Leitgewebe systemisch infizierter *N. benthamiana*-Blätter kleine weiße Chlorosen beobachtet werden, die auf die Degradation der Chloroplasten zurückgeführt werden konnten. In cpHsc70-gesilencten Pflanzen akkumulierte zudem signifikant weniger *single-stranded* DNA (ssDNA), als in den Kontroll-Proben, so dass über einen Effekt von cpHsc70-silencing auf virale ssDNA spekuliert werden kann. Eine Interaktion von cpHsc70 mit dem AbMV-Transportkomplex als Grundlage für den Transport von geminiviraler DNA über Membranen wird diskutiert.

Über die Eigenschaften des AbMV Transkriptions-Transaktivator Proteins (TrAP) als Silencing-Suppressor ist noch nichts bekannt. Die Funktion als Silencing-Suppressor oder dessen Interferenz mit der Silencing-assoziierten Maschinerie sollte charakterisiert werden, um gezielt Resistenz oder Toleranz zu etablieren. Eine GFP-Expressionskassette wurde zwischen zwei virale Replikationsursprünge inseriert und stabil in eine *N. benthamiana*-Pflanze als Transgen integriert, so dass durch Infektion mit AbMV ein Transreplikon aus dem Transgen mobilisiert werden konnte, das GFP exprimiert. In AbMV-infizierten Pflanzenzellen konnten GFP-Signale im Leitgewebe beobachtet werden und bestätigten damit die Mobilisierung und Replikation des Transreplikons. In Infiltrationsexperimenten mit Agrobakterien, die die Expression des AbMV Replikation-assoziiertem Proteins (Rep) und TrAP vermitteln, konnte gezeigt werden, dass die Mobilisierung des Transreplikons ausschließlich Rep-induziert ist. Während die Co-Expression von Rep und TrAP, genauso wie

Agrobakterium-vermittelte Transfektion mit dem infektiösen AbMV DNA A Plasmid, die Mobilisierung des Transreplikons unterdrückt. Das gut charakterisierte Silencing-Suppressor Protein p19 des *Cymbidium ringspot virus*, eines nicht verwandten RNA Virus, neutralisierte diesen Effekt, so dass von einer siRNA-vermittelten Unterdrückung der Transreplikation ausgegangen werden konnte.

Durch Darstellung der Topoisomere der viralen DNA konnte auf verschiedene Kondensierungszustände geminiviraler Minichromosomen geschlossen werden. Für AbMV konnte festgestellt werden, dass das Chromatin mit fortschreitender Infektionsdauer kondensierter und damit möglicherweise Transkriptions-inaktiver wird. Eine Überexpression von TrAP inhibierte diesen Effekt, was auf eine chromatinmodulierende Eigenschaft hinweist, während eine Funktion als Silencing-Suppressor eher unwahrscheinlich ist.

Summary

In recent years, geminiviruses have emerged as leading plant pathogens that cause severe crop losses worldwide. They infect a broad range of plant species, including tomatoes, cucurbits, cassava, maize, beans and cotton. Many attempts to generate geminivirus resistance have met with limited success. As a consequence, it is essential to develop new resistance strategies and to understand viral counterdefense mechanism.

RNA interference (RNAi), also called post-transcriptional gene silencing (PTGS), is a sequence-specific RNA degradation mechanism that silences a targeted gene. It is part of the natural virus defence system, whereas transcriptional gene silencing (TGS) is thought to be a defence against transposons or other invasive DNA elements. In addition, RNAi is a powerful tool for elucidating gene functions. We have developed a system based on the bipartite geminivirus *Abutilon mosaic virus* (AbMV) to identify host factors essential for viral infectivity. As a proof of concept, we replaced the AbMV coat protein gene by a fragment of the *Nicotiana benthamiana* phytoene desaturase (PDS) gene. Extensive PDS silencing was produced during further growth of inoculated plants. To use AbMV as a versatile tool to study gene function *in vivo*, it is desirable to use it simultaneously as a virus-based plant expression vector for foreign proteins. Plant virus-based vectors as transient gene expression systems are an attractive alternative to conventional breeding and transformation technology. Therefore, the open reading frame (ORF) of *mGFP4* was inserted in place of the coat protein, leading to GFP signals in infected cells, giving the opportunity for online monitoring of virus movement through distal parts of a

plant, and studying tissue specificity. The analysis confirmed that AbMV existed in tissues no other than the internal and external phloem of vascular bundle.

The C-terminal portion of *Arabidopisis thaliana* cpHsc70-1 was found to interact with the N-terminal region of *Abutilon mosaic virus* (AbMV) movement protein in a yeast two hybrid assay (Dr. T. Kleinow, pers. communication). Therefore a geminivirus-based gene silencing vector harboring the cpHsc70-fragment was constructed to silence the nuclear encoded and chloroplast-localized heat shock protein of 70 kDa (cpHSC70) in *N. benthamiana* plants. Systemically infected leaves showed punctated photo-bleached areas similar to those induced by phytoene desaturase (PDS) silencing indicating an interference with chloroplast stability. CpHsc70-silenced plants accumulated less viral DNA, in particular single-stranded DNA (ssDNA), than PDS-silenced or AbMV-infected plants. An involvement of cpHSC70 in geminiviral movement is discussed.

An AbMV-based transreplicon was constructed to monitor infection and to identify plant tissues specificity. A green fluorescent protein (GFP) expression cassette driven by the constitutive 35S promoter of *Cauliflower mosaic virus* (CaMV) was embedded in a truncated AbMV DNA A partial dimer and transferred to *N. benthamiana* plants as a transgene. Upon AbMV infection, the transreplicon was released and resulted in GFP overexpression. Agroinfiltration of these transgenic plants with a construct that expressed the AbMV replication-associated protein Rep (or AC1) alone showed, that Rep is necessary and sufficient to induce bright GFP fluorescence in the infiltrated area. Co-expression of AbMV Rep and TrAP (or AC2), a protein that has been identified as transcriptional transactivator as well as silencing suppressor protein for other begomoviruses, surprisingly suppressed transgene transreplication. This effect however, was neutralized by the strong p19 silencing suppressor of the unrelated, RNA-containing *Cymbidium ringspot virus* (CymRSV)

suggesting an involvement of small interfering RNA (siRNA). In order to investigate whether TrAP has an influence on the viral chromatin condensation as a response to silencing, the distribution of topoisomers of monomeric viral circular double-stranded DNA at different stages of infection was visualized. Transreplicon and viral minichromosomes were found to exist in structures dependent on Rep and/or TrAP expression. The topoisomers distribution pattern indicated the influence of AbMV TrAP in altering viral chromatin conformation, whereas suppression of transcriptional gene silencing (TGS) can presumably be excluded.

Einleitung

RNA-Interferenz

RNA-Interferenz (RNAi) ist ein evolutionär konservierter, ubiquitär verbreiteter eukaryotischer Mechanismus, der sequenz-spezifisch Transkription und Translation inhibiert und die Genexpression auf Chromatinebene reguliert. In seiner Funktion dient RNAi sowohl als zelluläres „Immunsystem“ gegen invasive genetische Elemente, wie Viren und Transposons, als auch als zelleigenes, regulatorisches Element der Genaktivität. Als Schlüsselmoleküle gelten kleine *double-stranded* RNAs (dsRNA), die als *micro*RNA (miRNA) oder *small interfering* RNA (siRNA) klassifiziert werden. Sie entstehen durch RNaseIII-Aktivität des Dicer (Dicer-like)-Komplexes, der dsRNA im Cytoplasma erkennt, bindet und schneidet. Die entstandenen mi- oder siRNAs werden in den *RNA Induced Silencing Complex* (RISC) oder *RNA Induced Transcriptional Silencing* (RITS) -Komplex aufgenommen und führen zur Degradation, zur Translationsblockade von komplementärer mRNA oder zur Methylierung und Abschaltung des entsprechenden Gens (Baulcombe, 2004; Carrington und Ambros, 2003; Meister und Tuschel, 2004; Voinnet, 2005). Somit wird die Proteinbiosynthese gestoppt; doch im Gegensatz zum „*Knock-Out*“ durch Genunterbrechung wird die nicht immer vollständige Repression durch RNAi als „*Knock-Down*“ bezeichnet. Die Aufklärung der RNAi-Maschinerie und möglicher natürlicher Funktionen standen in den letzten Jahren im Mittelpunkt. Für die Entdeckung des Mechanismus der RNA-Interferenz erhielten die beiden US-Wissenschaftler Andrew Z. Fire und Craig C. Mello den Nobelpreis für Physiologie oder Medizin 2006, doch hatte die Forschergruppe um Mol und Jörgensen das Phänomen zum ersten Mal beschrieben.

In den frühen 90ern versuchten sie durch Überexpression des Gens Dihydroflavonol-Reduktase die Blütenfärbung von Petunien zu verstärken (v.d. Krol *et al*., 1990; Napoli *et al*., 1990). Überraschenderweise zeigten die meisten transgenen Petunien-Pflanzen gar keine oder weniger starke Blütenfärbung. Zunächst Co-Suppression benannt, wird dieses Phänomen heute bei Pflanzen auch als *Post-transcriptional Gene Silencing* (PTGS) bezeichnet. Als biologische Funktion erkannte man zuerst das Potential als Abwehrmechanismus gegen Viren. So resultierte zum Beispiel eine Infektion von *Nicotiana clevelandii* mit *Tomato blackring nepovirus* (TBRV) anfänglich in einer symptomatischen Phase, in der sich das Virus systemisch ausbreitete. Neues Gewebe regenerierte sich, war symptomlos und virusfrei. Regeneriertes Gewebe war auch resistent gegen eine erneute Infektion mit TBRV oder eng verwandten Stämmen (Ratcliff *et al*., 1997). Ebenso verursachte eine Infektion von CaMV in *Brassica napus* PTGS unabhängig von einem homologen Transgen (Al-Kaff *et al.,* 1998). Diese zwei Arbeiten zeigten, dass höchst unterschiedliche Viren PTGS auslösen können und dass die Pflanze durch diesen antiviralen Abwehrmechanismus die Ausbreitung der Infektion eindämmen kann.

Schnell wurde klar, dass das Phänomen PTGS, unter verschiedenen Fachbegriffen beschrieben, ubiquitär im Pflanzen- und Tierreich zu finden ist (Cogoni und Macino, 2000). So wurden PTGS-ähnliche Phänomene in Pilzen, speziell in *Neurospora crassa*, entdeckt und *Quelling* genannt (Cogoni und Macino, 1997). Auch in *Drosophila*, *Trypanosoma*, Zebrafisch, Planarien und in Säugetierzellen konnte das Phänomen RNAi demonstriert werden. Eine interessante Ausnahme stellt hier *Saccharomyces cerevisiae* dar (Aravind *et al.*, 2000).

Als Auslöser von RNAi wurde dsRNA charakterisiert, die auf unterschiedliche Art und Weise ins Cytoplasma der Zelle gelangen oder dort erst entstehen kann. Fire und Mitarbeiter postulierten, dass nicht das *Antisense*-Transkript, sondern

kontaminierende dsRNA das inaktivierende Agenz darstellt (Fire *et al., 1998*). Sie konnten zeigen, dass die Injektion von substöchiometrischen Mengen an dsRNA in *C. elegans* einen besseren Silencing-Effekt hatte als die Injektion großer Mengen von *Antisense*-Transkripten. Auch in *Drosophila* (Pal-Bhadra *et al*. ,1997) und in Pflanzen (Chuang und Meyerowitz, 2000) konnte dsRNA als Auslöser von RNAi bzw. PTGS charakterisiert werden und man erkannte durch die zentrale Rolle von dsRNA die Verbindung und Redundanz zwischen PTGS, *Quelling* und RNAi.

Durch den Replikationszyklus von RNA-Viren entstehen dsRNAs als Intermediate, sind damit eine weitere natürliche Ursache zur Auslösung von RNAi und verdeutlicht die Funktion in der Pathogenabwehr. Als weiterer Auslöser gilt abberante RNA, ein Nebenprodukt, das bei der Überexpression von Transgenen oder viralen Transkripten entsteht.

Zum Auszulösen von RNAi, hat man verschiedene Methoden entwickelt und angewandt, um dsRNA-Konstrukte in die Zelle oder in den ganzen Organismus einzubringen. *C. elegans* kann man zum Beispiel mit dsRNA füttern, dsRNA injizieren oder den Wurm kurz in eine dsRNA-Lösung einlegen. Kultivierte *Drosophila*-Zellen kann man in dsRNA-Medium halten, dsRNA direkt in *Drosophila*-Embryos spritzen oder transgene Fruchtfliegen herstellen, die dsRNA-Konstrukte transkribieren. Ähnliche Methoden funktionieren auch bei Säugetierzellen und bei Säugetieren (Zamore, 2002). Bei Pflanzen haben sich der Goldpartikelbeschuss (Klahre *et al.,* 2002), die Transfektion von Protoplasten (Kanno *et al.*, 2000) und das Einschleusen von Plasmiden, die dsRNA-Konstrukte kodieren, mittels *Agrobacterium tumefaciens* (Wesley *et al*., 2001) bewährt.

Im Cytoplasma einer Zelle wird dsRNA durch das Enzym Dicer erkannt und in viele kleinere Fragmente von 21-28 Nukleotiden Länge zerschnitten. Menschen und *C. elegans* kodieren für nur einen Dicer-Komplex, *Drosophila* für zwei (Dcr1 und 2) und

in *Arabidopsis thaliana* wurden vier Dicer annotiert und als Dicer-like 1 – 4 (Dcl1 – 4) benannt. Alle Dicer-Proteine sind Ribonukleasen der RNaseIII-Superfamilie mit mehreren Domänen (RnaseIII-, PAZ-, dsRBD- und Helikase/ATPase-Domäne). Sie schneiden dsRNA über intramolekulare Dimerisierung der beiden RNaseIII-Domänen in kleine dsRNA-Moleküle mit 2 Nukleotiden-Überhang am 3'-Ende (Hammond, 2005). Es wurde gezeigt, dass speziell miRNAs weiter modifiziert werden, indem die Methyltransferase HEN1 das letzte Nukleotid an der 2'-OH-Gruppe methyliert, um vor Uridylierung zu schützen (Yu *et al*., 2005). Es wurde zudem postuliert, dass alle kleinen RNAs zur Stabilisierung und zum Degradationsschutz modifiziert werden. So konnten Abergenov und Mitarbeiter (2006) zeigen, dass siRNAs in Pflanzen am 5'-Ende phosphoryliert und auch am 3'-Ende modifiziert werden.

Lee und Mitarbeiter (2004) konnten den Dicer-Enzymen aus *Drosophila* Dcr-1 und Dcr-2 unterschiedliche Funktionen zuordnen und zeigten, dass Dcr-1 dsRNA in miRNAs prozessiert, während Dcr-2 siRNAs produziert. Die Biogenese der miRNAs besteht aus zwei Prozessierungsschritten: Nach der Transkription des miRNA-Gens bildet sich ein Transkript mit partieller dsRNA-Sekundärstruktur und Haarnadelschleife, die pri-miRNA. Diese wird im Zellkern durch den Enzymkomplex Drosha/Pasha erkannt, zur prä-miRNA prozessiert, über einen Exportin 5/RanGTP-vermittelten Transport in das Cytoplasma exportiert und zur reifen miRNA gespalten.

Ähnlich verhält es sich bei Pflanzen. Pflanzen-miRNAs entstehen ebenfalls aus primären miRNA-Transkripten mit partieller dsRNA-Sekundärstruktur mit Haarnadelschleife als Produkt der Dcl1-Aktivität. Sie besitzen eine Länge von nur 21-22 Nukleotiden und vermitteln die Hemmung der Translation oder die Degradation von mRNA, ähnlich wie siRNAs. So steuern sie eine Vielzahl von zellulären Prozessen, wie z.B. die Regulation von Wachstum und Blütenbildung bei Pflanzen (Wang *et al*., 2004; Palatnik *et al*., 2003; Schwab *et al*., 2006). Auch Viren kodieren

eigene miRNAs, die regulierend in die Genaktivität der Wirtszelle eingreifen und sogar bei einigen virus-bedingten Krebserkrankungen involviert zu sein scheinen (Pfeffer und Voinnet, 2006).

Dcl-1 und -4 konnten als GFP-Fusionsproteine im Zellkern nachgewiesen werden. Dcl-2 und Dcl-3 von *A. thaliana* tragen im Gegensatz zu Dcl-1 und -4 keine Kernlokalisationssequenz (Hiraguri *et al*., 2005). Dcl-2 und -3 sind in der Biogenese von endogenen und viralen siRNAs beteiligt (Xie *et al*., 2004). Für Dcl-2 wurde gezeigt, dass es siRNAs gegen *Turnip crinkle virus* (TCV) produziert, allerdings nicht bei einer Infektion mit *Cucumber mosaic virus strain Y* (CMV-Y) und *Turnip mosaic virus* (TuMV). Auch in dcl-1- und dcl-3-Defektmutanten von *A. thaliana* war die Replikation von CMV-Y und TMV nicht beeinträchtigt, so dass von Dcl-4 als aktive Silencing-Komponente gegen CMV-Y und TMV ausgegangen wird. Mit den dcl-3 Defektmutanten konnte zusätzlich gezeigt werden, dass endogene siRNAs nicht mehr gebildet wurden und mit Verlust von heterochromatischen Stellen im Genom assoziiert war. Dcl-3-produzierte siRNAs (*repeat-associated* siRNAs; ra-siRNAs) sind 24 Nukleotide groß, meist aus repetitiven DNA-Abschnitten im Genom generiert und vermitteln die Etablierung und Aufrechterhaltung von Heterochromatin durch RNA-abhängige DNA-Methylierung und Histon-Modifikation (Matzke und Birchler, 2005).

Boutla *et al.* (2002) konnten mit einer RNA-Präparation aus einer GFP-gesilenceten Pflanze die GFP-Expression eines GFP-transgenen *C. elegans* silencen. Diesen Experimenten lagen die Beobachtungen zugrunde, dass sich ein lokal ausgelöstes PTGS über ein mobiles Signal systemisch in einer Pflanze ausbreiten kann (Voinnet und Baulcombe, 1997). Pfropfungsexperimente (Palauqui *et al.,* 1997; Fagard und Vaucheret, 2000) ergaben, dass PTGS durch das Auftreten von 21-26 nt dsRNAs (siRNA) diagnostiziert werden kann (Hamilton und Baulcombe, 1999) und dass diese siRNAs PTGS bzw. RNAi auslösen (Boutla *et al.,* 2001; Caplen *et al*., 2001). Obwohl

das Silencing-Signalmolekül noch nicht genau spezifiziert werden konnte, haben Untersuchungen gezeigt, dass Dcl-4 in *A. thaliana* 21 Nukleotid kleine siRNAs produziert. Sie gelten als Komponenten des Zell-zu-Zell-Silencing-Signals (Dunoyer *et al*., 2005). 24-26 Nukleotid große siRNAs repräsentieren wahrscheinlich das systemische Signal, das über längere Strecken im Phloem transportiert wird (Hamilton *et al*., 2002). Für den Zell-zu-Zell-Transport (10-15 Zellen) des Silencing-Signals ist die RNA-abhängige RNA-Polymerase 6 (RDR6) essentiell, die auch mit Dcl-4 interagiert (Himber *et al*., 2003). RDR6 amplifiziert wahrscheinlich das Silencing-Signal, fungiert quasi als Relais, warnt und schützt damit vor Pathogenen (Schwach *et al*., 2005). In Pflanzen werden vermutlich vier homologe RDRs exprimiert. Die RDR1 scheint direkt an der Abwehr einiger RNA Viren beteiligt zu sein. Die RDR2 ist an Zellkernprozessen beteiligt und ist für die "*RNA-directed DNA methylation*" (RdDM) essentiell (Meister und Tuschl, 2004; Vaucheret, 2006).

SiRNAs werden nach dem Dicer-Schritt mittels Helikaseaktivität aufgewunden und entweder in den Proteinkomplex RISC (*RNA-induced silencing complex*) oder RITS (*RNA-induced transcriptional silencing complex*) eingebaut. Mit Hilfe der inkorporierten RNA-Fragmente bindet RISC an einen komplementären Bereich einer mRNA, der Ziel-mRNA, und kann diese dann degradieren, während der beladene RITS-Komplex komplementäre DNA-Bereiche im Zellkern stilllegt. Die Ziel-mRNA wird durch RNaseH-Aktivität des *Argonaute*-Enzyms (Slicer-Aktivität) nukleolytisch verdaut (Hammond, 2005), falls der siRNA/mRNA-Duplex ohne Fehlbasenpaarung hybridisieren kann. Fehlbasenpaarung beobachtet man meist bei miRNA/mRNA-Duplexen und führt hier zu einer Inhibierung der Translation ohne Degradation der Ziel-mRNA.

RISC, isoliert aus Drosophila-Zellen, ist ein multimerer Enzymkomplex bestehend aus dem Argonaute 2 (Ago2) Protein, einem RNA-Bindeprotein (VIG), dem

Drosophila-*Homolog Fragile X* Protein (dFXR), einer Helikase und einer Nuklease (SNase) (Caudy *et al*., 2002; 2003; Rivas *et al*., 2005). Experimente haben jedoch ergeben, dass Ago2 alleine Slicer-Aktivität besitzt und die SNase nicht notwendig ist. Ago2 besitzt eine Piwi- und PAZ-Domäne, wobei die Piwi-Domäne mit RNaseH-Motiv den siRNA/mRNA-Duplex mittig katalytisch prozessiert (Hammond, 2005).

Weitere Arbeiten zeigten, dass Gene nicht nur auf der Ebene der Transkription abgeschaltet werden, sondern auch auf der Ebene der Translation, das *Transcriptional gene silencing* (TGS) genannt wird. TGS lässt sich dadurch erkennen, dass schon im Zellkern keine mRNA detektiert wird, während bei PTGS erst im Cytoplasma die RNA degradiert wird (Kooter *et al.,* 1999; Stam *et al.,* 1998). Weitere Unterschiede sind eine Hypermethylierung des entsprechenden DNA-Bereichs, die Verpackung des DNA-Bereichs in Heterochromatin (Verdel und Moazed, 2005) und die Fähigkeit, die Information über das gesilencete Gen zu vererben (Park *et al*., 1996). Beides findet man bei PTGS nicht. Da TGS auf der Ebene der DNA und PTGS auf der Ebene der RNA wirkt, geht man davon aus, dass TGS ein Abwehrmechanismus gegen Transposons ist (Kooter *et al.,* 1999; Vaucheret *et al.,* 2001), aber auch eine wichtige Rolle in Chromosomensegregation, Genomstabilität und Genregulation spielt. Zur RNAi-vermittelten Heterochromatinisierung der Centromere oder dem *Silent mating type* Locus hat man in der Spalthefe *Schizosaccharomyces pombe* zwei Komplexe charakterisiert: RITS und RDRC (*RNA-directed RNA polymerase complex*). Der RITS-Komplex besteht aus dem *Chromodomain* Protein Chp1, dem *Argonaute* Protein 1 (Ago1) und Tas3, wobei einzelne Funktionalitäten noch nicht spezifisch zugeordnet werden konnten. Für *S. pombe* wurden zwei Modelle vorgestellt, wie der siRNA-beladene RITS-Komplex die spezifischen chromosomalen Bereiche erkennt: Das erste Modell favorisiert eine direkte siRNA-DNA-Bindung, das zweite Modell schlägt eine siRNA-

Bindung an gerade neu synthetisierte mRNA vor. Der RDRC regeneriert *de novo* mit der RNA-abhängigen RNA-Polymerase 1 (Rdrp1) neue dsRNA und hält dadurch die siRNAs auf RNAi-induzierendem Level (Verdel und Moazed, 2005).

TGS kann aber auch artifiziell von dsRNA-Konstrukten ausgelöst werden. Matzke und Mitarbeiter zeigten, dass dsRNA-Konstrukte eines Nopalinsynthase-Promotors einen homologen Promotor *in trans* silencen konnte, was mit der Methylierung beider Promotoren einherging (Mette *et al.*, 1999). In einer Folgearbeit konnten Mette *et al*. (2000) den Auslöser des Silencing-Effekts optimieren. Ein dsRNA-Konstrukt mit einer Haarnadelsschleifen-Struktur (*hairpin-loop*) stellte sich als besonders effizient heraus. Wesley *et al.* (2001) verfeinerten den Ansatz, indem sie einen Vektor herstellten, von dem ein dsRNA-Konstrukt mit einem gespleißten *Hairpin-Loop* transkribiert wird. Trug dieses dsRNA-Konstrukt mindestens 98 Nukleotide der Ziel-mRNA, konnten 90-100% der behandelten Pflanzen gesilencet werden.

Viren als Auslöser für RNAi zu nutzen, ist in der Literatur als *Virus-induced Gene Silencing* (VIGS) bekannt und eine alternative Methode, um wirtseigene Proteine herunter zu regulieren und Proteinfunktionen oder Prozessierungswege aufzuklären. Die zeitgewinnenden und kostensenkenden Vorteile dieser Strategie machen Transformationsschritte zur Integration mutagenisierter Nukleotidsequenzen in die chromosomale DNA der Pflanze durch Transposon- oder T-DNA-Transfer unnötig. Es lassen sich also Genfunktionen der Pflanze schnell und effektiv identifizieren, ohne dabei klassische *knockout*-Phänotypen zu produzieren und letale Mutanten ausschließen zu müssen.

In das Genom von Viren wird an geeigneter Stelle ein Teil der cDNA-Sequenz des Gens eingebaut, das in der Pflanze ausgeschaltet werden soll. Durch den RNAi-vermittelten Abwehrmechanismus gegen das rekombinante Virus entstehen siRNAs. Ein Teil der siRNAs ist komplementär zur mRNA des endogenen Gens, der Ziel-

mRNA. Diese mRNA wird dann wie oben beschrieben abgebaut und das entsprechende Protein kaum noch gebildet. Effizientes Silencing wurde bereits anhand mehrerer RNA- oder DNA-Viren-basierender Vektoren gezeigt (Robertson, 2004; Carrillo-Tripp *et al*., 2006). Kumagai und Mitarbeiter konnten 1995 den kompletten eukaryotischen Biosyntheseweg für Carotinoide inhibieren, indem sie cDNA von Phytoensynthase und -desaturase mit dem viralen Genom von TMV transkriptionsfähig fusionierten und *N. benthamiana* mit diesem chimären Virus infizierten. Systemisch infizierte Blätter waren weiß und cytoplasmatische mRNA von Phytoensynthase oder -desaturase konnte nicht nachgewiesen werden (Kumagai *et al.,* 1995). Die meist-benutzten auf RNA-Viren basierenden VIGS-Vektoren sind *Tobacco rattle virus* (TRV) (Ratcliff *et al*., 2001; Liu *et al*., 2002; Chung *et al*., 2004; Brigneti *et al*., 2004; Chen *et al*., 2005) und *Potato virus X* (PVX) (Voinnet und Baulcombe, 1997). Um monokotyledone Pflanzen zu silencen wurde *Barley stripe mosaic virus* (BSMV) als VIGS-Vektor umkonstruiert (Lacomme *et al*., 2003). Auch Satelliten-Viren, wie *Satellite tobacco mosaic virus*, können benutzt werden. Kjemtrup und Mitarbeiter (1998) nutzten als Erste ein DNA-Virus, *Tomato golden mosaic* virus (TGMV), um VIGS auszulösen und erreichten eine mit RNA-Viren vergleichbare RNAi-Effizienz. *Cabbage leaf curl virus*, *African cassava mosaic virus, Pepper Huasteco yellow vein virus* sowie der Satellit von *Tomato yellow leaf curl China virus* (TYLCCNV) sind in der Literatur als VIGS-Vektoren beschrieben (Carrillo-Tripp *et al*., 2006). Alle gehören zu der Familie der Geminiviren (Stanley *et al*., 2005) und nutzen drei entscheidende Vorteile: Beim geminiviralen Satellit von TYLCCNV konnte man den ORF C1, kodiert auf der β-DNA, gegen ein cDNA-Fragment der Ziel-mRNA austauschen (Tao und Zhou, 2004). Bei TGMV wurde ein cDNA-Fragment der Ziel-mRNA in die kleine intergene Region der DNA B inseriert (Peele *et al*., 2001). Die meist-gewählte Strategie, nutzt allerdings den Vorteil, dass das Hüllprotein der

beschriebenen bipartiten Geminiviren austauschbar ist, ohne die Infektivität wesentlich zu beeinflussen (Muangsan *et al*., 2004; Fofana *et al*., 2004). So sind in den vergangenen 10 Jahren für die wichtigsten Labor-Pflanzen, wie *Arabidopsis*, Tabak und Tomate, verschiedene Silencing-Vektoren mit unterschiedlichen Strategien hergestellt worden. Interessanterweise konnte dieses System bis jetzt noch nicht auf das tierische und humane Modell erweitert werden. Hier werden Viren, wie z.B. in der Gentherapie, lediglich als Transportvehikel genutzt, um RNAi-auslösende siRNA-expremierende Kassetten *in vivo* in die Ziel-Zellen einzuschleusen (Stewart *et al*., 2003). Im Zellkultursystem verhält es sich wesentlich einfacher. Es hat sich gezeigt, dass einfache Transfektion von siRNAs effizient RNAi auslösen kann und den Nachteil von dsRNA-Konstrukten umgeht, die im Zellkultursystem sequenz-unspezifische Degradation von mRNA auslösen kann (Elbashir *et al*., 2001).

Viren haben in der Evolution auch Eigenschaften herausgebildet, die das PTGS unterdrücken (Silencing-Suppressoren). Diese Suppressoren können sowohl auf das Entstehen als auch auf die Ausbreitung des PTGS-Signals Einfluss nehmen (Moissiard und Voinnet, 2004). Entscheidend sind dabei multifunktionelle Virusproteine, wie zum Beispiel das P1-Protein von *Rice yellow mottle virus*, das P19-Protein von *Tomato bushy stunt virus*, Hc-Pro von *Potato virus Y*, das 2b-Protein von *Cucumber mosaic virus* oder das AC2-Protein von *African cassava mosaic virus* (ACMV). Aber auch bei tierischen Viren konnte man Silencing-Suppressoren nachweisen, wie das B2-Protein vom *Flock house virus*, das sowohl in *Drosophila* S2 Zellen als auch in Pflanzen funktionell ist (Li *et al*., 2002). Erste Versuche zeigten, dass das Tat-Protein von HIV-1 Silencing-Suppressor-Eigenschaften besitzt (Bennasser *et* al., 2005). Der umgekehrte Versuch verdeutlichte die nahe Verwandtschaft der RNAi-Maschinerie zwischen Tier- und Pflanzenreich, da man den

phytoviralen Silencing-Suppressor p19 auch in humaner Zellkultur einsetzen konnte (Calabrese und Sharp, 2006). P19 gilt als bestcharakterisierter Silencing-Suppressor. Mit seiner einzigartigen Eigenschaft spezifisch siRNAs zu binden, unterdrückt es das Zell-autonome Silencing (Silhavy *et al*., 2002). Mit dem Hefe-Zwei-Hybridsystem wurde gezeigt, dass HcPro mit rgsCaM (*regulator of gene silencing calmodulin-like protein*) interagiert und somit einen endogenen Silencing-Suppressor aktivieren kann (Anandalakshmi *et al*., 2000).

Baulcombe und Mitarbeiter konnten als Erste zeigen, dass AC2 des Geminivirus ACMV ein Suppressor von PTGS ist (Voinnet *et al.,* 1999), das nicht wie das P1-Protein das Zell-zu-Zell-Silencing-Signal unterdrückt, sondern ähnlich dem 2b-Protein das Langstrecken-Silencing-Signal (Himber *et al*., 2003). Mutationsanalysen an dem Positions-homologen C2-Protein von *Tomato yellow leaf curl virus* (TYLCV) haben gezeigt, dass nicht die potentiellen Phosphorylierungsstellen entscheidend sind, sondern die Zink- bzw. DNA-Bindedomäne (van Wezel *et al*., 2002; 2003). Die Suppressoreigenschaft wurde auch als Interaktion von AC2 mit der Chromatinorganisation gedeutet (Trinks *et al*., 2005), denn bis dahin war nur die Eigenschaft von AC2 als Transkriptionsaktivator-Protein (TrAP) bekannt (Sunter und Bisaro, 1991). Transkriptom-Analysen haben gezeigt, dass AC2 von *Mungbean yellow mosaic virus-Vigna* (MYMV) und ACMV in *A. thaliana* verschiedene Gene hochreguliert, wie z. B. die *hypothetical 3'-5' exonuclease* WEL-1 (At3g12460) oder das *cold- and ABA-inducible protein* KIN1 (At5g15960), und dadurch ähnlich dem HcPro wirtseigene Silencing-Suppressoren induzieren könnte. Trinks und Mitarbeiter (2005) zeigten zudem, dass die Aktivierungs-, Zink-Binde-Domäne und die zweigeteilte Kernlokalisationssequenz von MYMV essentiell für Silencing-Suppressor-Aktivität sind. Kürzlich wurde gezeigt, dass AL2 (AC2) von TGMV im Hefe-Zwei-Hybridsystem mit zwei Proteinen interagiert, einer *serine/threonine kinase*

(SNF1) (Hao *et al*., 2003) und einer *adenosine kinase* (ADK) (Wang *et al*., 2003). Beide Kinasen werden durch AL2 inaktiviert und die Autoren vermuten, dass die Stilllegung dieser Schlüsselregulatoren für Zellmetabolismus und Methyl-Stoffwechsel die antiviralen Abwehreigenschaften der Silencing-Maschinerie indirekt hemmt. Überraschenderweise werden für Geminiviren immer neue Proteine mit Silencing-Suppressor-Charakter identifiziert (Vanitharani *et al.*, 2005). Neben AC2 von ACMV und C2 von TYLCV wurde AC4 bei ACMV-Cameroon (ACMV-[CM]) und *Sri Lankan cassava mosaic virus* (SLCMV) als Silencing-Suppressor charakterisiert (Vanitharani *et al.*, 2004). Gopal *et al*. (2006) wiesen für C4 und βC1 von *Bhendi yellow vein mosaic virus* (BYVMV) und Satellit Suppressor-Aktivität nach, während C2 nur als schwacher Silencing-Suppressor gedeutet werden konnte. Auch das βC1 des TYLCCNV Satelliten ist ein Silencing Suppressor (Cui *et al.*, 2005), sowie das V2 Protein des israelischen Isolats von TYLCV (Zrachya *et al.*, 2006).

Geminiviren

Geminiviren sind durch ihre einzigartige doppelikosaedrische und namensgebende Virionenstruktur charakterisiert (Böttcher *et al.*, 2004; Zhang *et al.*, 2001), in die einzelsträngige zirkuläre DNA verpackt ist. Das Zwillingskapsid besteht aus zwei mal 11 Capsomeren, das jeweils nur ein Molekül der zirkulären DNA aufnehmen kann. Die DNA ist mit maximal 3000 Basen klein und kodiert für 4 bis 7 ORFs. Alle Geminiviren vermehren sich im Zellkern. Die meisten sind phloemlimitiert und nur einige Vertreter (ACMV, BDMV, MSV, TGMV) befallen Gewebe wie Schwamm- oder Palisadenparenchym. Geminiviren sind eine Gruppe von Pflanzenviren, die weltweit einen großen ökologischen und ökonomischen Schaden anrichten (Moffat *et al.*, 1999; Polston und Anderson, 1997). Geminiviren befallen agrarökonomisch wichtige

Pflanzen, wie Tomate, Baumwolle, Mais, Zuckerrübe, Bohne und Maniok und führen zu teilweise enormen Ernteverlusten. Die Familie *Geminiviridae* wird in vier Genera (*Mastrevirus, Curtovirus, Topocuvirus und Begomovirus*) untergliedert, je nach Vektor, Wirtspflanze und Vermehrungsstrategie (Stanley, 2005) (Tabelle 1).

Tabelle 1: Einteilung der Geminiviren

Genus	**Genomorganisation**	**Wirtspflanzen**	**Vektoren**
Mastrevirus	monopartit	Mono- u. Dikotyle	*Cicadellidae*
Curtovirus	monopartit	Dikotyle	*Cicadellidae*
Topocuvirus	monopartit	Dikotyle	*Membracidae*
Begomovirus	mono- u. bipartit	Dikotyle	*Bemisia tabaci*

In den letzten Jahren nahm der durch Geminiviren verursachte ökologische und ökonomische Schaden, bedingt durch die zunehmende Verbreitung der Weißen Fliege und durch die schnelle Anpassung der Viren an Umweltbedingungen und Wirtspflanzen durch Rekombination und Pseudorekombination, zu. Unter Pseudorekombination versteht man dabei den Austausch der Genomteile bei nahverwandten bipartiten Begomoviren. Bedingung ist, dass beide DNA-Moleküle korrekt repliziert und transportiert werden. So führte eine Kombination der DNA A von *Abutilon mosaic virus* (AbMV) mit der DNA B von *Sida golden mosaic Costa Rica virus* (SiGMCRV) oder *Sida yellow vein virus* (SiYVV) in *N. benthamiana* zu einer Infektion (Frischmuth *et al.*, 1997, Unseld *et al.*, 2000b). Viren können dadurch auch neue Eigenschaften gewinnen. Pseudorekombination von AbMV DNA A mit der DNA B von *Bean dwarf mosaic virus* (BDMV) führte dazu, dass AbMV seine Phloemlimitierung verlor (Levy und Czosnek, 2003). Rekombination, z. B. zum Wildtypvirus konnte man *in vivo* beobachten, wenn man zwei verschiedene Defektmutanten zusammen inokulierte (Evans und Jeske, 1993). Eine Defektmutante

konnte auch durch ein Transgen ohne Mutation zum Wildtyp komplementiert werden (Frischmuth und Stanley, 1998). Geminiviren unterliegen zudem dem Phänomen der Größenreversion. Mutanten, die eine größere Deletion oder Insertion im Hüllprotein tragen, rekombinieren zu ihrer Wildtypgröße zurück, während der Austausch des Hüllproteins mit einer vergleichbar großen Sequenz stabil bleibt (Bisaro, 1994; Etessami *et al.*, 1989; Gilbertson *et al.*, 2003). Dies kann man als Vorteil nutzen, um Geminiviren zu viralen Expressionsvektoren umzufunktionieren, sofern die kritische Genomgröße nicht unter- oder überschritten wird. Verschiedene Pflanzenviren wurden zu diesem Zweck umkonstruiert, wie z.B. *Tomato golden mosaic virus* (TGMV) (Hayes *et al*., 1988), *Tobacco yellow dwarf virus* (TYDV) (Needham *et al*., 1998) und *Maize streak virus* (MSV) (Palmer *et al*., 1999). Um den Nachteil der Vektorinstabilität zu umgehen und um größere Transgene exprimieren zu können, wurden Replikon-basierende Expressionssysteme entwickelt. Eine Expressionskassette wurde zwischen zwei Ursprünge der viralen Replikation kloniert und stabil in eine Pflanze oder Pflanzenzelle als Transgen integriert, so dass durch Infektion mit dem homologen Virus oder nach Expression des Rep-Proteins ein Transreplikon aus dem Transgen mobilisiert werden konnte. Dadurch erhöhte sich die Kopienzahl der Expressionskassette in induzierten Pflanzenzellen um mehrere Hundert, so dass eine Überexpression beobachtet werden konnte (Morilla *et al.*, 2006; Tamilselvi *et al.*, 2004).

In allen DNA-Molekülen der Geminiviren gibt es eine Intergene Region (IR; Abb. 1), die den Ursprung der Replikation (ori) beinhaltet sowie zwei Promotoren, die bidirektional angeordnet sind. Der Rep-Promotor von *Cotton leaf curl Multan virus* (CLCuMV) exprimiert z.B. konstitutiv und ist sogar stärker als der 35S-Promotor von *Cauliflower mosaic virus* (Xie *et al.*, 2003), während die Promotoren von MYMV nur schwach konstitutiv exprimieren (Shivaprasad *et al.*, 2005). Der Hüllprotein-Promotor

von TGMV ist nach Aktivierung mit dem TrAP in Phloem- als auch in Mesophyllzellen aktiv (Sunter und Bisaro, 1997), und histochemische Färbungsversuche zeigten, dass der Promotor von *Wheat dwarf virus* nur in Phloemzellen aktiv ist (Dinant *et al.*, 2004). Eine ausschließliche Phloemaktivität findet man auch bei dem Promotor des Satelliten von *Tomato yellow leaf curl China virus* (TYLCCNV) (Guan und Zhou, 2006). Geminivirale Promotoren können nicht nur gewebespezifisch, sondern auch Zellzyklus-abhängig sein. Der Hüllprotein-Promotor von *Maize streak virus* (MSV) ist in der frühen G2-Phase am aktivsten, der Rep-Promotor in der S- und G2-Phase des Zellzykluses (Nikovics *et al.*, 2001).

Bei den Begomoviren befindet sich die Gemeinsame Region (*common region;* CR*)* innerhalb der IR. Die CR von ca. 180 Basen ist zwischen der DNA A und der DNA B

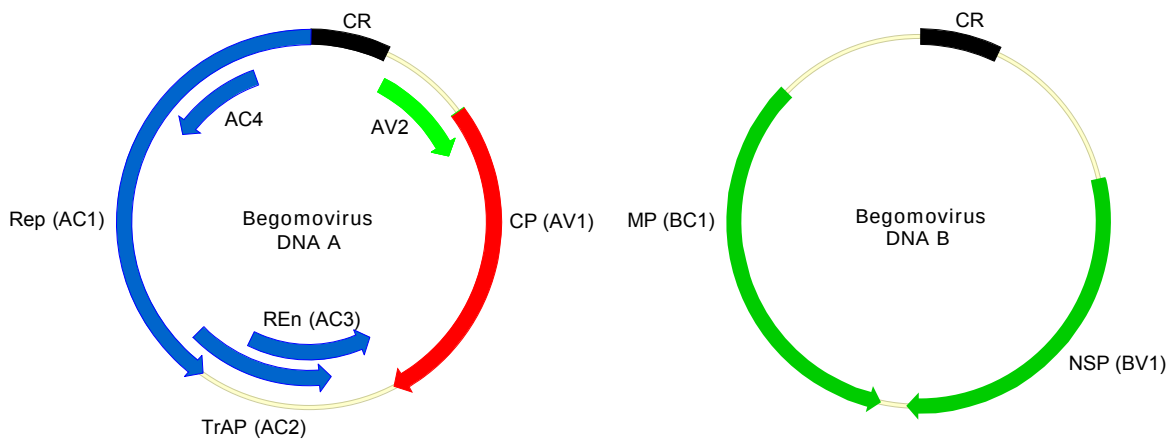

Abb. 1: Schematische Genomorganisation bipartiter Begomoviren.
Pfeile repräsentieren Offene Leserahmen (ORF) und deren Orientierung. Beschreibung der Funktionen der ORFs und der *common region* (CR) im Text.

eines Virus nahezu hundertprozentig gleich, aber wenig homolog zwischen verschiedenen Viren. Eine Ausnahme ist die Nonanukleotidsequenz (TAATATTAC) der Haarnadelschleife im ori, die in allen Geminiviren hochkonserviert ist (Hanley-Bowdoin *et al.*, 1999).

Die DNA A kodiert für bis zu sechs ORFs und zwar für Proteine der Replikation und Verpackung. Das kernlokalisierte Replikations-assoziierte Protein Rep (AC1) bindet innerhalb der CR an sogenannten Iterons, autoreprimiert dadurch seine eigene Transkription und initiiert die Replikation durch *nicking-closing* Aktivität am ori. Es steuert den G1-arretierten Zellzyklus zur S-Phase in differenziertem Gewebe durch Bindung an den Zellzyklusfaktor pRBR (*retinoblastoma-like* Protein) und regt dadurch zur DNA-Synthese an. Rep ist keine Replikase, daher sind Geminiviren auf Wirtspolymerasen angewiesen. Neben der *nicking-closing*- und DNA-Bindungs-Domäne wurde jetzt auch erstmals die Helikase-Domäne funktionell nachgewiesen. Die Helikase-Aktivität verläuft von 3' zu 5' und ähnelt dabei eher Helikasen, die in DNA-Reparatur involviert sind, als 5'-3'-Helikasen der Replikation (Clerot und Bernardi, 2006). Das Transkriptions-Transaktivator Protein TrAP (AC2) vom Hüllprotein CP (AV1) und *Nuclear shuttle protein* NSP (BV1) ist zudem für manche Begomoviren als Silencing-Suppressor charakterisiert worden. Es konnte keine sequenzspezifische Bindung am CP- und NSP-Promotor nachgewiesen werden, daher muss TrAP die Initiation der Transkription über Wirtsfaktoren vermitteln. Als essentiell für Silencing-Aktivität gilt die Zink- bzw. DNA-Bindedomäne, während potentielle Phosphorylierungsstellen ohne Funktionalitätsverlust mutiert werden konnten (van Wezel *et al*., 2002; 2003). TrAP unterdrückt das Langstrecken-Silencing-Signal (Himber *et al*., 2003), doch wie genau TrAP die Silencing-Maschinerie inhibiert, ist noch nicht aufgeklärt. Das positionshomologe C2 Protein von TYLCV besitzt eine Kernlokalisationssequenz und kann im Zellkern nachgewiesen werden (van Wezel *et al.*, 2001, Dong *et al.*, 2003), ebenso wie TrAP bei *Squash leaf curl virus* (SqLCV) (Sanderfoot und Lazarowitz, 1995). Das *Replication enhancer* Protein Ren (AC3) verstärkt die Replikation. Für das C3 Protein von TYLCV konnte eine Interaktion mit sich selbst, C1, PCNA (*proliferating cell*

nuclear antigen) und pRBR nachgewiesen werden, wobei die Interaktion von C3 mit pRBR für die Replikation nicht notwendig ist (Settlage *et al.*, 2005). Die Funktion von AC4 als miRNA- oder siRNA-Bindeprotein und Silencing-Suppressor ist bis jetzt nur für ACMV-[CM] gezeigt worden (Vanitharani *et al.*, 2004; Chellappan *et al.*, 2005). Das Hüllproteingen (CP o. AV1) wird erst durch TrAP aktiviert und gehört zur Gruppe der Proteine, die erst später gebildet werden. Es ist die einzige Hüll-Komponente des Virions, das aus zwei unvollständigen T1-Ikosaedern besteht. Das Hüllprotein ist kernlokalisiert und für MYMV konnte eine Interaktion mit Importin α nachgewiesen werden (Guerra-Peraza *et al.*, 2005). Das Hüllprotein determiniert zudem die Insektenübertragbarkeit (Briddon *et al.*, 1990). Der Austausch des Hüllproteins von AbMV mit dem von *Sida golden mosaic Costa Rica virus* (SiGMCRV) oder der Austausch von drei Aminosäuren im AbMV CP stellte die verloren gegangene Insektenübertragbarkeit wieder her (Höfer *et al.*, 1997, Höhnle *et al.*, 2001). Bei monopartiten Geminiviren übernimmt das CP auch die Transportfunktion. Manche Begomoviren besitzen einen ORF für AV2, der nur in Begomoviren der Alten Welt auftritt. Als GFP-Fusionsprotein zeigte AV2 eine mögliche Assoziation mit Plasmodesmata und Zell-zu-Zelltransport. AV2 hat daher möglicherweise die Funktion eines redundanten Transportproteins und ist eventuell ein genetisches Relikt eines monopartiten Begomovirus (Rothenstein *et al.*, 2007).

Die DNA B enthält nur zwei ORFs, einen für den Zell-zu-Zelltransport und das symptomatische Erscheinungsbild, das *Movement* Protein MP (BC1). NSP ist dagegen für den Transport vom Kern ins Cytoplasma und umgekehrt verantwortlich. Der geminivirale Transportkomplex konnte bis jetzt noch nicht aufgeklärt werden. In der Literatur werden zwei Transportmodelle vorgeschlagen: Das „*relay race model*" sieht vor, dass virale dsDNA mit Hilfe des NSP vom Kern in das Cytoplasma transportiert, die dsDNA an das MP übergeben und über Plasmodesmen in die

angrenzenden Zellen transportiert wird (Gilbertson *et* al., 2003). Das alternative "*couple-skating model*" favorisiert eine Interaktion des NSP/DNA-Komplexes mit dem MP, das gemeinsam in die nächste Zelle transportiert wird. AbMV MP konnte sich selbst nicht über Zellgrenzen hinweg bewegen, doch zusammen mit dem NSP wurde das MP mobilisiert und gemeinsam in die Nachbarzellen transportiert, falls sich die Zellen in einem bestimmten Entwicklungsstadium befanden. Es ist immer noch fraglich, ob der Transportkomplex alleine ausreicht, um von Zelle zu Zelle zu wandern, oder ob Wirtsfaktoren beim Transport beteiligt sein müssen. Das NSP interagiert in *A. thaliana* mit einer kernlokalisierten Acetylase (AtNSI) (McGarry *et al.*, 2003), drei Plasmamembran-Rezeptor-Kinasen NIK1-3 (Fontes *et al.*, 2004) und mit einer weiteren Kinase (NsAK) (Florentino *et al.*, 2006). Überexpression von AtNSI und Inhibierung der NIKs unterstützen die Infektion von CaLCuV. Ein NsAK-*knockout* reduzierte die Infektionsrate.

Da Geminiviren keine eigene Polymerase kodieren, ist die virale Replikation von der zellulären Replikationsmaschinerie abhängig. Die Replikation kann sowohl über einen Rolling-Circle-Mechanismus (RCR) (Hanley-Bowdoin *et al.*, 1999) als auch über eine Rekombinations-abhängige Replikation (RDR) im Zellkern erfolgen (Jeske *et al.*, 2001; Preiss und Jeske 2003). Das Rep-Protein setzt in der Haarnadelschleifensequenz einen Einzelstrangbruch, verbindet sich kovalent mit dem freien 5'-Ende und ligiert nach einer Replikationsrunde das 5'- und 3'-Ende (*nicking-closing*-Aktivität). Bei der RDR reparieren und replizieren Geminiviren unvollständige virale Moleküle durch homologe Rekombination. Während Rep für die RCR essentiell ist, sind Faktoren der RDR noch nicht aufgeklärt.

Die replikative und transkriptionsaktive DNA-Form des AbMV liegt als Minichromosom vor. Auf der DNA A und B konnten jeweils Nuklease-hypersensitive Stellen in der für beide homologen Gemeinsamen Region, in der Promotorregion vor

dem AC2-Gen und in einem der zwei Promotorbereiche vor dem BC1-Gen lokalisiert werden (Pilartz und Jeske, 2003).

AbMV gehört zur Gattung *Begomovirus*, hat aber die sonst typische Insektenübertragbarkeit durch die Weißen Fliege *Bemisia tabaci* GENN. verloren. Das AbMV verursacht bei der Malvenart *Abutilon sellovianum* REGEL ein gelbgrünes Mosaik auf den Laubblättern, es beeinträchtigt aber weder das Wachstum noch die Fortpflanzung. Daher wird infizierte *Abutilon sellovianum* gern gärtnerisch vermehrt.

Da AbMV anders als ACMV phloemlimitiert ist (Abouzid *et al.*, 1988; Horns und Jeske, 1991) und ACMV mit seinem AC2 einen effizienten Suppressor des PTGS besitzt, könnte man als Ursache der Gewebespezifität von AbMV und ACMV die Abwehrreaktion der infizierten Pflanze vermuten. Vorstellbar wäre, dass AbMV zwar prinzipiell das Phloem verlassen kann, aber dann außerhalb wegen eines weniger funktionstüchtigen AC2s gesilencet wird, so dass eine weitere Ausbreitung von Zelle zu Zelle verhindert wird. Defektmutanten von AC2 können sich nicht mehr in der Pflanze ausbreiten (Evans und Jeske, 1993). Dieses Phänomen wurde mit dem Fehlen der Proteine AV1 und BV1 erklärt (Jeffrey *et al*., 1996), könnte aber auch auf fehlenden Suppressor beruhen. C2-transgene *N. benthamiana* und *tabacum cv. Samsun* von *Beet curly top virus* (BCTV) zeigten nach einer Infektion mit einem anderem Geminivirus erhöhte Empfänglichkeit gegenüber Wildtyp-Pflanzen. Zudem ist C2 von BCTV als Transaktivator des CP nicht notwendig (Sunters *et al.,* 2001), was die Vermutung nur bestärkt, dass C2 ein multifunktionelles Protein ist.

Die Herstellung artifizieller, autonom replizierender und sich systemisch ausbreitender Silencing-Konstrukte sollte die Unterdrückung geminiviraler Infektionen erlauben. Dazu sollten Silencing-Konstrukte gegen eine AbMV-Infektion entwickelt und eingesetzt werden, um letztendlich Wirtsfaktoren zu charakterisieren, die für eine

geminivirale Infektion essentiell sind, zugleich aber die Wirtspflanze nicht in ihrer Entwicklung hemmen.

Die Funktion von AC2 als Silencing-Suppressor oder dessen Interferenz mit der Silencing-assoziierten Maschinerie sollte charakterisiert werden, um gezielt Resistenz oder Toleranz zu etablieren.

Ergebnisse und Diskussion

Konstruktion und Prüfung eines Abutilon Mosaik Virus-basierenden *Gene Silencing*- und Proteinexpressions-Vektors

(s.a. Manuskript 1: *Abutilon mosaic virus as a VIGS and protein expression vector*, Seite: 40)

Im Rahmen der Dissertation wurde ein auf dem Abutilon-Mosaik-Virus basierender Silencing Vektor hergestellt und zunächst an Modellpflanzen (*Nicotiana benthamiana*) erprobt. Die Vorteile dieser Strategie machen Transformationschritte zur Integration mutagenisierter Nukleotidsequenzen in die chromosomale DNA der Pflanze durch Transposon oder T-DNA-Transfer unnötig. AbMV wurde als Ausgangskonstrukt gewählt, da es im Gegensatz zu anderen Begomoviren eine schwache Symptomatik im experimentellen Wirt *N. benthamiana* zeigt. Das erlaubt, *Knock-down*-Phänotypen eher erkennen zu können. AbMV ist zusätzlich als Replikon attraktiv, weil es viele agrarwissenschaftlich relevante Wirtspflanzen, wie Tabak und Tomate, infizieren kann.

Um gezielt Pflanzengene ausschalten zu können, wurde die AbMV-DNA A als geminiviraler Vektor modifiziert. Das Basis-Konstrukt besteht aus einem infektiösen partiellem Dimer (Bitmer) von AbMV DNA A, in der das Hüllprotein-Gen AV1 deletiert wurde. Es hat sich gezeigt, dass diese Deletionsmutante in *N. benthamiana*-Pflanzen stabil replizieren kann und infektiös ist. Im Vergleich zu mock-inokulierten Pflanzen wurde kein veränderter Phänotyp festgestellt. Eine in der Literatur beschriebene Rekombination zurück zur Wildtyp-Genomgröße (Bisaro, 1994) konnte nicht beobachtet werden. Die Genomgröße des replizierenden Replikons blieb

unverändert und stabil. Dies begünstigt AbMV in der Wahl als Silencing-Vektor gegenüber anderen Viren.

In eine neu generierte *Bam* HI -Schnittstelle der Deletionsmutante wurde ein cDNA-Fragment von Phytoendesaturase (PDS) inseriert. *N. benthamiana*-Pflanzen zeigten 2-3 Wochen nach der Inokulation (wpi) chlorotische Bereiche in neu entwickelten Blättern, und 6-8 wpi waren Stengel und Blätter fast vollständig weiß. In systemisch-infizierten Blättern konnte durch *Southern Blot*- und *Rolling Circle Amplification* (RCA) – Analysen das PDS-Silencing Replikon nachgewiesen werden. Eine Infektion dieses modulierten AbMVs löste nicht nur PTGS gegen alle übrigen viralen Proteine aus, sondern auch gegen das Pflanzengen PDS. Ein *Knockdown* des Carotenoid-Stoffwechselwegs war die Folge, was sich durch Verlust des Chlorophylls und durch Degradation der Chloroplasten wegen mangelnder Photoprotektion in einem Albino-Phänotyp äußerte. Die Funktionalität von AbMV als Silencing-Vektor war damit gezeigt.

Es ist möglich, die DNA A von AbMV mit verschiedenen DNA B-Molekülen nahe verwandter Begomoviren zu kombinieren, um *in planta* Pseudorekombinanten mit neuen Eigenschaften zu generieren (Frischmuth *et al.*, 1997, Unseld *et* al., 2000b). So wurde das PDS-Silencing-Konstrukt zusammen mit der DNA B von *Sida yellow vein virus* (SiYVV) oder mit der DNA B von *Sida golden mosaic Costa Rica virus* (SiGMCRV) auf *N. benthamiana*-Pflanzen inokuliert. Beide Kombinationen sind infektiös, können Pflanzen systemisch infizieren und lösen massives PDS Silencing aus. In beiden Kombinationen konnte PDS Silencing zu einem früherem Zeitpunkt, schon 1 wpi, festgestellt werden. In den infizierten Pflanzen entwickelten sich jedoch auch die typischen, schweren Symptome wie Blattrollen und Zwergwuchs.

AbMV hat ein breites Wirtsspektrum und infiziert neben *N. benthamiana* auch *N. tabacum* und *Solanum lycopersicum*. Das PDS-Silencing-Konstrukt konnte in

Kombination mit SiGMCRV DNA B *N. tabacum*-Pflanzen systemisch infizieren und PDS-Silencing auslösen, während *in S. lycopersicum*-Pflanzen PDS-Silencing lokal begrenzt blieb und weder eine systemische Infektion noch ein systemisches PDS-Silencing erreicht werden konnte.

Virus-basierende Vektoren zur heterologen Genexpression in Pflanzen stellen eine vielversprechende Alternative zur klassischen Züchtung und Transgen-Technologie dar. In die infektiöse Hüllprotein-Deletionsmutante von AbMV wurde das Gen für das *Green Fluorescent Protein* (GFP) inseriert, so dass es unter der Kontrolle des Hüllprotein-Promotors exprimiert werden kann. Agrobakterien, die Pflanzenzellen mit dem GFP-Expressions-Konstrukt transfizieren können, wurden in *N. benthamiana*-Blätter infiltriert. Zwei Tage nach der Infiltration leuchtete infiltriertes Gewebe unter UV-Licht grün, während das übrige Gewebe die typische rote Eigenfluoreszenz von Chlorophyll zeigte. Mikroskopische Untersuchungen ergaben, dass die GFP-Expression unter dem Hüllprotein-Promotor unabhängig vom infiltrierten Gewebe war, so dass der Promotor als Gewebe-unspezifisch und das GFP-Expressions-Konstrukt als funktionell charakterisiert werden konnte.

Molekularbiologische Untersuchungen an jungen Blättern 3 wpi ergaben zudem, dass das aus dem GFP-Expressions-Konstrukt entstandene GFP-Replikon nachgewiesen werden konnte. Dünne Schnitte längs der Blattadern systemisch infizierter Blätter wurden im Fluoreszenz-Mikroskop untersucht. GFP-Signale konnten ausschließlich in Phloem-Zellen beobachtet werden und bestätigten dadurch die Phloemlimitierung von AbMV. Mit Hilfe dieses rekombinanten Virus kann man den Infektionsverlauf innerhalb der Pflanze optisch einfach nachvollziehen und die Infektionsdynamik aufklären. In Co-Inokulationsexperimenten mit Silencing-Konstrukten lassen sich Wirtsproteine charakterisieren, die z.B. für Gewebsspezifität

von AbMV und damit auch für andere phloemlimitierte Begomoviren verantwortlich sind.

Diese Ergebnisse zusammengefasst lassen den Schluß zu, dass AbMV als Proteinexpressions- und *Gene Silencing*-Vektor benutzt werden kann. Es lassen sich also zukünftig Genfunktionen der Pflanze schnell und effektiv identifizieren, ohne dabei klassische *Knock out*-Phänotypen zu produzieren und lethale Mutanten ausschließen zu müssen.

Virus-induziertes Gene Silencing (VIGS) eines chloroplastidären Hitzeschock-Proteins

(s.a. Manuskript 2: *Virus-induced gene silencing of chloroplast-localized heat shock protein results in reduced amount of viral ssDNA and photo-bleached phenotype*, Seite: 64)

Es sollten im Rahmen dieser Arbeit auch *Silencing*-Konstrukte entwickelt werden, die effizient und spezifisch die systemische Infektion von AbMV *in vivo*, wie z.B. in *N. benthamiana*, unterdrücken können. Dabei sollten Wirtsproteine charakterisiert werden, die essentiell für das Virus, aber nicht für den Wirt sind. Diese Untersuchungen stellen damit die Basis dar, um mittels VIGS schnell und einfach Pflanzenproteine zu bestimmen, die in der Ausprägung geminiviraler Infektionen involviert sind. Durch PTGS-vermittelte Resistenz wären neuentwickelte Gewebe möglicherweise virusfrei und die Pflanze vor einer Neuinfektion geschützt.

Mit Hilfe des Hefe-2-Hybrid-Systems konnte Dr. T. Kleinow nachweisen, dass die N-terminale Domäne des *Movement Protein* (MP; BC1) von AbMV mit dem C-terminalen Bereich des Chloroplasten-lokalisierten und Zellkern-kodierten Hitzeschock-Proteins von 70 kDa (cpHsc70) aus *Arabidopsis thaliana* interagiert.

Um zu überprüfen, wie sich ein cpHsc70-*knock down* Phänotyp auf eine AbMV-Infektion auswirkt, wurde ein cDNA-Fragment von AtcpHsc70 in den AbMV-basierten Silencing-Vektor inseriert. Inokulierte *N. benthamiana*-Pflanzen zeigten 3 wpi in jungen Blättern kleine weiße Chlorosen entlang der Leitgewebe, in denen das cpHsc70-Silencing-Replikon mittels RCA- und Gewebeabdruck-Analysen nachgewiesen werden konnte. Fluoreszenz-mikroskopische Untersuchungen bestätigten, dass die Chlorosen auf den Verlust von Chlorophyll zurückzuführen waren, das Gewebe aber noch intakt und nicht nekrotisch war. Durch Extraktion der RNA aus weißem Blattgewebe liessen sich die für PTGS typischen siRNAs gegen cpHsc70 und der Verlust des cpHsc70-Transkripts durch semi-quantitative RT-PCR nachweisen. Die kleinen, weißen Chlorosen entlang der Leitgewebe können dem cpHsc70-Silencing Phänotyp zugeordnet werden. Es wird vermutet, dass der Verlust an Chloroplasten-lokalisierten Hitzeschock-Proteins einen essentiellen Eingriff in den Chloroplasten-Stoffwechsel darstellt, der zur Degradation dieser Zellorganellen führt. Damit resultieren PDS- und cpHsc70-Silencing im selben Albino-Phänotyp. Im Gegensatz zum PDS-Silencing, das sich im Blattgewebe ausbreiten kann, scheint das cpHsc70-Silencing lokal begrenzt zu bleiben. Mit einer *Southern Blot*-Analyse wurden PDS-, cpHsc70- gesilencete- und AbMV-infizierte Pflanzenproben miteinander verglichen. In cpHsc70-gesilencten Pflanzen akkumulierte signifikant weniger ssDNA als in den Kontroll-Proben. Andere DNA-Formen, wie offen zirkuläre- oder kovalent-geschlossen zirkuläre DNA, waren mengenmäßig unverändert, so dass über eine Interaktion von cpHsc70 und viraler ssDNA spekuliert werden kann. In früheren Arbeiten wurde berichtet, dass ssDNA von AbMV in Chloroplasten nachweisbar war (Gröning *et al*., 1987; 1990). Da cpHsc70 als Schlüsselenzym für den Protein-Import in Chloroplasten verantwortlich ist, kann somit vermutet werden, dass der AbMV-Transportkomplex über die Protein-Interaktion von cpHsc70 und MP

aktiv in Chloroplasten geleitet wird. Diese Modell kann damit als Grundlage für den Transport von geminiviraler DNA über Membranen dienen und zur Aufklärung des Zell-zu-Zell-Transport beitragen.

Studien zur Charakterisierung des Transkriptions-Transaktivator und Silencing-Suppressor Proteins AC2 von AbMV

(s.a. Manuskript 3: The complex role of Abutilon mosaic virus (AbMV) AC2 in remodeling of viral and transreplicon minichromosomes, Seite: 85)

Die auf das Leitgewebe begrenzte Ausbreitung einiger Geminiviren kann verschiedene Ursachen haben. Sie könnte auf dysfunktionalen Transportproteinen beruhen. Biolistische Experimente zeigten allerdings, dass AbMV-BC1 den Zell-zu-Zell-Transport auch in Epidermiszellen befördern kann, soweit *Sink*-Zellen getroffen wurden (Zhang *et al*., 2001). Eine zweite mögliche Erklärung wäre, wenn AbMV im Vergleich zu anderen, nicht-phloemlimitierten Geminiviren unterschiedlich unter den Abwehrmechanismen der Pflanzen leiden würde. Hierbei könnten TGS und PTGS zentrale Rollen spielen. *African cassava mosaic virus* (ACMV) besitzt mit seinem AC2-Protein einen charakterisierten Suppressor des PTGS (Hamilton *et al*., 2002). Über die Eigenschaften des AbMV-AC2 ist in dieser Hinsicht noch nichts bekannt.
Eine GFP-Expressionskassette wurde zwischen zwei Ursprünge der viralen Replikation kloniert und stabil in eine *N. benthamiana*-Pflanze als Transgen integriert, so dass durch Infektion mit AbMV ein Transreplikon aus dem Transgen mobilisiert werden kann. Dadurch erhöht sich die Kopienzahl der GFP-Expressionskassette in AbMV-infizierten Pflanzenzellen um mehrere Hundert, so dass ein starkes GFP Signal beobachtet werden kann. Dünne Schnitte längs der Blattadern systemisch infizierter Blätter wurden im Fluoreszenz-Mikroskop untersucht. GFP-Signale konnten

ausschließlich im Leitgewebe festgestellt werden und bestätigten prinzipiell die Mobilisierung und Replikation des Transreplikons. Im Gegensatz zur Fluoreszenz-Mikroskopie ließ sich in allen Proben das Transreplikon weder durch RCA- noch durch Southern Blot-Analyse nachweisen.

In Infiltrationsexperimenten mit Agrobakterien, die die Expression von AbMV Rep und TrAP (Dr. T. Kleinow, unpubliziert) vermitteln, konnte gezeigt werden, dass die Mobilisierung des Transreplikons Rep-abhängig ist. Die Co-Expression von TrAP mit Rep, genauso wie Agrobakterium-vermittelte Transfektion mit dem infektiösen AbMV DNA A Plasmid (Frischmuth *et al*., 1990), unterdrückt die Vermehrung des Transreplikons. Der Silencing-Suppressor p19 (Silhavy *et al*., 2002) eines nichtverwandten RNA Virus neutralisierte diesen Effekt, so dass von einer siRNA-vermittelten Unterdrückung der Transreplikation ausgegangen werden kann. Folgerichtig wurde nur in Pflanzenproben GFP-siRNA detektiert, in denen die Vermehrung des Transreplikons unterdrückt wurde.

Durch Darstellung der Topoisomere der viralen DNA kann auf verschiedene Kondensierungszustände geminiviraler Minichromosomen geschlossen werden. Mit Hilfe des Interkalators Chloroquin in der Gelektrophorese kann der Kondensierungs-grad von viralem Chromatin exakt ermittelt werden (Jeske *et al*., 2001). Die Anzahl der Nukleosomen pro DNA-Zirkel bildet sich in der Verteilung der superhelikalen Windungen der Topoisomere zirkulärer DNA ab. Für AbMV konnte festgestellt werden, dass das Chromatin mit fortschreitender Infektionsdauer stärker kondensiert vorliegt und damit möglicherweise weniger transkriptionsaktiv wird. Eine Überexpression von TrAP inhibierte diesen Effekt und konnte durch densiometrische Analyse erstmalig visualisiert werden.

Darstellung der Ergebnisse als Publikationsmanuskripte in englischer Sprache

1. Abutilon mosaic virus as a VIGS and protein expression vector
Seite: 40

2. Virus-induced gene silencing of chloroplast-localized heat shock protein results in reduced amount of viral ssDNA and photo-bleached phenotype
Seite: 64

3. The complex role of Abutilon mosaic virus (AbMV) AC2 in remodeling of viral and transreplicon minichromosomes
Seite: 85

Manuskript 1:

Abutilon mosaic virus as a VIGS and protein expression vector

Abstract

Virus-induced gene silencing (VIGS), a type of RNA interference, is initiated by viral vectors carrying fragments of host genes to knock down gene expression in plants by degrading the homologous transcripts. Although geminiviruses have been already used for this purpose, *Abutilon mosaic virus* (AbMV) has some advantages. AbMV was chosen as a VIGS vector because of its mild symptoms in *Nicotiana benthamiana*. It has the capability to infect a broad range of important dicotyledonous crops, but is naturally not transmissible by insects or mechanical inoculation, making it a safe vector to handle. AbMV coat protein (CP) is dispensable for replication and systemic movement in *N. benthamiana* and can be replaced by sequences of interest. To proof the concept, a phytoene desaturase fragment from *N. benthamiana* (NbPDS) was inserted into an infectious AbMV DNA A, which was able to trigger PDS silencing. If *mGFP4* replaced the coat protein ORF, GFP signals in infected cells allowed an online monitoring of virus movement through the whole plant and to study tissue specificity. In combination with transgenic *N. benthamiana* plant carrying a dimer repeat of the AbMV DNA B component, the DNA A-based VIGS vector is easily to apply and makes this system an attractive and innovative tool for gene function studies in *N. benthamiana*.

Keywords: geminivirus, virus-induced gene silencing, viral plant expression vector, rolling circle amplification, recombination

Introduction

RNA interference, also called post-transcriptional gene silencing (PTGS), is a sequence-specific RNA degradation mechanism that silences a targeted gene (Al-Kaff *et al.*, 1998). It is part of the natural virus defence system, whereas transcriptional gene silencing (TGS) is thought to be a defence against transposons or other invasive DNA elements. Trigger of the gene silencing mechanism is dsRNA (Mello and Conte, 2004), which is recognized and cleaved by Dicer-like protein(s), a multi protein-complex, to generate siRNAs of different size and fate (Akbergenov *et al.*, 2006). SiRNAs are incorporated into the RNA-induced silencing complex (RISC) to degrade specifically homologous mRNA (Hammond, 2005) into the RNA-induced transcriptional gene silencing complex (RITS) (Verdel and Moazed, 2005) to cause heterochromatin formation, or to function as a mobile silencing signal (Mlotshwa *et al.*, 2002).

Virus-induced gene silencing (VIGS) is a method to transiently interrupt gene function through RNA interference which is initiated by viral vectors carrying fragments of host genes. This was first demonstrated with an RNA virus by inserting sequences into *Tobacco mosaic virus* (TMV) (Kumagai *et al.*, 1995), and then for a DNA virus by replacing the coat protein gene, which is dispensable for some geminiviruses, with a silencing target sequence (Kjemtrup *et al.*, 1998). The ability to suppress specific genes is a powerful tool to assign biological function to uncharacterized genes of various plant species. Several RNA viruses have been successfully used as VIGS vectors, to knock down transgenes as well as endogenous plant genes, like TMV, TRV and PVX (Kumagai *et al.*, 1995; Ratcliff *et al.*, 2001; Ruiz *et al.*, 1998). Using TMV carrying a partial cDNA fragment of the tomato phytoene desaturase (PDS) gene, Kumagai *et al.* (1995) were able to manipulate the carotenoid biosynthetic

pathway in *N. benthamiana*. The construct induced a photo-bleaching white phenotype due to the effective silencing of the endogenous PDS.

Geminiviruses, like *Tomato golden mosaic virus* (TGMV) or *Cabbage leaf curl virus* (CaLCuV) have been used to generate silencing vectors (Carrillo-Tripp *et al.*, 2006; Fofana *et al.*, 2004; Turnage *et al.*, 2002). The family *Geminiviridae* comprises four genera: Mastrevirus, Curtovirus, Begomovirus and Topocuvirus, divided according to their genome organization and transmission vector (Stanley *et al.*, 2005). Begomoviruses are serious plant pathogens infecting dicotyledonous plants, among them important crop plants, but induces also ornamental mosaic without harming the plant, like *Abutilon mosaic virus* (AbMV). Most begomoviruses comprise bipartite genomes (DNA A and B), encapsidated in twin particles of approximately 20 × 30 nm (Böttcher *et al.*, 2004) and are transmitted by the whitefly-vector *Bemisia tabaci*. Their genomes consist of two single-stranded circular DNA molecules, each 2.5-3.0 kb in size. DNA replication occurs using host enzymes and double-stranded viral DNA intermediates via recombination-dependent replication and rolling circle replication (Alberter *et al.*, 2005; Hanley-Bowdoin *et al.*, 1999; Jeske *et al.*, 2001; Preiss and Jeske, 2003). Some bipartite geminiviruses can move systemically without the coat protein gene, which can be replaced by foreign sequence of approximately 800 bp (Sudarshana *et al.*, 1998). AbMV was chosen because of its mild symptoms in *N. benthamiana* and *N. tabacum*. AbMV infects a broad range of plant species. Families containing susceptible hosts are *Cucurbitaceae*, *Leguminosae-Papilionoideae*, *Malvaceae* and *Solanaceae*, but AbMV is naturally not transmissible by an insect vector or mechanical inoculation, making it a safe vector to handle (Höfer *et al.*, 1997; Höhnle *et al.*, 2001). The products of rolling circle amplifications (RCA) of the designed silencing vectors have been used as inoculum, indicating the infectivity of the RCA and the possibility of cell-free cloning of silencing

vectors. RCA was also used as reliable diagnosis tool, using actively replicating geminiviruses as ideal substrate (Haible *et al.*, 2006). In combination with transgenic *N. benthamiana* plants carrying a dimer repeat of the AbMV DNA B component (Pohl and Wege, unpublished), the DNA A-based VIGS vector is easily applied and makes this system an attractive tool for gene function studies. As a proof of concept, a fragment of the *N. benthamiana* phytoene desaturase (NbPDS) gene replaced the AbMV coat protein gene.

To use AbMV as a versatile tool to study gene function in vivo, it is desirable to use it as a viral-based plant expression vectors for foreign proteins. Plant virus-based vectors as transient gene expression systems are an attractive alternative to conventional breeding and transformation technology. ORF of *mGFP4* (Haseloff *et al.*, 1997) replaced the coat protein, leading to GFP signals in infected cells, giving the opportunity for monitoring virus movement through distal parts of a plant and studying tissue specificity. Analysis confirmed for AbMV existing in tissues no other than the internal and external phloem of vascular bundle (Wege *et al.*, 2001).

In experiments, recombination occurred quickly if two handicapped geminivirus constructs were co-inoculated (Evans and Jeske, 1993), e.g. with mutated genes for the control of replication or transcription, or if a single construct deviated considerably from viral genome size (Bisaro, 1994). Recombination was also detected between a mutated virus and its homologous transgene, as shown for *African cassava mosaic virus* (ACMV) in *N. benthamiana* plants (Frischmuth and Stanley, 1998). It was reported that geminiviruses underlie the phenomenon of size reversion, which means, that recombinant geminiviruses of genomic size different from wildtype, revert to wildtype size. Cloned viral constructs with large defects in the coat protein gene tend to reconstitute genomic size DNA for unknown reasons (Bisaro, 1994). Sequence analysis of the size revertants revealed footprints of an illegitimate

recombination (Etessami *et al.*, 1989). Transient protein expression from recombinant geminiviral-based vectors is therefore limited. Gilbertson *et al.* (2003) reasoned a scenario where a wide range of recombinants are produced in the nucleus. Only an optimal size class of recombinants are efficiently trafficked from cell to cell. In this report, we show, that recombinant AbMV vectors of smaller size than wildtype remain small, whereas only larger components revert to genomic wildtype size of 2.6 kbp. Interestingly, only AbMV-based vectors biolistically inoculated were able to revert in contrast to agrobacteria-mediated inoculation. Last mentioned lost their infectivity.

Material and Methods

Microorganisms and plants

Virus strains used in this work were agroinfectious clones of *Abutilon mosaic virus* (AbMV; X15983, X15984) (Frischmuth *et al.*, 1990) and DNA Bs of *Sida yellow vein virus* (SiYVV; Y11101; formerly named *Sida golden mosaic Honduras virus - yellow vein*) and *Sida golden mosaic Costa Rica virus* (SiGMCRV; X99551) (Frischmuth *et al.*, 1997; Unseld *et al.*, 2000).

Escherichia coli strain DH5α was used for subcloning. *Nicotiana benthamiana* Domin, *Nicotiana tabacum* cv. Samsum nn L. and *Solanum lycopersicum* L. cv. Moneymaker were grown in an insect-free S2 greenhouse with supplementary lighting (Wege *et al.*, 2001).

Cloning procedures

Recombinant binary plasmids were introduced into *A. tumefaciens* GV3101 (Koncz *et al.*, 1994) by chemical transformation. *A. tumefaciens* GV3101 were grown overnight in 50 ml YEB media at 28 °C till an OD_{600}=0.5, centrifugated (15 min, 4 °C, 4000 rpm), the pellet was resuspended in 500 µl ice-cold YEB media. 200 µl agrobacteria-suspension were transformed with 1 µg of pBIN-TR22(x) plasmid preparation, shock-frozen in liquid N_2 for 3 sec and incubated 5 min at 37 °C. After addition of 1 ml pre-warmed YEB media, cells were shaken at 28 °C for 4 h and 1/10 vol. finally plated under selection conditions.

A. tumefaciens GV3101 was transformed with the constructs pBIN-TR22, pBIN-TR224(GFP), pBIN-TR225(PDS↓) and pBIN-TR229(PDS↓). PCR analysis was performed to check construct integrity.

Recombinant DNA techniques were performed as described by Sambrook and Russell (2001). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

DNA sequences were determined using Thermo Sequenase Primer Cycle Sequencing kit (Amersham Pharmacia Biotech) with universal IRD800 labeled forward primer and reverse primer (Table 1). Sequence reactions were analyzed on a LI-COR sequencer (MWG-Biotech, Germany). Sequence alignment was done with Vector NTI program (Invitrogen).

VIGS constructs for mechanical inoculation

Generating pBK3c, a 2056bp fragment was amplified by PCR (Taq polymerase, Quiagen) with primers BK3AbA and SmAIR-c (Table 1, Fig. 1) from AbA1.5 (Frischmuth *et al.*, 1990). To construct pBK5v, a 509bp fragment of AbA1.5 (Frischmuth *et al.*, 1990) was amplified using primers BK5AbA and SmAIR-v (Table 1, Fig. 1). Both PCR fragments were inserted into pGEM-T according to manufacturer's recommendations (Promega). pBK5v was digested by *Bam* HI and *Bgl* II. The resulting fragment was inserted into *Bam* HI-linearized pBK3c to gain pBK-TR22 (Fig. 1), an DNA A bitmer cassette with a deletion of the AV1 ORF and a single *Bam* HI restriction site.

To generate pBK-TR224(GFP), the ORF of *mGFP4* (Haseloff *et al.*, 1997) was inserted into pBK-TR22, to obtain expression under the control of the AV1 promoter. A 1013bp PCR fragment with primer pair BKPDS5' (*Bam* HI) and BKPDS3'(*Bam* HI) (Table 1) of the genomic NbPDS was first subcloned into pGEM-T and then transferred into pBK-TR22 to obtain pBK-TR225(PDS↓). Primer pair BKPDS5' (*Bam* HI) and BKPDS3'(*Bam* HI) (Table 1) were designed based on sequence (AJ616742)

from Tao and Zhou (2004). Whereas pBK-TR229(PDS↓) carries a cDNA fragment of NbPDS, amplified by RT-PCR (Invitrogen) with primers BKPDS5'1070 and BKPDS3'(*Bam* HI) (Table 1). Primer BKPDS5'1070 was designed based on a sequence homology result of a multiple sequence alignment with availbale PDS sequence entries in available databases.

Table 1
Primers used for amplification and sequencing of viral AbMV DNA A , NbPDS and *mGFP4*.
[a] Restriction sites within PCR primers are underlined
[b] AbMV DNA A numbering according to NCBI Acc. NC001928, pGEM-T (Promega), mGFP4 (U87624) and NbPDS (DQ469932).
[c] Sequencing primer, DY-781 or IDR-800 labelled.

Primer name	**Sequence 5`→ 3`** [a]	**Location** [b]
forward primer [c]	CGCCAGGGTTTTCCCAGTCACGAC	pGEM-T nt 1 – 24
reverse primer [c]	AGCGGATAACAATTTCACACAGGA	pGEM-T nt 240 – 263
BK5AbA	ATGGGGGATCCCGCCTAGGTA	AbMV A (c) nt 359 – 379
SmAIR-C	TGGAGTCGACGGGCTTCCTGTACATGGGCC	AbMV A (c) nt 482 – 511
BK3AbA	CTTTGAGGATCCGAATCTA	AbMV A (v) nt 1070 – 1092
SmAIR-V	TGGAGTCGACTTAGCTCCCTGAATGTTCGG	AbMV A (v) nt 2348 – 2377
BKPDS5' (*Bam* HI)	GGATCCGGCACTCAACTTTATAAACC	NbPDS nt 849 – 874
BKPDS3'(*Bam* HI)	GGATCCCTTCAGTTTTCTGTCAAACC	NbPDS nt 1244 – 1269
BKPDS5'1070	GGATCCTGCACCCTTAAATGGAATATGGGC	NbPDS nt 643 – 668
BKmGFP45' (*Bam* HI)	GGATCCGCGCCACCATGAGTAAAGGAGAAGAACTTTTC	*mGFP4* nt 1 – 44
BKmGFP43' (*Bam* HI)	GGATCCGCCGCTTATTTGTATAGTTCATCCATGCC	*mGFP* nt 714 –743

Constructs for agroinoculation

All pBIN-TR22(x) derivates were constructed by releasing the different AbMV DNA A ΔAV1-multimeric cassettes from the pBK-TR22(x)-clones using *Sal* I and inserting them into single *Sal* I site of pBINplus (van Engelen *et al.*, 1995).

Rolling circle amplification - Restriction fragment length polymorphism (RCA-RFLP)

RCA was performed using the TempliPhi DNA amplification kit (GE Healthcare, formerly Amersham Bioscience) following manufacturer's instructions as described in Haible *et al.* (2006) using plasmid DNAs as templates at 28 or 30 °C for at least 16 h. The reaction was stopped at 65 °C for 10 min, and RCA products were analyzed by restriction fragment length polymorphism (RFLP).

Inoculation of plants

N. benthamiana plants in the 4-5-leaf stage were biolistically inoculated using a particle gun (PDS1000/HE; Bio-Rad). Gold particles (1 µm) were coated with RCA products or plasmid DNAs and bombarded using 900 psi rupture discs under a vacuum pressure of 27 inch. Hg (Unseld *et al.*, 2001).

Alternatively, *N. benthamiana* plants were inoculated with agroinfectious clones (Klinkenberg *et al.*, 1989).

Agroinfiltration assay

Agrobacteria were grown overnight, pelleted, resuspended in 10 mM $MgCl_2$ and 100 µM of acetosyringone to an OD_{600}=0.5 and incubated for at least 2 h at room

temperature. *N. benthamiana* leaves were injured with a needle and the agrobacteria-suspension was infiltrated with a 5 ml syringe (Morilla *et al.*, 2006).

Analysis of viral DNAs

Total cellular nucleic acids were extracted from systemically infected plant tissues with extraction buffer (100 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM EDTA, 1% SDS). Proteins were extracted by phenol-chloroform (1:1), nucleic acids were ethanol-precipitated, and dissolved in 100 µl sterile water. From each plant, 100 ng total DNA were separated on agarose gels using 1× TBE buffer, transferred to Hybond NX membranes (Amersham) (Wege and Siegmund, 2007). Viral DNAs were detected using digoxigenin-labeled probes (Roche, Mannheim, DIG-High Prime Kit).
For RCA (10 ng) total nucleic acids from systemically infected plant tissues served as template.

Microscopy

Plants were screened for GFP expression under the Axiophot fluorescence microscope (ZEISS, Oberkochen, DE; with filter G 365; FT 395; LP 420). Macroscopic detection of GFP fluorescence was performed by using a hand-held, long-wave UV lamp (4 W) and photographed with a Canon Powershot 1 with yellow filter. Images were processed using Paint Shop Pro.

Results

Construction of AbMV-based vectors for reporter protein expression and VIGS

A partial dimer (bitmer) of AbMV DNA A was constructed in order to delete AV1 and to introduce an unique *Bam* HI insertion site (pBK-TR22, Fig. 1). The deletion starts 19 nt upstream of the AV1 start codon and ends 29 nt upstream of the stop codon of the AV1 ORF, to enable insertion of foreign ORFs with their own start and stop codons, and to preserve the overlaping AbMV transcription termination signals (Frischmuth *et al.*, 1991). *In planta*, an autonomous replicon will be generated via replicational release from this clone. pBK-TR22 was bombarded once on three AbMV DNA B-transgenic *N. benthamiana* plants ("DNA B plants", Pohl and Wege, unpublished). The plants were phenotypically indistinguishable from mock-inoculated plants. The replicon was, nevertheless, detected by RCA in systemically infected leaves in all three plants at 21 dpi, and no reversion to AbMV DNA A wildtype size was observed (data not shown).

The bitmer DNA A ΔCP cassette was transferred to plasmid pBINplus (van Engelen *et al.*, 1995) (pBIN-TR22), transformed into agrobacteria, and inoculated onto five DNA B plants. pBIN-TR22 was able to systemically infect these plants without inducing viral symptoms. Whereas Southern Blot analysis (Fig. 2b) showed the expected viral DNA in four of five plants, RCA-RFLP (Fig. 2c) confirmed that all plants were infected. Remarkably, the inoculated AbMV DNA A ΔCP had spread through DNA B plants without altering its size (Fig. 2b, c). Three further DNA B plants were agro-inoculated with pBIN-TR22 and confirmed these results.

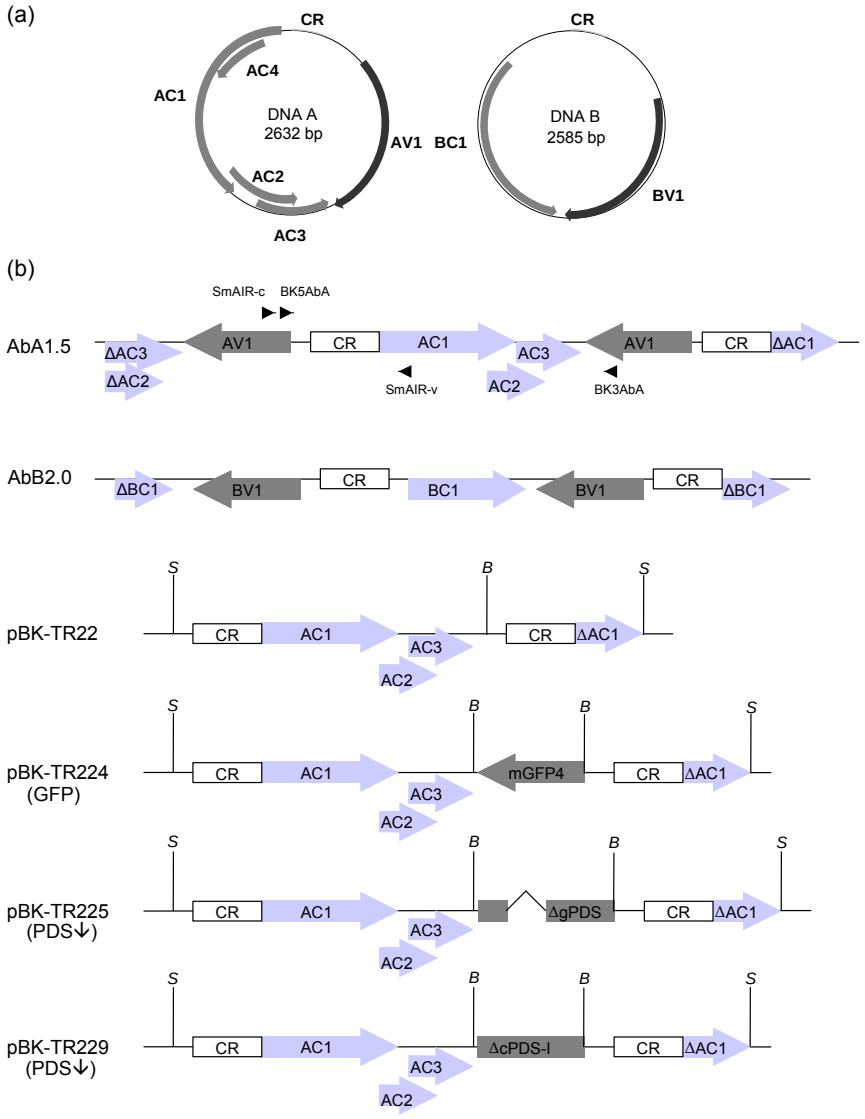

Figure 1. Genome organization of *Abutilon mosaic virus* DNA A and DNA B (a), used and constructed vectors (b).
Arrows indicate open reading frames (AC1: replication-associated protein; AC2: transcriptional activator protein; AC3: replication enhancer; AV1: coat protein; BC1: movement protein; BV1: nuclear shuttle protein and CR: common region). Black arrowheads show position and orientation of used primers (Table 1). pBK-TR22 is a 1.3 bitmer of AbMV DNA A flanked by *Sal* I (S) restriction sites, with an AV1 deletion, an introduced *Bam* HI (B) cloning site. *mGFP4* was inserted into pBK-TR22 and referred to as pBK-TR224. A portion of the NbPDS gene (ΔgPDS, including an intron), was inserted into pBK-TR22, resulting in pBK-TR225. And a 625 bp cDNA fragment of NbPDS (ΔcPDS-I) was inserted to gain pBK-TR229. (GFP) indicates the GFP expression and (PDS↓) PDS silencing vectors.

GFP expression

In order to test, whether the AbMV-based vector is suitable for foreign protein expression, we used the gene of green fluorescent protein (*mGFP4*) (Haseloff *et al.*,

1997). *mGFP4* was inserted into pBK-TR22 revealing pBK-TR224(GFP) (Fig. 1b) by altering the genome size of the replicon only slightly as compared to wildtype AbMV DNA A (2632 vs. 2684bp).

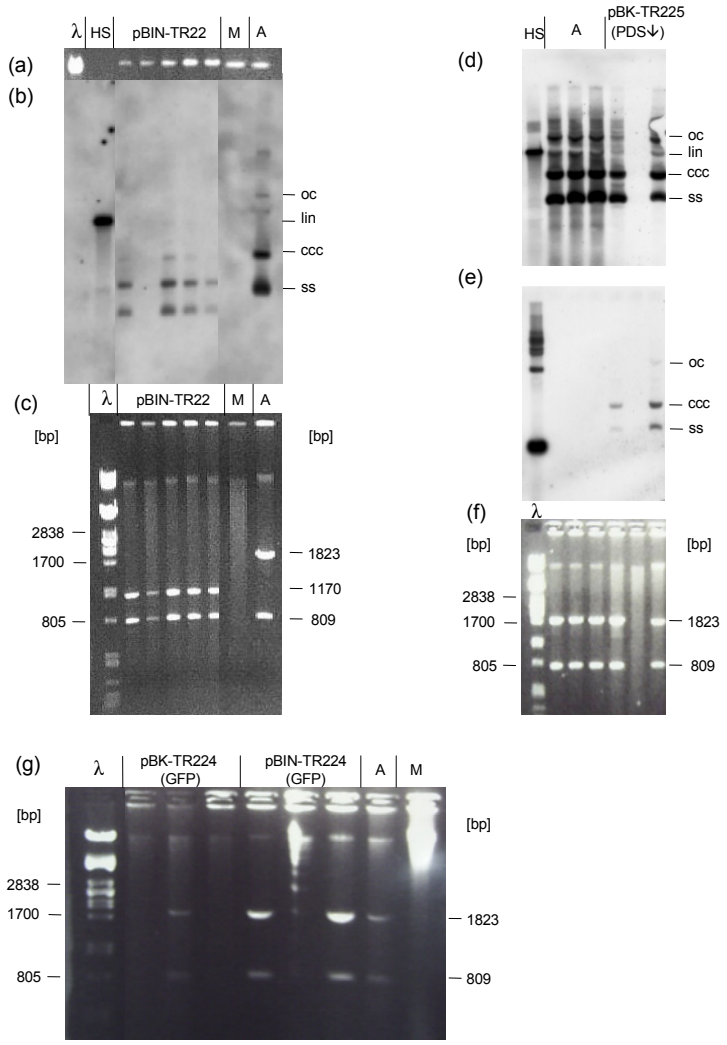

Figure 2. Characterization of replicon DNA induced by pBIN-TR22, -TR224(GFP), pBK-TR224(GFP) and – TR225(PDS↓).
Loading control, ethidium bromide (EtBr) stained host DNA of samples extracted from DNA B plants infected with AbMV DNA A (A) or pBIN-TR22 by agroinoculation 21 dpi (a), Southern blot analysis probed with AbMV DNA A (b). λ indicates *Pst* I digested λ DNA as a molecular marker, HS AbA an 100pg of linearized AbMV DNA A as hybridisation standard and M: mock-inoculated. RCA-RFLP with *Eco* RI restriction enzyme of same samples used in Southern Blot separated in an 1% agarose gel (c).
The DNA progeny of GFP or PDS expressing AbMV vectors are shown in (d-g). DNA B plants infected with AbMV DNA A (A), pBK-TR225(PDS↓) or pBK-TR224(GFP) by biolistic- or pBIN-TR224(GFP) by agroinoculation. Southern blot analysis probed with AbMV DNA A (d) or NbPDS-specific probe (e) and RCA-RFLP using *Eco* RI separated in an 1% agarose gel (f, g). Southern blots were performed with 1% agarose gels and in the presence of EtBr. The positions of open circular (oc), linear (lin), covalently closed-circular (ccc) and single-stranded (ss)DNA forms are indicated. The sizes of the expected fragments and marker are indicated [bp].

Particle bombardment of pBK-TR224(GFP) into DNA B plants showed no local GFP fluorescence in bombarded tissue, no systemic GFP signals in younger leaves, although pBK-TR224(GFP) replicons were detected by Southern blot hybridization (data not shown) and RCA-RFLP (Fig. 2g). These plants displayed less severe symptoms than wildtype AbMV-infected plants and were phenotypically nearly identical to wildtype *N. benthamiana* plants. pBK-TR224(GFP) was bombarded on DNA B plants seven times in total in two independent experiments. Furthermore, three leaves were biolistically inoculated with pBK-TR224(GFP) and two with pBIN-TR224(GFP). No leaf showed GFP signals. Unfortunately, GFP wasn't maintained stably, although the constructs were within the size-limitations that are tolerated without producing size reversion (Gilbertson *et al.*, 2003). The lack of systemic GFP signals are mostly due to recombinants, able to spread and systemically infect *N. benthamiana* plants, but presumably with dysfunctional *mGFP4* ORF.

To analyze the construct under more efficient inoculation conditions, the replicon cassette of pBK-TR224(GFP) was transferred into pBINplus and transformed into agrobacteria. An agroinfiltration assay (Fig. 3f, g) showed bright GFP fluorescence in most cells of infiltrated tissue, at 2 dpi, and GFP signals in individual cells of newly developed, systemically infected younger leaves, at 21 dpi (Fig. 3h-j). GFP signals in systemically infected leaves were exclusively found close to the veins as expected for the phloem-limited AbMV (Horns and Jeske, 1991; Wege *et al.*, 2001). RCA-RFLP confirmed the presence of viral DNA in systemically infected tissue (Fig. 2g). A mean value of at least 114 signals were counted in an area of approximately 5 cm^2 in 3 systemic leaves.

Virus-induced gene silencing (VIGS)

In order to test the suitability of the vector construct for VIGS, a 1014bp partial genomic DNA fragment of the NbPDS gene including an intron, was inserted into pBK-TR22 (pBK-TR225(PDS↓), Fig. 1b). DNA B plants were inoculated by particle bombardment using RCA products of pBK-TR225(PDS↓). At six dpi, inoculated leaves showed single white spots which increased with time until they reached a major vein (Fig. 3b). At 2-3 weeks post inoculation (wpi) newly developed leaves showed photobleached areas, mostly veins and adjacent cells. A nearly complete PDS silencing of whole leaves and stems was observed at 6-8 wpi, leaving some intercostal areas green (Fig. 3c, d). The PDS silencing was maintained during the whole plant lifetime observed.

Systemically infected leaves showing symptoms of PDS silencing were tested for the presence of construct DNA 2-3 wpi using Southern blot hybridization, probing first DNA A and then for the PDS (Fig. 2d, e). AbMV DNA A-inoculated control plants showed the typical band pattern of wildtype infected plants, if probed with DNA A but no signals if probed with PDS, whereas both probes detect the silencing replicon in pBK-TR225(PDS↓)-inoculated plants. An RCA-RFLP showed the expected restriction fragments of 1823 and 809bp (Fig. 2f). The PDS silencing vector pBK-TR225(PDS↓) is capable to infect DNA B plants systemically and to cause systemic PDS silencing (Fig. 3b-d). Interestingly, only size revertants were detected in systemically infected leaves (Fig. 2f). The replicon should comprise 3001 bp, 363 bp more than wildtype AbMV DNA A, but DNA of only 2.6 kbp was observed (Fig. 2f). The RCA products of one of the size revertants was biolistically inoculated on *N. benthamiana* plants and caused the same PDS silencing phenotype as for pBK-TR225(PDS↓)-inoculated plants (data not shown).

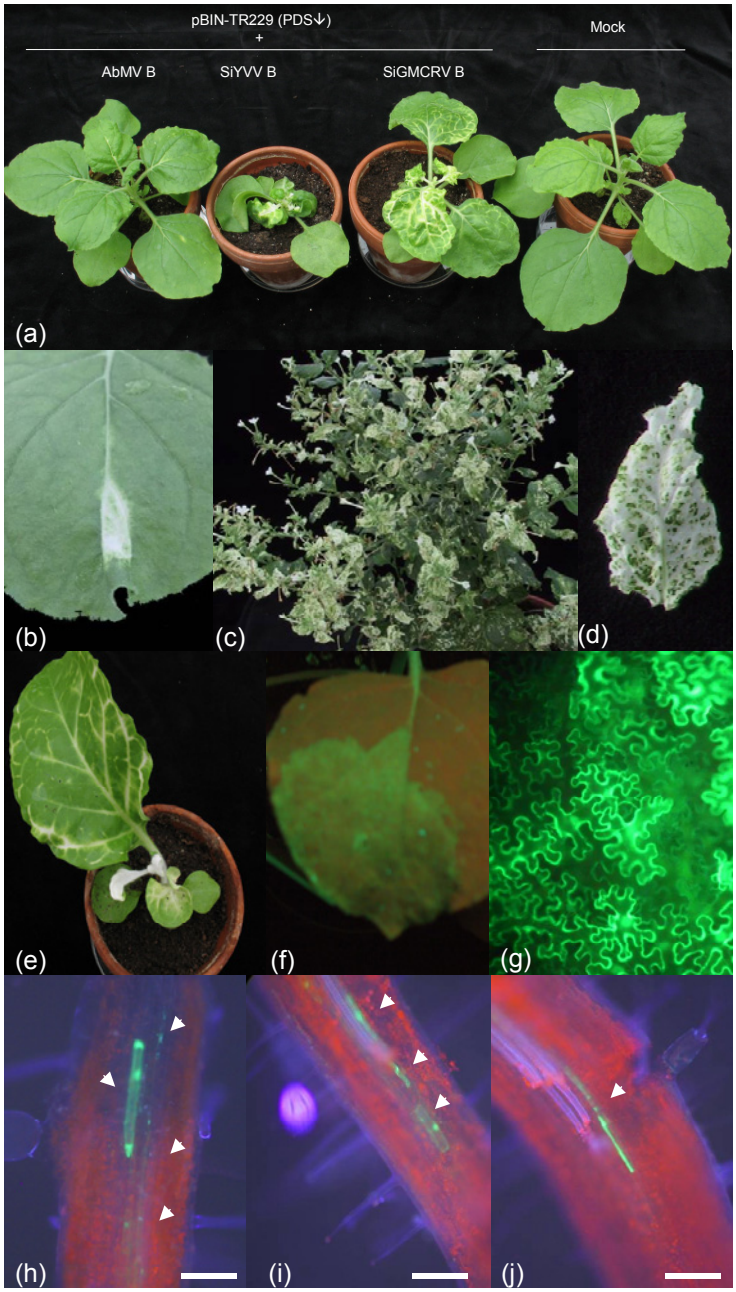

Figure 3. RNA silencing effects on *N. benthamiana* and *N. tabacum* and GFP expression in pBIN-TR224(GFP)-inoculated plants.
N. benthamiana plants infected with pBIN-TR229(PDS↓) by agroinoculation 21 dpi with either the homologous AbMV DNA B (AbB) or in a pseudorecombinant background with DNA Bs of SiYVV or SiGMCRV. Whereas the co-inoculation of pBIN-TR229(PDS↓) and AbB is only able to cause mild PDS silencing in few spots and major veins at that time point, the pseudorecombinant combinations are already able to trigger severe PDS silencing (a). pBK-TR225(PDS↓) bombarded leaf of DNA B plant 20 dpi showing PDS silencing spot (b), whole plant at 8 wpi (c) and systemically infected leaf at 8 wpi (d).
N. tabacum plant infected with pBIN-TR229(PDS↓) and heterologous SiGMCRV DNA B by agroinoculation at 21 dpi. PDS silencing in the beginning of infection is restricted to major veins, but results in PDS silencing of the whole leaf areas of young emerging leaves (e).
pBIN-TR224(GFP)-agroinfiltrated DNA B plant leaf, viewed under UV light at 2 dpi (f). Infiltrated area viewed under fluorescence microscope (g). Razor blade cuttings along major veins from pBIN-TR224(GFP)-infected young plant leaves, viewed under fluorescence. White arrows show GFP expressing cell(s) (h-j).

In order to restore nearly the original genome size of 2632bp, a second AbMV-based PDS silencing vector (pBK-TR229(PDS↓)) was designed by inserting a smaller, 625bp cDNA fragment of NbPDS into pBK-TR22. The bombardment of this construct into DNA B plants resulted in systemic PDS silencing with no differences observed in comparision to the bombardment with pBK-TR225(PDS↓) (data not shown). In addition, both constructs (pBK-TR225(PDS↓), pBK-TR229(PDS↓)) were co-bombarded with an AbMV DNA B -dimer containing vector (Frischmuth *et al.*, 1990) into wildtype *N. benthamiana* plants, and induced the same VIGS-phenotype as described above (data not shown).

After transfer of both bitmer-cassettes into pBINplus (van Engelen *et al.*, 1995), agroinoculation (Klinkenberg *et al.*, 1989) of DNA B plants caused systemic PDS silencing in plants inoculated with pBIN-TR229(PDS↓), but surprisingly not with the larger construct pBIN-TR225(PDS↓). Although infectious after particle bombardment, this construct lost its infectivity during transmission by agrobacteria. Presumably, size restriction is more stringent in this transfer system.

It is possible to combine AbMV DNA A with the related but distinct DNA Bs of *Sida yellow vein virus* (SiYVV) and *Sida golden mosaic Costa Rica virus* (SiGMCRV) to generate pseudorecombinants (Frischmuth *et al.*, 1997; Unseld *et al.*, 2000). pBIN-TR229(PDS↓) was therefore inoculated together with SiYVV DNA B or with SiGMCRV DNA B. Both co-inoculations caused massive PDS silencing in *N. benthamiana* and the onset of PDS silencing occurred earlier, within 6-7 days (Fig. 3a). Due to the increase of infectivity in comparison to AbMV DNA B, systemic PDS silencing was accelerated with the pseudorecombinants, but the silencing phenotype was accompanied by more severe symptoms. Within 6 days major veins of newly

developed leaves showed photobleaching and silencing was achieved after 2 weeks in whole leaf and stem (Fig. 3a).

AbMV-based silencing vector pBIN-TR229(PDS↓) was tested on *N. tabacum* Samsun nn and *Solanum lycopersicum* Moneymaker. Only *N. tabacum* plants showed systemic PDS silencing at 9 dpi (Fig. 3e), although AbMV is able to infect tomato (Wege and Siegmund, 2007). Interestingly, PDS silencing in tomato could be observed at areas of agroinoculation 9 dpi (data not shown), leading to the suggestion that local replication was not compromised, but viral movement. It has to be shown, if the AV1 coat protein is necessary to systemically infect *Solanum lycopersicum* plants or if other factors are sufficient to suppress viral spread. The homology between NbPDS and NtPDS is high enough to cause PDS silencing. The inserted NbPDS fragment in pBIN-TR229(PDS↓), which triggers the PDS silencing, has 98% homology to the NtPDS sequence provided by the Genbank (NCBI Genbank number: AJ616742).

Discussion

To examine the ability of AbMV to function as a silencing and expression vector, the AV1 gene was deleted in the context of an AbMV DNA A bitmer and a series of silencing and expression clones were engineered. The AbMV-based vectors pBK-TR22 and pBIN-TR22, both missing the coat protein gene, and therefore producing replicons with considerably small size compared to wildtype virus replicated and spread in *N. benthamiana* and *N. tabacum* plants. In contrast to other geminiviruses (Bisaro, 1994), the smaller genomic size has not been reversed to wildtype genomic size (Fig. 2b, c) (Gardiner *et al.*, 1988; Gilbertson *et al.*, 2003).

It is consistent to use pBK- and pBIN-TR22 as basic silencing or as expression vectors. Remarkably, symptoms produced with pBK- and pBIN-TR22 were delayed and attenuated compared to those induced by the already mild wildtype AbMV, a desirable property of silencing vectors.

As shown with pBK-TR225(PDS↓) and pBIN-TR229(PDS↓) a nearly complete PDS silencing can be observed 6-8 wpi either via particle bombardment or agrobacteria-mediated inoculation, when a fragment of NbPDS is introduced into pBK-TR22. But surprisingly, pBIN-TR225(PDS↓), which carries the pBK-TR225(PDS↓) replicon in the plant transformation vector pBINplus, failed to cause PDS silencing, if delivered via agroinoculation. The increased size of additional 363 bp compared to wildtype AbMV DNA A size seems to hinder the replicon to spread systemically from cell to cell. The pBIN-TR229(PDS↓) replicon, with only less than 20 bp than the AbMV DNA A genome, showed no restrictions in cell-to-cell movement, if mediated by agrobacteria. Systemic PDS silencing in pBK-TR225(PDS↓)-bombarded plants showed always replicons with size reversion in systemic leaves, suggesting that only size revertants are able to spread properly *in planta* and only these are able to cause

PDS silencing. Gilbertson *et al.* (2003) reasoned a scenario where a wide range of recombinants are produced in the nucleus. Only an optimal size class of recombinants are efficiently trafficked by BC1 from cell to cell, which result(s) in an enrichment of size revertant(s) in adjacent cells, followed by rapid systemic spread of those.

It is more likely that constructs mediated by agrobacteria underlie recombination in that way, that with the chromatin-integrated AbMV multimeric cassette a proper template is always present which gives the recombinants the opportunity to restore using the cellular DNA repair machinery. Whereas vectors mediated by particle bombardment are missing a proper copy to repair. That should presumably be the reason why pBIN-TR225(PDS↓) is able to function if bombarded, but failed by agroinoculation. It is currently under investigation which cellular or viral components are responsible for a size reversion and why replicons released from a DNA A bitmer cassette carried by agrobacteria failed to convert to AbMV DNA A genome size. The problem of size reversion caused by the size limitation of cell-to-cell movement is a disadvantage when it comes to systemic protein expression by AbMV-based vectors, because no ORF larger than 1000 bp could be expressed. But size reversion is a major advantage of AbMV as a silencing vector, because fragments of any size can be inserted into pBK-TR22, bombarded into *N. benthamiana* plants to gain target silencing after a few weeks. Our results with the recombinant AbMV-PDS vectors indicated that the AbMV-based VIGS vector induced efficient and reliable gene silencing in *N. benthamiana* and *N. tabacum*.

Plant virus-based vectors for expressing heterologous proteins in plants present promising tools to support conventional breeding and transgenic technology. DNA viruses have not been extensively used as expression vectors due to the size constraints (Palmer and Rybicki, 2001). However, a nonmobile maize streak virus-

derived vector has been successfully used for long-term production of protein in maize cell cultures (Palmer *et al.*, 1999). This study demonstrates for the first time that an AbMV-based vector is suitable for an efficient expression of a foreign protein in *N. benthamiana*. Under the control of the AV1 promoter, strong GFP signals were observed in infiltrated areas, if inoculated with agrobacteria (Fig. 3f, g). GFP signals were also observed in systemically infected cells (Fig. 3h-j), allowing to monitor virus movement and studying tissue tropism. It is also thinkable to combine the GFP expression vector within a VIGS assay, in knock-out or knock-down plants to assess involvement of host genes in viral replication, movement or resistance.

Inoculating DNA B plants combine demands of high security and simplicity, because only one non-infectious vector element is necessary to induce silencing. The vector is also not transmissible by insects (Höfer *et al.*, 1997; Höhnle *et al.*, 2001). Without nearly any side effect of viral symptoms silencing phenotypes can be easily observed. But in combination with DNA Bs of SiYVV or SiGMCRV systemic silencing can be accelerated accepting the disadvantage of more severe symptoms.

N. benthamiana plants were successfully inoculated with RCA products of plasmids or of viral replicons from plants which allow a cell-free construction of silencing vectors and direct sequencing.

pBK-TR22 will be improved with a more convenient multi cloning site, the possibility to tag expressed proteins with an HA-epitope, i.e., and to generated GFP fusion proteins, despite the limited size of systemic protein expression. It is reasonable to screen cDNA- or genomic libraries with the aid of AbMV vectors for functional genomics.

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Manuskript 2:

Virus-induced gene silencing of chloroplast-localized heat shock protein results in reduced amount of viral ssDNA and photo-bleached phenotype

Abstract

The C-terminal portion of *Arabidopisis thaliana* cpHSC70-1 was found to interact with the N-terminal region of *Abutilon mosaic virus* (AbMV) movement protein (MP; BC1) in a yeast two hybrid assay (Dr. T. Kleinow, pers. communication). Therefore a geminivirus-based gene silencing vector harboring the AtcpHsc70-fragment was constructed to silence the nuclear encoded and chloroplast-localized heat shock protein of 70 kDa (cpHSC70) in *Nicotiana benthamiana* plants. Systemically infected leaves showed punctated photo-bleached areas similar to those induced by phytoene desaturase (PDS) silencing indicating an interference with chloroplast stability. CpHsc70-silenced plants accumulated less viral DNA, in particular single-stranded DNA (ssDNA), than PDS-silenced or AbMV-infected plants. CpHsc70 is an essential component of chloroplast stability and can be used as a novel silencing marker besides PDS. An involvement of cpHSC70 in geminiviral movement is discussed.

Keywords: geminivirus, virus-induced gene silencing, chloroplast, heat shock protein, movement

Introduction

Members of the heat shock protein 70 (HSP70) family have been found in almost all organisms, from archaebacteria to eubacteria and eukaryotes, and represent one of the most conserved protein families known to date (Boorstein *et al.*, 1994). In eukaryotes, multiple members of the HSP70 family are known. Partial genomic sequences for three cytosolic members of the *Arabidopsis* hsp70 gene family were first described nearly 20 years ago (Wu *et al.*, 1988). With the completion of genome sequencing, 12 full-length *Arabidopsis* hsp70 genes were identified, five encoding cytosolic, three endoplasmic reticulum luminal, and two each plastidal- or mitochondrial proteins. HSP70 isoforms in higher plant chloroplasts, are located either in the chloroplast outer envelope (Ko *et al.*, 1992; Schnell *et al.*, 1994) or in the stroma (Marshall and Keegstra, 1992). The stromal HSP70s are more closely related to the eubacterial and mitochondrial proteins, whereas the envelope-associated HSP70s exhibit higher similarity to eukaryotic HSP70s. The best-documented function of plastidal HSP70s is the import of nuclear-encoded polypeptides into plastids (Schroda *et al*., 1999).

A subclass of cytoplasmic *Cucurbita maxima* heat shock cognate 70 (CmHsc70) chaperones was shown to interact with the plasmodesmal non-cell-autonomous translocation pathway (Aoki *et al.*, 2002). Microinjection experiments demonstrated that both CmHsc70-1 and CmHsc70-2 were interacting with plasmodesmata, increasing the size exclusion limit and mediating their own cell-to-cell transport.

A role of heat shock proteins for viral movement was proposed, because Closteroviruses encode a protein with homology to the HSP superfamily, termed Hsp70h, i.e. p65 of the *Beet yellows closterovirus*. Hsp70h played a direct role in the formation of the viral movement-complex (Agranovsky *et al.*, 1998; Alzhanova *et al.*,

2000; Alzhanova *et al.*, 2001; Peremyslov *et al.*, 1999). In plant tissues, the cell-to-cell spread of viral infection occurs via plasmodesmata, and, consistent with the utilization of this pathway, Hsp70h has been immunolocalized to these intercellular channels (Medina *et al.*, 1999; Prokhnevsky *et al.*, 2005; Prokhnevsky *et al.*, 2002).

Movement of geminiviruses within host plants has been studied extensively (Gafni and Epel, 2002; Gilbertson *et al.*, 2003; Lazarowitz and Beachy, 1999; Waigmann *et al.*, 2004). Geminiviruses replicate in cell nuclei and, consequently, have to cross two borders during spread, the nuclear envelope and the plasma membrane. In the case of bipartite begomoviruses, genes required for systemic spread are located on DNA B. ORF BV1 (syn. BR1) encodes for the nuclear shuttle protein (NSP) and ORF BC1 (syn. BL1) for the movement protein (MP). MP mediates cell-to-cell transport, whereas NSP is responsible for the nuclear import and export of viral DNA from the nucleus. A “relay race model” of transport was proposed in which NSP transfers viral dsDNA from the nucleus to the cytoplasm, from which it is delivered to MP for plasmodesmata crossing. Gilbertson *et al*. (2003) suggested DNA size selection during cell-to-cell transport. An alternative “couple-skating model” has been proposed for *Abutilon mosaic virus* (AbMV) in which viral DNA is shuttled between the nucleus and the cytoplasm in an NSP-containing complex, which then cooperates with MP to move the NSP/DNA complex from cell to cell. The NSP of AbMV, a strictly phloem-limited bipartite geminivirus in Abutilon (Abouzid *et al.*, 1988; Horns and Jeske, 1991; Jeske *et al.*, 1977) as well as in the experimental host *N. benthamiana* (Wege *et al.*, 2001), has been detected inside nuclei by immuno-histochemical staining and GFP tagging (Zhang *et al.*, 2002). AbMV BC1 alone never moved to neighbouring cells, but did so upon co-bombardment with BV1-encoding plasmids. BC1 mobilized BV1 from the nucleus to the cell periphery and into the next cell in a subpopulation of competent cells exclusively in host sink leaves. In these particular cells, BC1 entered

the neighbouring cells, irrespective of whether it was fused to GFP at its N- or C-terminus. If formation of the movement complex alone is sufficient to transport itself through plasmodesmata or if host factor(s) is (are) required is still a question.

In a yeast two-hybrid screen (Fields and Song, 1989) of an *Arabidopsis* cDNA library the N-terminus of AbMV BC1 was found to bind the protein encoded by the 748 bp C-terminal fragment of *Arabidopsis thaliana* cpHsc70-1 (AL078637, At4g24280), which is a nuclear encoded but chloroplast-localized heat shock protein of 70 kDa (T. Kleinow, personal communication).

To investigate a presumable function of cpHsc70 in geminiviral movement, we have silenced cpHsc70 by virus-induced gene silencing (VIGS) using AbMV in *N. benthamiana* (Krenz *et al*., 2007a). VIGS, a type of RNA interference, is initiated by viral vectors carrying fragments of host genes to knock down gene expression in plants by degrading the homologous transcripts (Dinesh-Kumar *et al.*, 2003).

Material and Methods

Microorganisms and plants

Virus strains used in this work were agroinfectious clones of *Abutilon mosaic virus* (AbMV; X15983, X15984) (Frischmuth *et al.*, 1990), DNA B of Sida golden mosaic Costa Rica virus (SiGMCRV; X99551) (Frischmuth *et al.*, 1997; Unseld *et al.*, 2000) and pBIN-TR229 (Krenz *et al*., 2007a).

Escherichia coli strain DH5α was used for subcloning. *Nicotiana benthamiana* Domin were grown in an insect-free S2 greenhouse with supplementary lighting (Wege *et al.*, 2001).

Cloning procedures

Recombinant binary plasmid pBIN-TR227 (Fig. 1a) was introduced into *A. tumefaciens* GV3101 (Koncz *et al.*, 1994) by chemical transformation. PCR analysis was performed to check construct integrity.

Recombinant DNA techniques were performed according to Sambrook and Russell (2001). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

DNA sequences were determined using Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech, now GE Healthcare) with universal IRD800 labeled forward primer and reverse primer (Table 1). Sequence reactions were analyzed on a LI-COR sequencer (MWG-Biotech, Germany). Sequence alignment was done with Vector NTI program (Invitrogen).

VIGS constructs for agroinoculation

The 748 bp C-terminal fragment of *Arabidopsis thaliana* cpHsc70-1 (AL078637, At4g24280) of *A. thaliana* cDNA library was released by *Bam* HI and *Bgl* II restriction digestion and inserted into *Bam* HI linearized pBK-TR22 (Krenz *et al*., 2007a), to generate pBK-TR227.

pBIN-TR227 was constructed by releasing the AbMV DNA A ΔAV1: ΔcpHsc70-bitmer cassette from the pBK-TR227 using *Sal* I and inserting it into single *Sal* I site of pBINplus (van Engelen *et al.*, 1995) (Fig. 1a).

Rolling circle amplification - Restriction fragment length polymorphism (RCA-RFLP)

RCA was performed at 28 or 30 °C for at least 16 h using the TempliPhi DNA amplification kit (GE Healthcare, formerly Amersham Bioscience) following manufacturer's instructions as described (Haible *et al.*, 2006) using plasmid or viral DNAs as templates. The reaction was stopped at 65 °C for 10 min, and RCA products were analyzed by RFLP.

Inoculation of plants

Transgenic AbMV DNA B *N. benthamiana* ("DNA B") plants (Pohl and Wege, unpublished) were inoculated with agroinfectious clones (Klinkenberg *et al.*, 1989).

Analysis of viral DNAs

Total cellular nucleic acids were extracted from systemically infected plant tissues with extraction buffer (100 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM EDTA, 1%

SDS, 100 mM DTT). Proteins were removed by phenol-chloroform (1:1) treatment, and ethanol precipitated nucleic acids were dissolved in 100 µl sterile water. From each plant, 100 ng total DNA were separated on agarose gels using 1× TBE buffer, transferred to Hybond NX membranes (Amersham) (Wege and Siegmund, 2007). Viral DNAs were detected using digoxigenin-labeled probes (Roche, Mannheim, DIG-High Prime Kit). DNA amounts were quantified using 4',6-diamidino-2-phenylindole (DAPI) (Tanious *et al.*, 1992) with the fluorescence normalized against a standard DNA of known concentration. For densitometric analysis the Sigma ScanPro software (Systat Software GmbH, Erkrath, Germany) was used and pixel intensities were plotted against pixel distance.

Tissue print blots of pBK-TR227-, AbMV- and mock-inoculated DNA B plants were prepared on nylon membrane. Fresh sections of stems, cut with a sterile razor blade, were gently printed onto the membrane, which was then air-dried prior to detection procedure described above.

For RCA-RFLP (10 ng) total nucleic acids from systemically infected plant tissues served as template.

Microscopy

Plants were investigated for DAPI and chlorophyll fluorescence using Axiophot fluorescence microscope (ZEISS, Oberkochen, DE; filter: G 365; FT 395; LP 420). Images were processed using Adobe Photoshop.

Reverse Transcription – PCR (RT-PCR) and small interfering RNA (siRNA) isolation and detection

For RT-PCR, total plant RNA was extracted from photo-bleached areas of young leaves in the case of pBIN-TR227 and –TR229-inoculated DNA B plants and from young leaves in the case of AbMV- and mock-inoculated DNA B plants (100 mg). Leaves were ground in liquid nitrogen and extracted by TRIZOL reagent (Invitrogen) following the manufacturer's protocol. 2 µg RNA was used as template for first-strand cDNA synthesis using d(T)18-primer and supercript reverse transcriptase (Invitrogen). Primers (Table 1) that anneal the region targeted for silencing were used to ensure that endogenous gene transcripts were assayed. The *N. benthamiana* gene for ribulose 1,5-bisphosphate carboxylase small subunit (RbcS; X02353) served as an internal control. PCR products were examined by electrophoresis in 1% agarose gel, inserted into pGEM-Teasy (Promega) and sequenced.

For siRNA isolation and detection, total RNA was extracted from 100 mg plant tissue using a TRIZOL reagent (Invitrogen) according to the manufacturer's protocol and dissolved in 15 µl loading buffer (95% formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 70 °C for 5 min, and separated on 15% polyacrylamide mini-gel (acrylamide:bis-acrylamide 19:1, 7 M urea) at 70 V for 4 h. RNA was transferred to Hybond NX membrane by electroblotting in 0.5x TBE buffer for 1 h (Semy-dry-Blotting-Apparatus, BioRAD). Hybridization was performed according to Papefthimiou *et al.* (2001) using digoxigenin-labeled RNAs (DIG-RNA labeling kit, Roche, Germany) as probe. The blot was washed twice with 2x SSC, 0.5% SDS for 30 min at 58°C. Chemiluminescent probe detection via disodium 3-(4-methoxyspiro{1,2-dioxethane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenylphosphate (CSPD, Roche

Diagnostics) followed the manufacturer's protocols (DIG Application Manual for Filter Hybridization; Roche).

Table 1
Primers used for amplification and sequencing of NbcpHsc70-1 and NbRbcS.
[a] pGEM-T (Promega), NbRbcS (X02353) NbHsp70cp-1 (AB181295).
[b] Sequencing primer, DY-781, or IDR-800 labelled.

Primer name	Sequence 5`→ 3`	Location [a]
forward primer [b]	CGCCAGGGTTTTCCCAGTCACGAC	pGEM-T nt 1 – 24
reverse primer [b]	AGCGGATAACAATTTCACACAGGA	pGEM-T nt 240 – 263
NbRbcS for	CCTCTGCAGCAGTTGCCACC	NbRbcS nt 1067 - 1086
NbRbcS rev	CCTGTGGGTATGCCTTCTTC	NbRbcS nt 1882 - 1901
NbHsp70cp-1for	GTGGAGTCATGACCAAAATTATCCCAAG	NbHsp70cp-1 nt 1- 26
NbHsp70cp-1rev	GAAGTCTGCATCGATAACTTCTCCATC	NbHsp70cp-1 nt 664 - 690

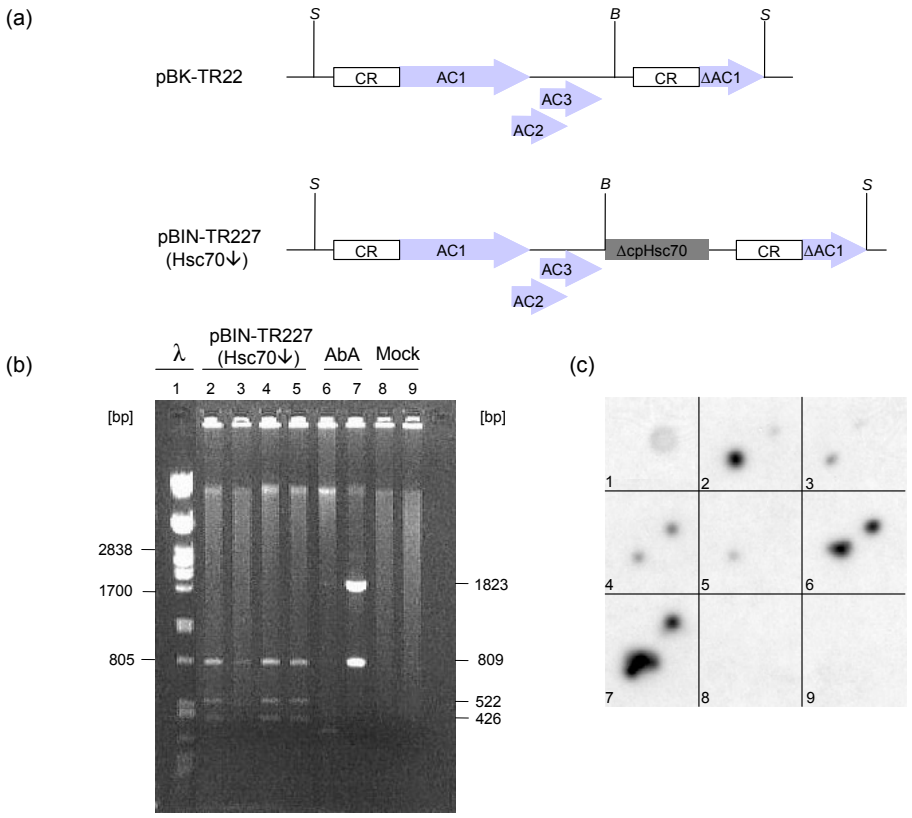


Figure 1. Recombinant virus constructs and their infection potential. pBK-TR22 is a partial dimer of AbMV DNA A flanked by *Sal* I restriction sites (S), in which AV1 is deleted and an additional *Bam* HI cloning site (B) introduced. A 748 bp fragment of *Arabidopsis thaliana* cpHsc70-1 released by *Bam* HI and *Bgl* II was inserted into *Bam* HI linearized pBK-TR22 to generate pBK-TR227. The resulting replicon is 103 bp larger than AbMV wildtype DNA A. This cassette was transferred into pBINplus and named pBIN-TR227, respectively (a). Assigned gene functions of indicated open reading frames: AC1: replication-associated protein, Rep; AC2: transcriptional activator protein, TrAP; AC3: replication enhancer, Ren; AV1: coat protein, CP; CR: common region.
Samples extracted from DNA B plants infected with AbMV DNA A (AbA) or pBIN-TR227 by agroinoculation were analyzed at 21 dpi by RCA-RFLP using *Eco* RI separated on an 1% agarose gel, stained with ethidium bromide (EtBr) (b). λ indicates *Pst* I-digested Lambda DNA as a molecular marker. AbMV DNA A is digested into two fragment of 1823 and 809 bp and the cpHsc70-silencing replicon into four fragments of 809, 522, 426 and 117 bp (Fig. 1b, lowest band of 117 bp is not visible). AbMV DNA B is unaffected by *Eco RI* enzyme. Tissue blot analysis probed with AbMV DNA A (c). (1) 10 pg of linearized AbMV DNA A as hybridization standard, (2-5) DNA of pBIN-TR227-inoculated DNA B plants, (6,7) DNA of AbMV DNA A-inoculated AbMV DNA B plants and (8,9) DNA of mock-inoculated plants. (Hsc70↓) indicates cpHsc70-silencing.

Results

Plant inoculation with pBIN-TR227 (Hsc70↓)

pBIN-TR227(Hsc70↓)-infected DNA B plants systemically. All four inoculated plants showed the expected viral band pattern upon RCA-RFLP using *Eco* RI at 21 days

post inoculation (dpi) (Fig. 1b). Tissue blot analysis (Fig. 1c, 2-5), probed with AbMV DNA A, confirmed these results. This experiment was repeated with three DNA B plants with the same result.

pBIN-TR227-inoculated were indistinguishable from mock-inoculated DNA B plants at 21 dpi, except that single white spots in major veins of newly developed leaves appeared in the infected plants (Fig. 2, white arrows). Fluorescence microscopy of the white spots showed that DAPI staining revealed nuclei fluorescence as in green tissue (data not shown), whereas chorophyll autofluorescence was considerably decreased (Fig. 3a, b).

Figure 2. pBIN-TR227 (Hsc70↓)-, pBIN-TR229 (PDS↓)-, AbA- and mock-infected leaves of DNA B plants 60 dpi showing the difference of cpHSC70 or PDS silencing. White arrows indicate single white spots in major veins. (Hsc70↓) indicates cpHsc70 silencing and (PDS↓) PDS silencing.

To determine whether the bleaching phenotype was caused by reduced cpHsc70 expression, a semi-quantitative RT-PCR was performed using gene-specific primers that amplify the C-terminal fragment of the NbcpHsc70 gene. NbcpHsc70-specific mRNA was detected in mock-, pBIN-TR229(PDS↓)- (an AbMV-based PDS silencing vector carrying a cDNA fragment of *N. benthamiana* phytoene desaturase (NbPDS)) (Krenz *et al*., 2007a), and AbMV DNA A-infected DNA B plants, but it was absent from pBIN-TR227(Hsc70↓)-infected DNA B plants (Fig. 3c). Complementary, pBIN-

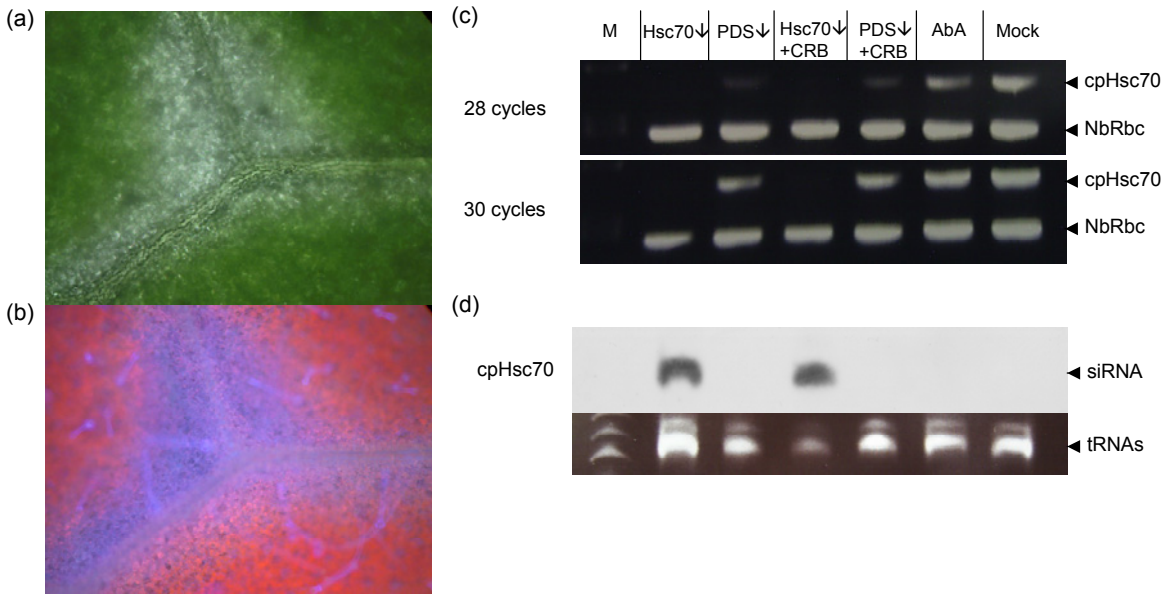


Figure 3. A systemically infected leaf area of a DNA B plant inoculated with pBIN-TR227 (Hsc70↓) (60 dpi) viewed under brightfield (a) or UV light(b). Multiplex semi-quantitative RT-PCR of NbcpHsc70 and NbRbcS cDNA after 28 and 30 cycles was separated on an 1% agarose gel and stained with EtBr (c). Northern analysis to detect siRNAs. Samples were hybridized with a cpHsc70-specific probe (d). Lane M, 50bp ladder; lane Hsc70↓, pBIN-TR227(Hsc70↓)-inoculated DNA B plant, lane PDS↓, pBIN-TR229(PDS↓)-inoculated DNA B plant; lane Hsc70↓+CRB, pBIN-TR227(Hsc70↓)- and SiGMCRV DNA B-inoculated nontransgenic *N. benthamiana* plant; lane PDS↓+CRB, pBIN-TR229(PDS↓)- and SiGMCRV DNA B-inoculated nontransgenic *N. benthamiana* plant; lane 5, AbMV-infected; and lane 6, mock-inoculated DNA B plant. EtBr-stained tRNA bands are shown as loading control.

TR227(Hsc70↓)-infected plants showed siRNAs specific for cpHsc70 which were absent from mock-, pBIN-TR229(PDS↓) and AbMV DNA A-infected DNA B plants (Fig. 3d). These results suggest that VIGS of Hsp70 expression has interferred with chloroplasts stability in plant cells adjacent to veins (Fig. 2, 3a and b).

In order to investigate why cpHsc70 silencing was restricted to spots instead of spreading to adjacent tissues, six DNA B plants were each inoculated with either pBIN-TR227(Hsc70↓), -TR229(PDS↓), or AbA. At 60 dpi, a nearly complete photo-bleaching phenotype of whole leaves and stems was observed for five of six DNA B

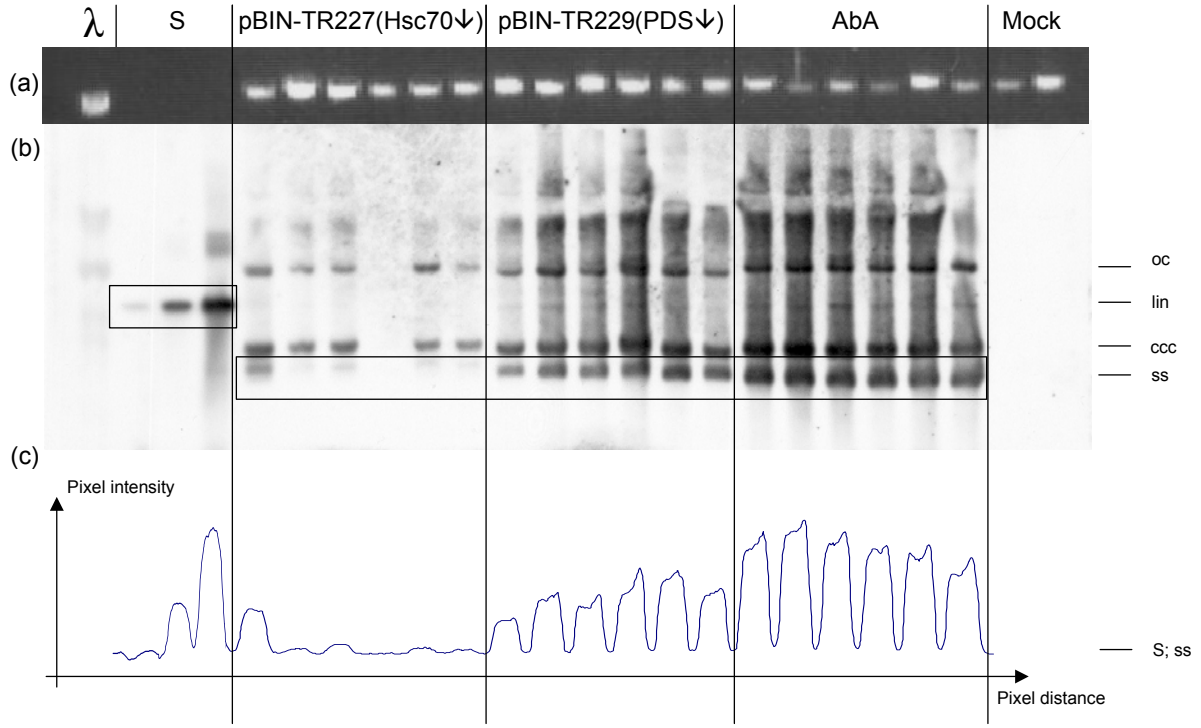


Figure 4. Six independent samples each extracted from DNA B plants inoculated with pBIN-TR227(Hsc70↓), -TR229(PDS↓), or AbMV DNA A (AbA) were separated on an 1% agarose gel and stained with EtBr to compare genomic plant DNA as loading control (a). Southern blot analysis of same gel probed with AbMV DNA A (b). Horizontal densitometric analysis of pixel intensities of hybridization standard S of 1, 10 and 100 pg of linearized AbMV DNA A, or all ssDNA bands plotted against pixel distance using Sigma ScanPro software (c). λ indicates *Pst* I-digested Lambda DNA as a molecular marker and S AbA 1, 10 and 100 pg of linearized AbMV DNA A as hybridization standard. The position of open circular (oc), linear (lin), covalently closed-circular (ccc) and single-stranded (ss)DNA forms are indicated.

plants which were inoculated with pBIN-TR229(PDS↓) (Krenz *et al*., 2006a). pBIN-TR227(Hsc70↓)-inoculated DNA B plants, however, only showed bleached spots, except of one plant. The white spots did not increase as observed for PDS silenced DNA B plants, during the course of the experiment (Fig. 2).

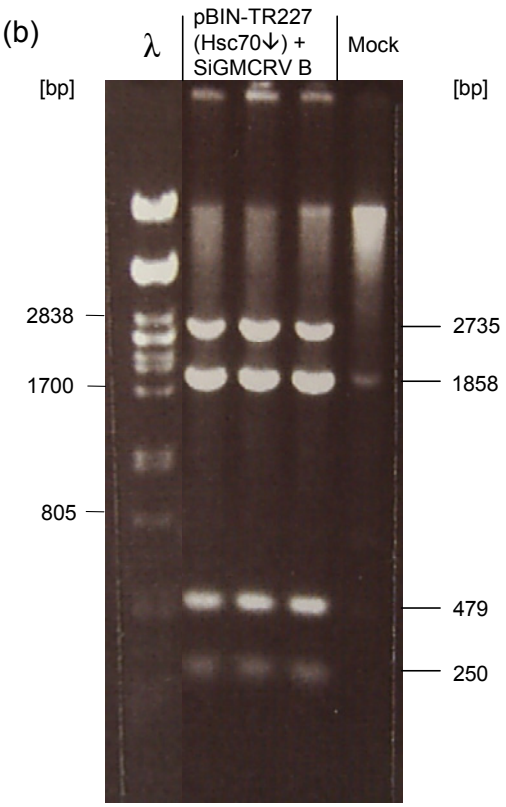
A semi-quantitative Southern blot analysis of nucleic acids of these plants revealed a reduction of viral DNA in the systemic infected leaves of all pBIN-TR227(Hsc70↓) as

compared to that of pBIN-TR229(PDS↓)- or AbA-infected DNA B plants (Fig. 4b). The amounts of viral single-stranded DNA (ssDNA) were significantly less in four of five pBIN-TR227(Hsc70↓)-infected DNA B plants compared to open-circular and covalently closed-circular DNAs (ocDNA, cccDNA) (Fig. 4b). A densitometric analysis (Pilartz and Jeske, 2003) confirmed reduction in ssDNA accumulation in pBIN-TR227(Hsc70↓)-infected leaves (Fig. 4c). This experiment was repeated with three pBIN-TR227(Hsc70↓)-agroinoculated DNA B plants with the same result.

It is possible to combine AbMV DNA A with the related but distinct DNA B of *Sida golden mosaic Costa Rica virus* (SiGMCRV B; X99551) (Frischmuth *et al.*, 1997; Unseld *et al.*, 2000) to generate pseudorecombinants. pBIN-TR227 was therefore inoculated together with SiGMCRV DNA B. Co-inoculations caused massive cpHsc70 silencing in DNA B plants and the onset of cpHsc70 silencing occurred earlier than with AbMV DNA B, within 6-7 days. Due to the increase of infectivity in comparison to AbMV DNA B, systemic cpHsc70 silencing was accelerated with the pseudorecombinants, but the silencing phenotype interfered with more severe symptoms. Within 6 days, major veins of newly developed leaves showed photobleaching and silencing was achieved after 2 weeks in whole leaves and stems (Fig. 5a). pBIN-TR227(Hsc70↓) together with SiGMCRV DNA B was able to systemically infect *N. benthamiana* plants. At 20 dpi, all three plants showed the expected viral band pattern upon RCA-RFLP analysis with *Bam* HI, by which the cpHsc70-silencing replicon was linearized (2735 bp) and SiGMCRV DNA B was cut three times (Fig. 5b). pBIN-TR227(Hsc70↓)-infected plants showed siRNAs specific for cpHsc70 whereas they were absent in mock-, pBIN-TR229(PDS↓)- and AbA-infected *N. benthamiana* plants (Fig. 3c, Hsc70↓). Southern blot analysis of nucleic acids of *N. benthamiana* plants revealed reduction of viral ssDNA in all pBIN-TR227(Hsc70↓) (Fig. 5c).

(a)

(b)



(c)

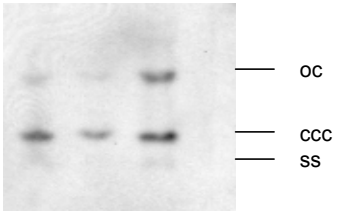


Figure 5. Systemically infected leaf of a wildtype *N. benthamiana* plant inoculated with pBIN-TR227(Hsc70↓) and SiGMCRV DNA B at 20 dpi (a). Three independent samples were analyzed at 20 dpi by RCA-RFLP using *Bam* HI (b). Products were separated on an 1% agarose gel and stained with EtBr. The cpHsc70-silencing replicon was linearized (2735 bp) and SiGMCRV DNA B was cut three times into fragments of 1858, 479 and 250 bp; λ indicates *Pst* I digsted Lambda DNA as a molecular marker. Southern blot analysis probed with AbMV DNA A (c). The position of open circular (oc), covalently closed-circular (ccc) and single-stranded (ss)DNA forms are indicated.

Discussion

The N-terminal part of AbMV movement protein BC1 was found to interact with the C-terminal portion of *A. thaliana* cpHsc70-1 gene in a yeast two hybrid screen (Dr. T. Kleinow, pers. communication). To examine the function of cpHsc70 in geminiviral movement, we used VIGS as a reverse genetic approach to silence cpHsc70. An AbMV-based cpHsc70 silencing vector was constructed, which infected DNA B plants systemically (Fig. 1b and c) and caused a photo-bleaching phenotype that was restricted to spots or short stripes along the major veins. This result confirmed that the *A. thaliana* cpHsc70-1 gene fragment inserted into an AbMV-based silencing vector was able to silence the heterologous NbcpHsc70 gene (Fig. 2 and 3). Similar to PDS (Brigneti *et al.*, 2004; Kumagai *et al.*, 1995; Ruiz *et al.*, 1998; Tao and Zhou, 2004) and FtsH (Saitoh and Terauchi, 2002) silencing, cpHsc70 silencing led to chloroplast degradation indicating an essential role of cpHSC70 in chloroplast stability. CpHsc70 can therefore be used as a silencing marker, like PDS, but with an effect restricted to the veins. To date, only one sequence of chloroplast-localized Hsc70 is known for *N. benthamiana* whether other members of this subclass are affected by pBIN-TR227(Hsc70↓) cannot be excluded, but it is improbable that cytoplasmatic Hsc70-1 was targeted, because Kanzaki *et al*. (2003) observed stunting for Hsc70-1-silenced *N. benthamiana* plants, which was absent for cpHsc70-silenced *N. benthamiana* plants in this study (Kanzaki *et al.*, 2003).

The cpHsc70-silenced plants accumulated less viral DNA, especially ssDNA, than PDS-silenced, or AbMV-infected plants (Fig. 4, 5). A general reduction in ssDNA is observed in geminivirus coat protein mutants (Unseld *et al.*, 2004) due to either enhanced instability of unencapsidated ssDNA in vivo or during extraction, or some regulatory effect of the coat protein on the switch from viral dsDNA replication to

ssDNA replication (Stanley and Townsend, 1986). ssDNA is even more decreased in cpHsc70-silenced DNA B plants that may indicate an involvement of cpHsc70 in geminiviral intracellular movement, because plastidic HSP70 functions in the import of nuclear-encoded polypeptides. Gröning *et al*. (1987, 1990) isolated DNA from intact plastids of AbMV-infected and noninfected plants. Plastids from infected plants were shown to contain single-stranded AbMV DNA (Gröning *et al.*, 1987; Gröning *et al.*, 1990). It is possible that cpHSC70 interacts with AbMV movement complex and mediates transport through membranes into the plastid lumen.

pBIN-TR227(Hsc70↓) was also able to silence cpHsc70 in *N. benthamiana* plants, if co-inoculated with related DNA B of SiGMCRV (Fig. 5). Silencing phenotype was not restricted to spots of major veins, whole leaves and stems were bleached, but accumulation of ssDNA was also impaired. Presumably, movement of pseudorecominants with SiGMCRV DNA B is not affected by cpHsc70-silencing indicating an interaction of AbMV BC1 with chloroplast-localized heat shock protein of *N. benthamiana* plants. Interference with replication can also be ruled out, because accumlation of dsDNA intermediates were not altered.

It is rather speculative whether cpHSC70 also mediates cell-to-cell transport through plasmodesmata but it is possible that other members of the HSC70 family function in geminiviral movement. A subclass of cytoplasmic *Cucurbita maxima* heat shock cognate 70 (CmHsc70) chaperones had the capacity to interact with plasmodesmata, increased the size exclusion limit, and mediated their own cell-to-cell transport (Aoki *et al.*, 2002). This subclass was isolated from pumpkin phloem sap. A further hint for the relevance of heat shock proteins for viral movement was found for *Closteroviridae*. They facilitate their cell-to-cell movement with a viral protein displaying homology to the HSP70 family. However, no equivalent portion could be detected in CmHsc70-1 and -2. Interestingly, viral Hsp70 chaperones are more

similar to the endoplasmic reticulum-associated Bip chaperone than to the cytosolic Hsc70 subfamily (Karasev, 2000). Geminiviruses do not encode a protein displaying homology to the HSP70 family, like *Closteroviridae*, and may have, therefore, utilized host factors.

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Manuskript 3:

The complex role of *Abutilon mosaic virus* (AbMV) AC2 in remodeling of viral and transreplicon minichromosomes

Abstract

An *Abutilon mosaic virus* (AbMV)-based transreplicon was constructed to monitor infection and to identify plant tissues specifity. A green fluorescent protein (GFP) expression cassette driven by the constitutive 35S promoter of *Cauliflower mosaic virus* (CaMV) was embedded in a truncated AbMV DNA A partial dimer and transferred to *Nicotiana benthamiana* plant as a transgene. Upon AbMV infection, the transreplicon was released and resulted in GFP overexpression. Agroinfiltration of these transgenic plants with a construct that expressed the AbMV replication-associated protein Rep (or AC1) alone showed, that Rep is necessary and sufficient to induce bright GFP fluorescence in the infiltrated area. Co-expression of AbMV Rep (AC1) and TrAP (AC2), a protein that has been identified as transcriptional transactivator as well as silencing suppressor protein for other begomoviruses, surprisingly suppressed transgene transreplication. This effect however, was neutralized by the strong p19 silencing suppressor of the unrelated, RNA-containing *Cymbidium ringspot virus* (CymRSV) suggesting an involvement of siRNA. In order to investigate whether TrAP has an influence on the viral chromatin condensation as a response to silencing, the distribution of topoisomers of monomeric viral circular double-stranded DNA at different stages of infection were visualized. Transreplicon and viral minichromosomes were found to exist in structures dependent on Rep and/or TrAP expression. The topoisomers distribution pattern confirmed the influence

of AbMV TrAP in altering viral chromatin conformation, whereas suppression of transcriptional gene silencing (TGS) can be excluded.

Keywords: geminivirus, transreplicon, transcriptional transactivator, AC2, silencing suppressor, transcriptional gene silencing, rolling circle amplification, topoisomer

Introduction

The family *Geminiviridae* comprises four genera: Mastrevirus, Curtovirus, Begomovirus and Topocuvirus, divided according to their genome organization and transmission vector (Stanley *et al.*, 2005). Begomoviruses are serious plant pathogens infecting dicotyledonous plants, among them important crop plants, but they induce also ornamental mosaic without harming the plant, like *Abutilon mosaic virus* (AbMV). Geminiviruses are characterized by small geminate particles containing single-stranded circular DNA molecules (Böttcher *et al.*, 2004). The AbMV genome is divided into two components defined as DNA A and DNA B, and both are required for proper infectivity in plants. Proteins located on the DNA A are involved in viral replication and encapsidation, whereas the DNA B encodes movement proteins. Both molecules harbour a region of identical sequence, a common region (CR) of about 180 bp. The CR includes all cis-acting elements required for DNA replication as well as two bidirectional core promoters which regulate expression of the replication-associated protein Rep (AC1) gene in complementary orientation and the coat protein CP (AV1) in viral orientation (Usharani *et al.*, 2006). Rep also functions as a negative-feedback regulator of transcription for its own promoter by binding to iterative sequences (iterons) located within the CR between the TATA box and the Rep transcription start site (Haley *et al.*, 1992; Hong and Stanley, 1995). Geminiviruses amplify their genomes in the nuclei of host cells by rolling-circle replication (RCR) mechanism and recombination-dependent replication (RDR) (Alberter *et al.*, 2005; Hanley-Bowdoin *et al.*, 1999; Jeske *et al.*, 2001; Morilla *et al.*, 2006; Preiss and Jeske, 2003). The initial step of RCR is the specific binding of Rep to the cognate DNA involving a directly repeated Rep-binding motif, within the intergenic region (Arguello-Astorga *et al.*, 1994a; Arguello-Astorga *et al.*, 1994b;

Behjatnia *et al.*, 1998; Fontes *et al.*, 1994a; Fontes *et al.*, 1994b; Fontes *et al.*, 1992). Downstream of this motif, the virion-sense strand is nicked by Rep within the universally conserved nonanucleotide sequence (TAATATT/AC) within the loop of a hairpin structure (Laufs *et al.*, 1995a; Laufs *et al.*, 1995b; Stanley, 1995).

ORF AC2 encodes for the transcriptional activator protein (TrAP) (Haley *et al.*, 1992; Sunter and Bisaro, 1997) which regulates late viral gene AV1, the coat protein (CP), and BV1, the nuclear shuttle protein (NSP) expression. In addition, AC2 and its positional homologous (also named C2, AL2 or L2) from several begomoviruses have been shown to reverse RNA silencing in plants and, thus, suppress local silencing in transient assays (Bisaro, 2006; Vanitharani *et al.*, 2005). AL2 of *Tomato golden moaic virus* (TGMV) and the related L2 protein of *Beet curly top virus* (BCTV) inactivated an SNF1-related kinase and enhanced virus susceptibility (Hao *et al.*, 2003). They also interacted with and inactivated adenosine kinase (ADK), a cellular enzyme important for adenosine salvage and chromatin methylation maintenance. ADK activation may be viewed as a counterdefense against plant DNA viruses, because of interference with the transcriptional gene silencing pathway (Hao *et al.*, 2003; Wang *et al.*, 2005; Wang *et al.*, 2003). Other viral silencing suppressors like the p19 protein of *Cymbidium ringspot virus* (CymRSV) and related tombusviruses bind and sequester small interfering RNAs (siRNA) (Baulcombe and Molnar, 2004; Silhavy *et al.*, 2002). They prevent siRNA incorporation into the RNA-induced silencing complex (RISC) and RNA-induced transcriptional silencing complex (RITS), block the production of a systemic silencing signal in plants, and suppress thereby local silencing in transient assays. The siRNAs are key-factors in a plant defense system, named RNA interference (RNAi) or post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). RNAi is a sequence-specific RNA degradation mechanism that silences a targeted gene (Al-Kaff *et al.*, 1998), whereas

TGS is thought to be a defence against transposons or other invasive DNA elements. Trigger of the gene silencing mechanism is dsRNA (Mello and Conte, 2004), which is recognized and cleaved by Dicer-like protein(s), a multi protein-complex, to generate siRNAs of different size and fate (Akbergenov *et al.*, 2006). SiRNAs guide RISC to degrade specifically homologous mRNA (Hammond, 2005) and RITS (Verdel and Moazed, 2005) to cause heterochromatin formation, or to function as a mobile silencing signal (Mlotshwa *et al.*, 2002). Whether begomovirus AC2 interferes directly with this (P)TGS pathway like p19 or whether its function is rather indirect as indicated by results for TGMV and BCTV L2 protein cited above, is still a matter of investigation. To further elaborate the role of TrAP for silencing, we used an AbMV-based transreplicon to study viral chromatin condensation as a sign of chromatin remodeling. Upon AbMV infection, the transreplicon was replicationally released (Bisaro, 1994) as shown for *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Morilla *et al.*, 2006).

Geminiviral double-stranded DNA, in order to serve as a template for replication as well as for transcription, is assembled into host nucleosomes, yielding circular viral minichromosomes (Abouzid *et al.*, 1988; Pilartz and Jeske, 1992). AbMV minichromosomes possess a nucleosome-free space in the intergenic region and a second minichromosomal region, hypersensitive to nucleases, allowing the interaction of viral DNA with host factors. The most prominent DNase-hypersensitive site was assigned to the hairpin loop within the viral origin of replication and initiation sites of AC2/AC3 transcripts colocalized with the second hypersensitive site of DNA A. At least two and perhaps three different nucleosomal arrangements were found. These might reflect different temporal and/or spatial regulation of the genes (Pilartz and Jeske, 2003). Their condensation state could be inferred from topoisomer distributions using one-dimensional gels and Southern blot hybridization. Here, we

used this technique to investigate the role of Rep and TrAP on viral chromatin structure.

Material and Methods

Microorganisms, plants, and general methods

Virus strains used in this work are agroinfectious clones of *Abutilon mosaic virus* (Frischmuth *et al.*, 1990) (AbMV; NC 001928, NC 001929). Agrobacterial expression clones of CymRSV p19 was kindly provided by Dr. Silhavy (Silhavy *et al.*, 2002), AbMV HA:AC1 and HA:AC2 expression constructs are named pPCV812Menchu HA:AC1 (AC1) and -HA:AC2 (AC2) and kindly provided by Dr. T. Kleinow (unpublished).

Escherichia coli strain DH5α was used for subcloning. *Agrobacterium tumefaciens* was grown and selected with the appropriate antibiotics. *A. tumefaciens* GV3101 (Koncz *et al.*, 1994) was transformed with the construct pBIN-GFPexpress by chemical transformation. Construct integrity was analyzed by PCR and sequencing. Plants were grown in an insect-free S2 greenhouse with supplementary lighting as described previously (Wege *et al.*, 2001).

Cloning of viral constructs

Recombinant DNA techniques were performed according to Sambrook and Russell (2001). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

DNA sequences were determined using Thermo Sequenase Primer Cycle Sequencing kit (Amersham Pharmacia Biotech) with universal IRD800 labeled primers (forward primer 5`-CGCCAGGGTTTTCCCAGTCACGAC-3`, reverse primer 5´-AGCGGATAACAATTTCACACAGGA-3´). Sequence reactions were analyzed on a LI-COR sequencer. Sequence alignment was done with Vector NTI program.

Transreplicon cloning

To generate pBK-GFPexpress, the *mGFP4* expression cassette with 35S promoter and nos terminator (Haseloff *et al.*, 1997) was transferred into pBK-TR22 (Krenz *et al*., 2007a) via *Eco* RI and *Hind* III restriction sites (Fig. 1b, c). The recombinant cassette was released by *Sal* I digestion and inserted into *Sal* I-linearized pBINplus (van Engelen *et al.*, 1995), resulting in pBIN-GFPexpress.

Plant transformation and inoculation

N. benthamiana transgenic plants were generated by transformating leaf discs using *A. tumefaciens* GV3101 carrying the binary plasmids pBIN-GFPexpress and antibiotic selection (100 µg of kanamycin/ml MS media). Shoots from transgenic callus were placed on root and selection media to regenerate whole transgenic plants, which were grown in a controlled environment light chamber at 22 to 24° C with a 16 h photoperiod. Potential transformants were analyzed by PCR using *mGFP4* specific primers (Krenz et al., 2007a).

N. benthamiana plants were inoculated with agroinfectious clones (Klinkenberg et al., 1989).

Agroinfiltration assay

Agrobacteria were grown overnight, pelleted, resuspended in 10 mM $MgCl_2$ and 100 µM of acetosyringone to an OD_{600}=0.5 and incubated for at least 2 h at room temperature. *N. benthamiana* leaves were injured with a needle and the agrobacteria-suspension was infiltrated with a 5 ml syringe (Morilla *et al.*, 2006).

Rolling circle amplification - Restriction fragment length polymorphism (RCA-RFLP)

RCA was used to analyze viral DNA from inoculated plants. Total nucleic acid preparation from systemically infected plant tissues (10 ng) served as template in these RCA reactions. RCA was performed using the TempliPhi DNA amplification kit (GE Healthcare, formerly Amersham Bioscience) following manufacturer's instructions and described elsewhere Haible *et al.* (2006), with total cellular nucleic acids as template for at least 16 h at 28 °C. The reaction was stopped at 65 °C for 10 min. RCA products were investigated by restriction fragment length polymorphism (RFLP) using *Eco* RI restriction enzyme.

Analysis of viral DNA conformations

Total cellular nucleic acids were extracted from systemically infected plant tissues with extraction buffer (100 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM EDTA, 1% SDS, 10 mM N-ethylmaleimide (NEM) and 100 mM DTT). Proteins were extracted twice by phenol-chloroform (1:1), once with chloroform and nucleic acids were ethanol-precipitated. Nucleic acids were dissolved in 50 µl sterile water. From each plant, nucleic acids containing 300 ng total DNA were separated by electrophoresis in 1.4% agarose gel supplemented with 10 µg/ml chloroquine in the gel as well as in the buffer (1x TBE) tank and transferred to Hybond NX membranes (Amersham). Viral DNAs were detected by digoxigenin-labeled probes, according to manufacturer's recommendation (Roche, Mannheim, DIG-High Prime Kit). Chemoluminescent detection was done as described in Jeske *et al*. (2001).

Small interfering RNA (siRNA) isolation and detection

For siRNA isolation and detection, total RNA was extracted from 100 mg plant tissue using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol, and dissolved in 15 µl loading buffer (95% formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 70 °C for 5 min and separated on 12% polyacrylamide mini-gel (acrylamide:bis-acrylamide 19:1, 7 M urea) at 70 V for 4 h. RNA was transferred to Hybond NX membrane by electroblotting in 0.5x TBE buffer for 1 h (Semy-dry-Blotting-Apparatus, BioRAD). Hybridization was performed as described elsewhere (Papefthimiou *et al.*, 2001) using digoxigenin-labeled RNAs (DIG-RNA labeling kit, Roche, Germany) as probe. The blot was washed twice with 2x SSC, 0.5% SDS for 30 min at 58 °C. Chemiluminescent probe detection via disodium 3-(4-methoxyspiro{1,2-dioxethane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenylphosphate (CSPD, Roche Diagnostics) following the manufacturer's protocol (DIG Application Manual for Filter Hybridization; Roche).

Western blot analysis

Protein was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol and resuspended in SDS-PAGE loading buffer (2% SDS, 10% glycerol, 0.1 M DTT, 62.5 mM Tris–HCl, pH 6.8, 0.01% bromophenol blue). The solution was boiled for 5 min, and separated in 12.5% polyacrylamide gels (Laemmli, 1970). Proteins were transferred to nitrocellulose (Schleicher und Schuell) using a semidry transfer assembly (Bio-Rad).

GFP protein was detected using a 1:2500 dilution of GFP (FL): sc-8334 polyclonal antibody (Santa Cruz Biotechnology), and HA-tagged AC1 using a 1:500 dilution of

HA.11 monoclonal antibody (Covance). Blots were developed using HRP-linked anti-mouse and anti-rabbit from sheep or donkey (1:10 000) as secondary antibodies (GE Healthcare (formerly Amersham)).

Microscopy and GFP imaging

Plants were screened for GFP expression under the Axiophot fluorescence microscope (ZEISS, Oberkochen, DE; with filter G 365; FT 395; LP 420). Macroscopic detection of GFP fluorescence was performed by using a hand-held, long-wave UV lamp (4 W) and photographed with a Canon Powershot 1 with yellow filter. Images were processed using Paint Shop Pro.

Results

Trans-replication of GFPexpress replicon during AbMV infection

Transgenic *Nicotiana benthamiana* plant were created and named “GFPexpress plants” in the following. They carry a green fluorescent protein (GFP) expression cassette under the constitutive and strong 35S promoter of *Cauliflower mosaic virus* (CaMV) (Haseloff *et al.*, 1997) embedded into a truncated AbMV DNA A partial dimer derived from pBK-TR22 (Krenz *et al.*, 2007a) (Fig. 1b, c).

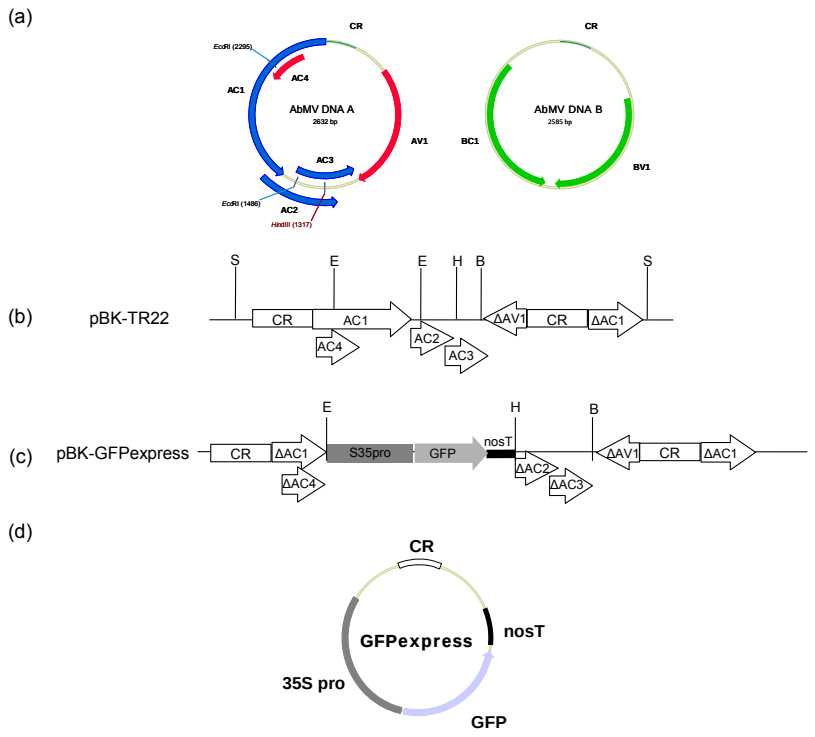

Figure 1. Genome organization of wildtype *Abutilon mosaic virus* DNA A and DNA B (a) with indicated open reading frames, assigned gene functions (AC1: replication-associated protein, Rep; AC2: transcriptional activator protein, TrAP; AC3: replication enhancer, Ren; AV1: coat protein, CP; BC1: movement protein, MP; BV1: nuclear shuttle protein, NSP and CR: common region) and restriction enzyme recognition sequences of *Eco* RI and *Hind* III. pBK-TR22 is a partial dimer (bitmer) of AbMV DNA A flanked by *Sal* I (S) restriction sites with AV1 deleted, and introduced *Bam* HI (B) cloning site (b). *Eco* RI (E) and *Hind* III (H) restriction sites are indicated. Introduced GFP expression cassette, generating pBK-GFPexpress (c). GFPexpress transreplicon expected upon replicational release (d).

The GFPexpress plants were tested positive for transgene presence by *mGFP4*-specific PCR and showed a low level of evenly distributed GFP fluorescence in leaves under UV light, due to constitutive GFP expression (data not shown). AbMV agroinoculation of wildtype and GFPexpress plants resulted in systemic infection in both cases accompanied with typical AbMV symptoms at 20 dpi (Wege *et al.*, 2001). Three weeks post-infection (wpi), enhanced bright GFP fluorescence was detected in single cells of the vascular bundle in GFPexpress plants (Fig. 2a), as expected for the phloem-limited AbMV.

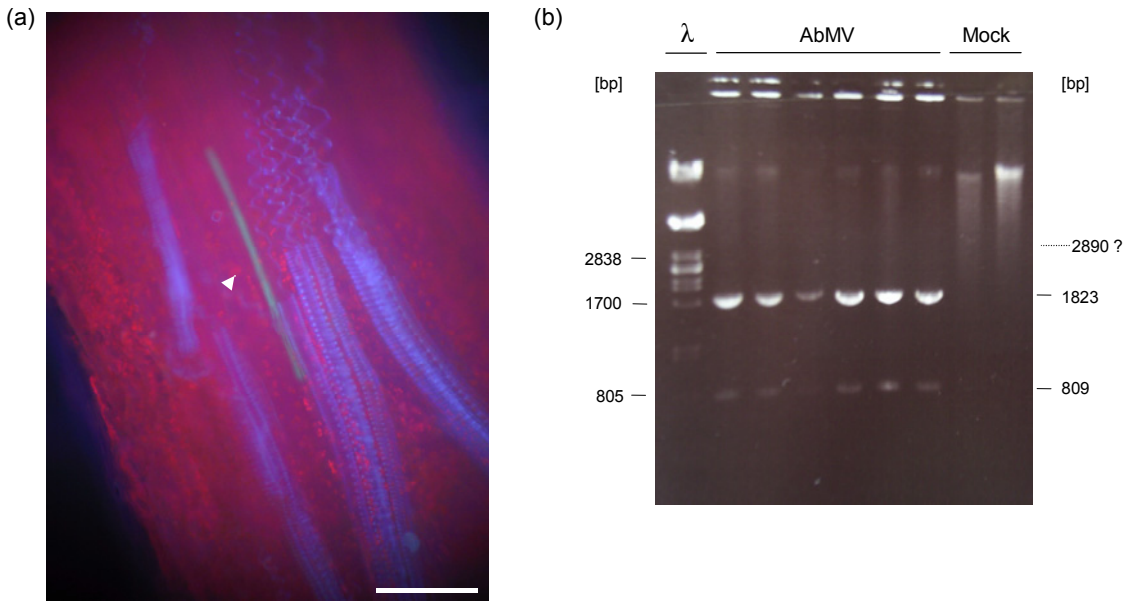

Figure 2. Handmade cross section along a major vein under UV light (a). White arrow marks GFP expressing cell of the vascular bundle, in which xylem elements appear blue due to its autofluorescence. Bar represents 50 µm. Six independent samples extracted from GFPexpress plants which were systemically AbMV-infected by agroinoculation and analyzed 14 dpi by RCA-RFLP using *Eco* RI (b). Digested products were separated in an 1% agarose gel stained with ethidium bromide (EtBr). λ indicates *Pst* I digsted Lambda DNA as a molecular marker. The sizes of the expected fragments and marker are indicated [bp].

Rolling circle amplification - restriction fragment length polymorphism (RCA-RFLP) of products of total nucleic acid preparations (Haible *et al.*, 2006) from systemic infected leaves of AbMV- and mock- inoculated GFPexpress plants were analyzed using *Eco* RI. The expected AbMV DNA A pattern, an 1823 bp and an 809 bp fragment,

indicated the presence of AbMV in younger leafs (Fig. 2b). The GFPexpress plants, however, did not show the additional third fragment of higher size (2890 bp), expected for the transreplicon (Fig. 1c), probably reflecting the low amount of transreplicon, which has already been observed in the similar experiment for

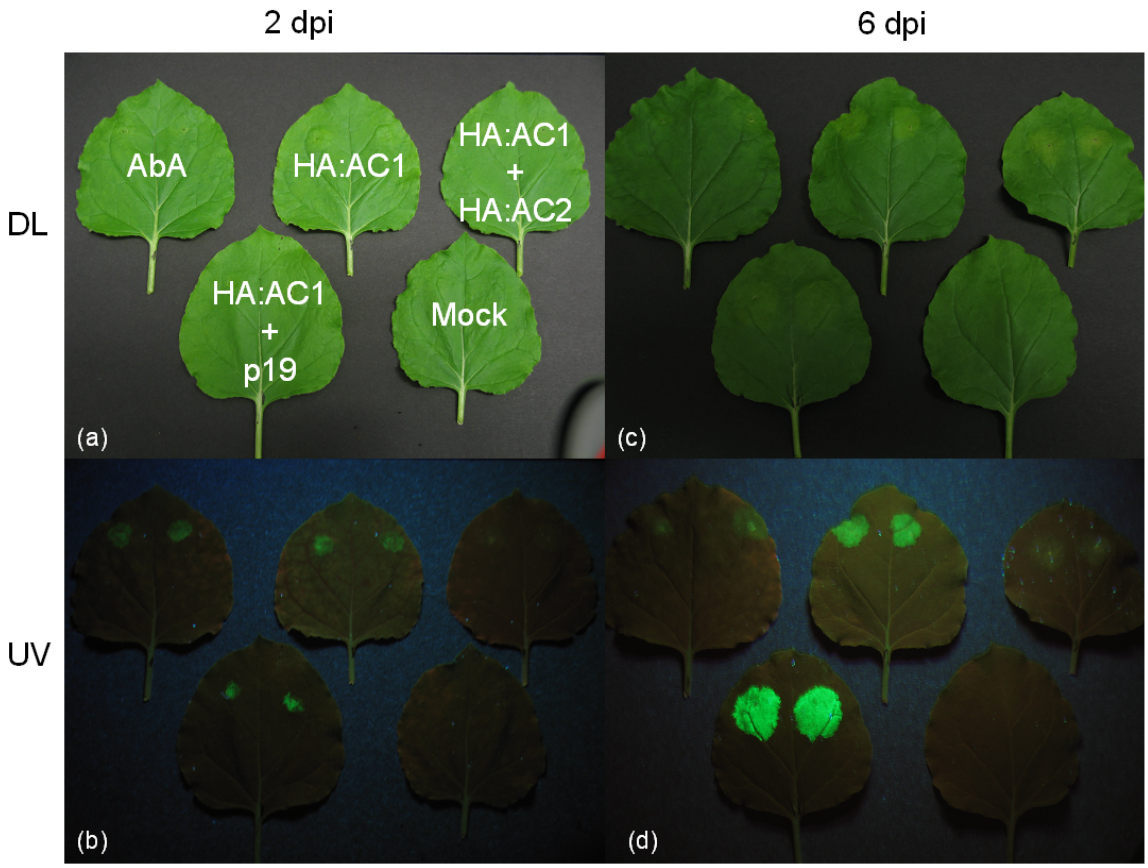

Figure 3: Agroinfiltration of GFPexpress plants with AbMV DNA A (AbA), pPCV812Menchu HA:AC1 (HA:AC1), -HA:AC2 (HA:AC2) (T. Kleinow, unpublished) and pBINp19 (p19) (Silhavy *et al*., 2002) observed at two and six dpi under daylight (DL) (a, c) or UV light (b, d), respectively.

TYLCSV (Morilla *et al.*, 2006).

Rep is necessary and sufficient to release GFPexpress replicon

In order to investigate whether the presence of AbMV AC1 was sufficient to transreplicate the GFPexpress replicon, leaves were agroinfiltrated with a construct expressing AbMV HA:AC1 (Rep) under the control of the 35S CaMV promoter. In this

construct AC1 is N-terminal fused to an HA-epitope via a splicable intron. Therefore the tagged protein is only detectable after expression in the eukaryotic cell, but not if expressed in agrobacteria. Two leaves of two plants were infiltrated twice, once on each leaf side. Two to six dpi, an increased GFP fluorescence was detected in all HA:AC1-infiltrated leaves, similar to the result when leaves were agroinfiltrated with an infectious clone of AbMV DNA A (AbA) (Fig. 3b, d). Interestingly, the fluorescence in AbA-infiltrated leaves was first equal (2 dpi) but lateron (6 dpi) lower than in HA:AC1-infiltrated leaves. Mock-infiltrated areas showed no increased GFP fluorescence. The increase of GFP expression correlated with the production of GFPexpress replicons as detected by an accumulation of the diagnostic restriction fragment by RFLP of RCAs (Fig. 4) in AbA-, HA:AC1-, but not in mock-inoculated leaves. Interestingly, GFPexpress replicon levels in AbA-infiltrated leaves were again significantly lower than those in HA:AC1-infiltrated ones. In summary, these results indicate that AbMV AC1 is necessary and sufficient to mobilize and replicate GFPexpress replicon from the transgene present in GFPexpress plants, and that

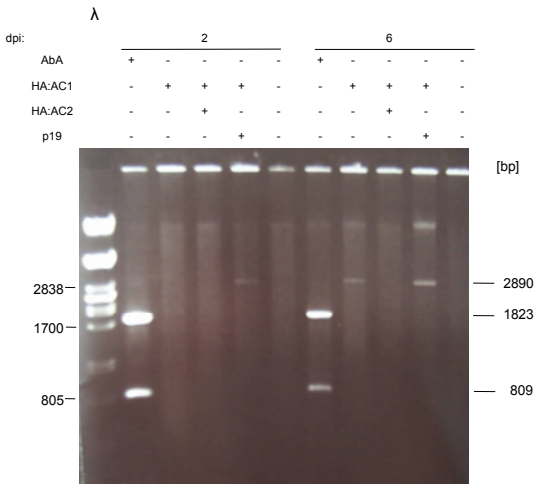

Figure 4. Analyzed by RCA-RFLP using *Eco* RI samples extracted two and six dpi from GFPexpress plants agroinfiltrated with constructs described in Fig. 3. Digestion products were separated in an 1% agarose gel stained with EtBr. Abbreviations as in Fig. 3.

HA:AC1 is even more efficient in this process than the complete AbMV DNA A.

Co-infiltration assays

To investigate why AbA-infiltrated leaves accumulate lower amounts of GFPexpress replicon than HA:AC1-infiltrated leaves, a co-infiltration assays with constructs expressing HA:AC2 and p19 were performed, the putative homologous and heterologous suppressor of silencing, respectively. Note, that the construct pPCV812Menchu HA:AC2 expresses AC2 as an N-terminal HA-tagged splicing-dependent fusion product. GFPexpress plants were agroinfiltrated with AbA, HA:AC1, HA:AC1 and HA:AC2, HA:AC1 and p19 and HA:AC1 and HA:AC2 and p19 and checked for GFP fluorescence macroscopically under UV light. Fig. 3 and 5 with assays on separate or the same leaves, respectively, showed that upon co-infiltrations of HA:AC1 and HA:AC2 GFP accumulated to the same low level like in AbA-infiltrated leaves. In contrast, the combination of HA:AC1 and p19 even increased the GFP fluorescence. The triple infiltration of HA:AC1 and HA:AC2 and p19 (Fig. 5b) showed a mediate GFP fluorescence between HA:AC1 and p19 co-infiltration at 5 dpi. Infiltrations with either HA:AC2 or p19 alone, were unable to increase GFP fluorescence (data not shown). In order to judge how far PTGS was responsible for the effect, siRNAs were detected by Northern analysis (Fig. 5c). GFP-specific siRNAs were detected for tissue areas infiltrated with AbA, HA:AC1 and HA:AC2 and HA:AC1 and HA:AC2 and p19, but they were undetectable in HA:AC1 or mock-infiltrated tissues. The accumulation of GFP siRNAs, thus, correlated with the inability of GFPexpress replicon to replicate properly. Western blot analysis confirmed the presence of GFP in the infiltrated leaf areas. Furthermore, HA:AC1 protein was exclusively detected in HA:AC1-infiltrated leaves, when HA antibodies were used, and accumulated to similar levels (Fig. 5e). Inhibition of HA:AC1

expression in co-infiltration experiments can, therefore, be ruled out as main cause of the differential effects of the co-inoculation. Nevertheless, the amount of transreplicated DNA (Fig. 4) correlated with the appearance of GFP and as highest

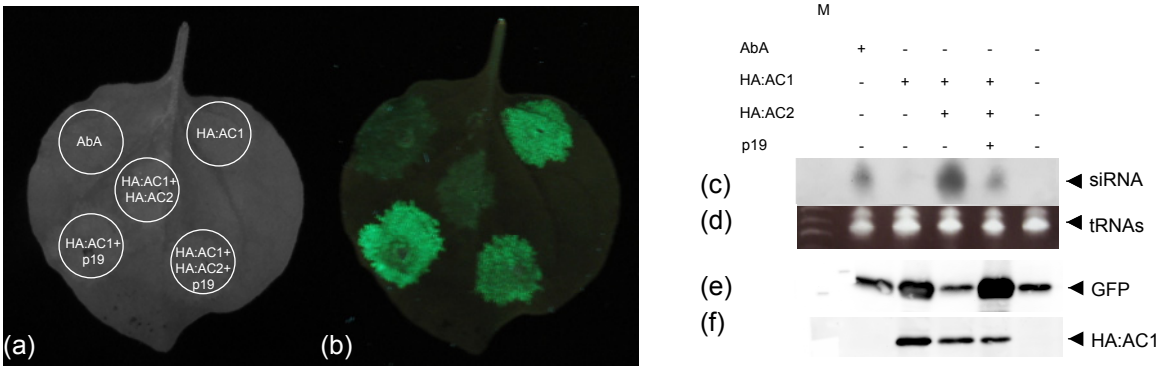

Figure 5. Agroinfiltration of GFPexpress plants with constructs described in Fig. 3 on the same leaf observed 5 dpi: infiltration scheme (a) and under UV light (b).
Northern analysis for detection of GFP-specific siRNAs (c). Samples were hybridized with a GFP digoxigenin-labeled RNA probe. Lane M, 50bp ladder from Fermentas; EtBr-stained tRNA bands are shown as loading control (d). Immunodetection of GFP with antiGFP antibodies (e) or HA-tagged AC1 with antiHA antibodies (f).

for HA:AC1 and p19, intermediate for HA:AC1 and undetectable for HA:AC1 and HA:AC2, AbMV DNA A and mock-inoculated specimen.

Analysis of topoisomer distribution to recognize changes in chromatin condensation

Total DNAs were prepared from the agroinfiltrated tissue areas of four different leaves (Fig. 5b) and were separated on one-dimensional gels, taking advantage of the intercalation of chloroquine to separate the topoisomers (Pilartz and Jeske, 2003) (Fig. 6a). After Southern blotting, transreplicon DNA was detected by a GFP-specific probe. Densitometric analysis, where pixel intensities were plotted against pixel distance, revealed that HA:AC1-infiltrated GFPexpress leaf areas accumulated topoisomers of faster mobility (Fig. 6b; except leaf 3; asteriks mark most intense bands), whereas co-infiltration of HA:AC1 with p19 results in increased amounts of

topoisomers of slower mobility, which is construed as condensed viral DNA conformation. Interestingly, HA:AC2 can reverse this effect and most minichromosomes are again less condensed. No or very low levels of GFPexpress transreplicon DNA was identified, as expected, in AbA and HA:AC1 and HA:AC2-infiltrated plant parts.

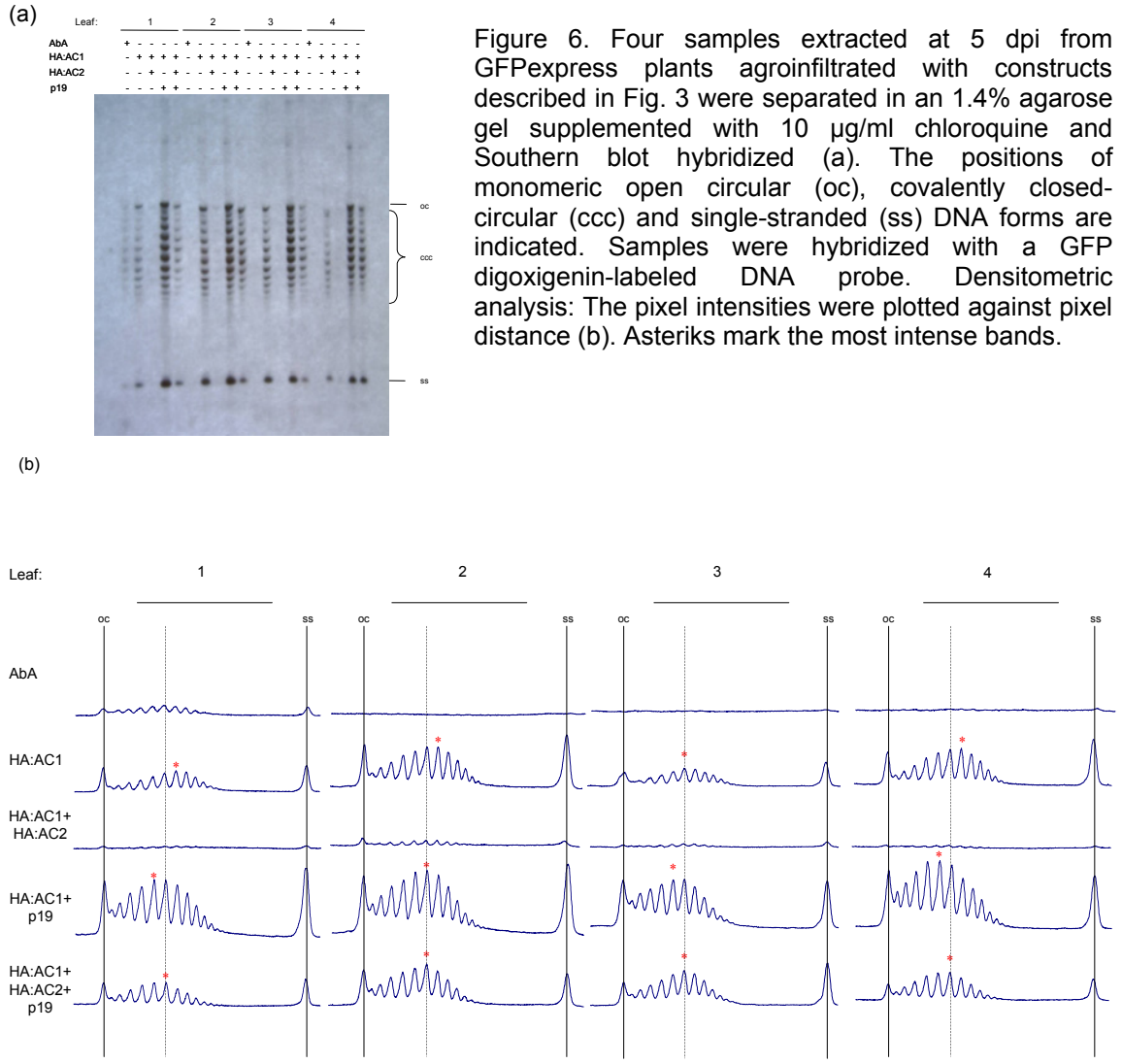

Figure 6. Four samples extracted at 5 dpi from GFPexpress plants agroinfiltrated with constructs described in Fig. 3 were separated in an 1.4% agarose gel supplemented with 10 µg/ml chloroquine and Southern blot hybridized (a). The positions of monomeric open circular (oc), covalently closed-circular (ccc) and single-stranded (ss) DNA forms are indicated. Samples were hybridized with a GFP digoxigenin-labeled DNA probe. Densitometric analysis: The pixel intensities were plotted against pixel distance (b). Asteriks mark the most intense bands.

Viral DNA from AbMV-infiltrated leaves of *N. benthamiana* plants, prepared in such a way, that infection process was followed at different time points post infiltration (3-6 dpi), showed a shift of the topoisomer patterns with time (Fig. 7). A detailed scanning analysis of the blot (Fig. 7b) revealed that infiltrated leaves examined after 6 dpi have

accumulated topoisomers of slower mobility. With progress of infection and virus life cycle, topoisomers reflecting a more condensed viral DNA conformation increased, whereas in infiltrated leaves investigated earlier after 3 and 4 dpi lower linking numbers were prevalent. This phenomenon is interpreted to be caused by a mixed effect of replication and transcription on viral nucleosome loading and minichromatin condensation (Pilartz and Jeske, 2003). In direct comparision, HA:AC2

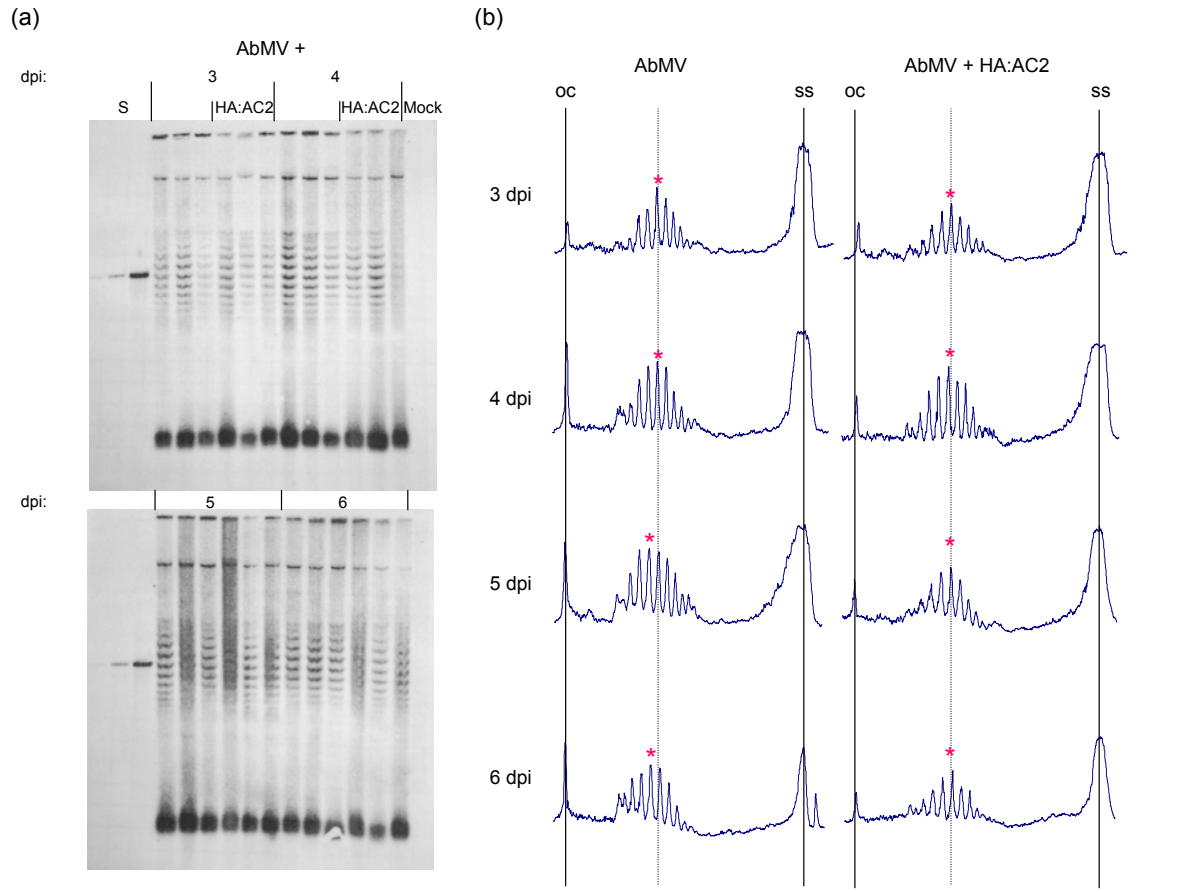

Fig. 7: Samples extracted at 3 to 5 dpi from *N. benthamiana* plants agroinfiltrated with AbMV or AbMV and HA:AC2 were separated as in Fig. 6 and Southern blot hybridized (a). Samples were hybridized with an AbMV DNA A digoxigenin-labeled DNA probe. Densitometric analysis: The pixel intensities were plotted against pixel distance (b). Asteriks mark the most intense bands.

overexpression prevent viral minichromosomes to condensate, keeping it accessable for the host transcription machinery.

Discussion

In this study, an AbMV-based transreplicon was constructed, which carries a green fluorescent protein (GFP) expression cassette (GFPexpress). Transgenic GFPexpress plants showed only a low level of basal GFP expression, although it is driven by the constitutive and strong 35S promoter from CaMV. The flanking common regions seems to hinder effective GFP expression as it has been observed for similar construct, based on TYLCSV (Morilla *et al.*, 2006). Transgene positional effects in the plant genome may add to this phenomenon. Inoculation with AbMV increased the GFP fluorescence only in a limited number of systemically infected cells (Fig. 2b), giving the opportunity to monitor viral infection without using recombinant virus. GFPexpress transreplicon (Fig. 1d) could not be detected in systemically AbMV-infected GFPexpress plants by either RCA-RFLP (Fig. 2a) or Southern blot analysis (data not shown). Transreplication is less efficient than for TYLCSV, where plant veins clearly exhibited GFP fluorescence and the GFP replicons were detectable in Southern blot analysis (Morilla *et al.*, 2006).

However, agroinfiltration assays with HA:AC1 demonstrated, that increase in GFP fluorescence is Rep dependent. HA:AC1 alone is sufficient to mobilize the GFPexpress replicon from the plant genome and to support its further autonomous replication, resulting in multiple copies of the GFPexpress replicon and therefore in an overexpression of GFP (Fig. 3 and 5b). Surprisingly, HA:AC1-infiltrated leaf areas showed brighter GFP fluorescence than AbA-infiltrated at 6 dpi. It could be speculated, that AbMV DNA A prefers to replicate itself instead of the transreplicon or GFPexpress transreplicon is repaired to wildtype AbMV DNA A, because the amount of viral AbMV DNA A was much higher than of the GFPexpress replicon. But co-infiltration of HA:AC1 and HA:AC2 demonstrated, that the presence of HA:AC2

hinders the proper transreplication of the GFPexpress replicon (Fig. 3, 5b and 6a). Ectopic expression of HA:AC1 was not impaired (Fig. 5f), but an interaction of HA:AC1 and HA:AC2 may occur that disfunctions HA:AC1 in replication or that the transactivation of the AV1 promoter hinders HA:AC1-mediated release of the GFPexpress replicon from the plant genome. It could also be, that HA:AC2 overexpression is somehow harmful for the plant and foils replication indirectly. The infiltration of p19 reversed this effect and secondly the replication of AbMV DNA A in AbA-infiltrated areas was not compromised. It is more likely, that the attribute of p19 as an effective silencing suppressor neutralize replication suppressor property of AC2 in this context. P19 is a well-characterized silencing suppressor, known to bind and sequester specifically siRNAs (Baulcombe and Molnar, 2004; Omarov *et al.*, 2006; Silhavy *et al.*, 2002). HA:AC2 expression induced siRNA-mediated suppression of transreplicon replication, which can be overcome by p19 co-expression.

Transcription in geminiviruses is bidirectional with the production of polycistronic mRNAs occurring from the CR, which contains the promoter sequences. These polycistronic mRNAs of opposite polarity could overlap at their 3'ends. The resulting overlap region can form a dsRNA, which could be potential enough to induce the plant's PTGS system (Chellappan *et al.*, 2004). Presumably, HA:AC2 transactivates the viral AV1 promoter and transcripts are generated complement to transcripts driven by the 35S promoter, although polyA-sites of AV1 should abolish creation of antisense transcripts. An unwanted read-through results in dsRNA fragments which triggers the post- and transcriptional gene silencing (PTGS and TGS) plant defense systems. Produced siRNAs are incorporated into the RISC and RITS complex leading to the dicing and slicing of homologous mRNA, but also in GFPexpress transgene silencing or heterochromatin formation, which disables the replicational release of the GFPexpress replicon. Previous work has shown that silencing of plant

transgenes in response to infection by viruses sharing promoter homology (virus-induced transcriptional gene silencing or VITGS) can be triggered by DNA and RNA viruses and results in heritable transgene silencing, due to transcriptional inactivation accompanied by hypermethylation of the transgene promoter (Al-Kaff *et al.*, 2000; Jones *et al.*, 2001; Seemanpillai *et al.*, 2003).

Replication of AbMV DNA A is not affected, propably due to its state as an episome and/or of missing homology to the produced siRNAs, which mainly should consist of the nopaline synthase terminator and *mGFP4*. The virus escapes this defense mechanism, possibly due to a differential methylation of the *de novo* synthesized viral and host plant DNA (Bian *et al.*, 2006). AbMV and ToLCV DNA has been found to replicate by a combination of rolling circle replication (RCR) and recombination dependent replication (RDR) (Alberter *et al.*, 2005; Jeske *et al.*, 2001). RCR is a semi-conservative process resulting in the production of DNA consisting of a *de novo* unmethylated strand and a preexisting methylated strand. By contrast, both DNA strands produced by RDR are synthesized *de novo* and initially lack methylation. Replication of geminiviral DNA by RDR may give rise to a population of unmethylated, transcriptionally active DNA that may become methylated subsequently. Whereas generation of GFPexpress transreplicon from the transgene by replication release is presumably arrested by transgene silencing.

It has to be shown whether the suppression of transreplicon replication release is accompanied with *de novo* histone, DNA transgene methylation and the establishment of heterochromatin formation. Consistently, Northern blot analysis of siRNAs detect GFP siRNAs in AbA-, HA:AC1 and HA:AC2, and HA:AC1 and HA:AC2 and p19-infiltrated leaves, so only in those where no GFPexpress replicon was detectable. It is well accepted, that AC2 itself is a silencing suppressor (Bisaro, 2006; Dong *et al.*, 2003; Hamilton *et al.*, 2002; van Wezel *et al.*, 2002; 2003), but not in this

experimental setup. The silencing activity is in general not ruled out, but a dominant function in transcriptional gene silencing suppression can presumably be excluded. Wang and coworkers described the interaction of AL2 with adenosine kinase (ADK), a cellular enzyme important for adenosine salvage and methylation maintenance (Wang *et al.*, 2005; Wang *et al.*, 2003). ADK inhibition by AL2 and L2 may be viewed as a counterdefense against this antiviral pathway, because ADK plays a critical role in sustaining S-adenosylmethionine-dependent methyltransferase activity and ADK-deficiency exhibits reduced methylation in yeast and plants. This interference does not suffice to inactivate the transreplicon replication suppression. This assay can be used to study viral or host factors who can counteract the AC2 mediated transgene silencing and might give new insights in the transcriptional gene silencing mechanism.

Chromosomal phasing of GFP transreplicon to model a synchronized infection system was achieved by HA:AC1 or HA:AC2-infiltration. Analyzing GFP transreplicon topoisomers of HA:AC1-infiltrated GFPexpress plant leaf areas indicated that minichromosomes predominantly existed in a more nucleosome-free conformation and reflected an early time point of infection. Co-infiltration together with p19 rearranged transreplicon minichromosomes to a heterochromatin-like structure resembling an occupation of all nucleosome-free spaces with nucleosomes and the presumable AC1 promoter shut-off.

Hefferon *et al.* (2006) have demonstrated that Rep and RepA perform different functions with respect to regulating *Bean yellow dwarf virus* (BeYDV) bidirectional promoter activity. In the early stages of BeYDV infection, both gene products are expressed from promoter Pc, apparently in the absence of other virus gene products. While high levels of Rep and RepA result in a shut-off of promoter Pc, RepA alone is responsible for transactivating late genes V1 and V2 as a single dicistronic

transcription unit from promoter Pv. Consequently, HA:AC2 transactivated the AV1 promoter in triple infiltration of HA.AC1 and HA:AC2 and p19, visible in a shift of the dominant topoisomer band reflecting a more accessable minichromosome structure.

The examination of topoisomers distribution in agroinfiltrated leaves revealed an involvement of HA:AC2 in chromatin remodeling keeping viral chromatin, organized in minichromosomes, in an accessable conformation for host transcription machinery excluding the AC1 promoter. HA:AC2 overexpression prevented complete AbMV minichromosome condensation. With respect to recent and current results, we like to emphasize the role of chromatin structure for geminiviral gene regulation.

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Ich erkläre hiermit, daß ich die vorliegende Arbeit ohne unzulässiger Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Stuttgart, den

Unterschrift

Curriculum Vitae

- Björn Krenz
- Geboren am 26.07.1976
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Ausbildung

Seit März 2003	Promotion in Technischer Biologie an der Universität Stuttgart am Biologischen Institut, Abt. Molekularbiologie und Virologie der Pflanzen Stipendiat der Landesgraduiertenförderung Baden-Württemberg im Jahr 2004 Stipendiat des Deutschen Akademischen Austauschdienst im Jahr 2006
Oktober 1997 – März 2003	Studium der Technischen Biologie an der Universität Stuttgart mit Abschluss Diplom-Biologe (t.o.) in den Hauptfächern: • Pflanzenvirologie • Bioinformatik • Industrielle Genetik • Technische Biochemie Diplomarbeit am Biologischen Institut, Abt. Molekularbiologie und Virologie der Pflanzen: Posttranskriptionellen Gen-*Silencing* (PTGS) und das *Abutilon mosaic virus*

Juli 1986 - Juni 1995	Besuch des Frobenius Gymnasium Hammelburg, Bayern; Abschluss Abitur

Auslandsaufenthalt

Im Rahmen des Projekts „Biotechnologie by Distance Learning“ BioDiLea einwöchige Seminare an den Universitäten von Hanoi und Ho Chi Minh City, Vietnam, im November 2001.

Dreimonatiger Forschungsaufenthalt (01.01. – 31.3.2006) an der University of Arizona, Tucson, USA.

Veröffentlichungen

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- Rothenstein, D., Krenz, B., Selchow, O., and Jeske, H. (2007). Tissue and cell tropism of Indian cassava mosaic virus (ICMV) and its AV2 (precoat) gene product. Virology. In press.