Maniok-assoziierte Begomoviren in Indien - Biodiversität, Gewebelokalisation und Funktionsanalyse

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Zusammenfassung

Die vorliegende Arbeit wurde im Rahmen des von der Europäischen Union geförderten *International* Scientific Cooperation Project „Improved screening of cassava against selected systemic arboviruses“ durchgeführt. Das Projekt untersucht die ökologische Bedeutung von Geminiviren als Pflanzenpathogene in Südindien und Zentralafrika. Geminiviren befallen wichtige Nutzpflanzen, wie Maniok, Mais, Zuckerrübe, Bohne und Tomate und führen zu teilweise enormen Ernteverlusten. In den letzten Jahrzehnten stieg die Zahl von Virusinfektionen an, einerseits durch die Verbreitung der Weißen Fliege (*Bemisia tabaci*, Genn.), den für die Übertragung verantwortlichen Vektor, andererseits durch die schnelle Anpassung der Viren an Umweltbedingungen und Wirtspflanzen durch verschiedene molekulargenetische Mechanismen, wie Mutation, Rekombination und Pseudorekombination. Der Maniokanbau in den von der Maniok Mosaikkrankheit (engl. Cassava mosaic disease CMD) betroffenen Regionen Afrikas brach vollständig zusammen und erholte sich erst nach Jahren wieder langsam. Untersuchungen über die Biodiversität von CMD-auslösenden Begomoviren in Indien sind bisher unvollständig. Diese Lücke wurde mit der vorliegenden Arbeit geschlossen, in der die biologische Varianz Maniok-infizierender Begomoviren in Südindien untersucht wurde.

Die Ergebnisse zeigen, dass neben dem schon bekannten *Indian cassava mosaic virus* (ICMV) auch das bisher nur in Sri Lanka nachgewiesene *Sri Lankan cassava mosaic virus* (SLCMV) in Indien weitverbreitet auftritt. Um Neuzüchtungen von Manioksorten auf Resistenz prüfen zu können, wurden Maniok-infektiöse Begomoviren kloniert. Aus einer künstlich infizierten Tabak-Pflanze wurden beide DNA Komponenten des bipartiten ICMV isoliert und kloniert. Dieser Klon induzierte sowohl in experimentell verwendeten *Nicotiana*-Spezies als auch im natürlichen Wirt Maniok systemische und symptomatische Infektionen.

Die Gewebelokalisation von ICMV in systemisch infiziertem Maniok und Tabak wurde durch *in situ* Hybridisierungsstudien als phloemlimitiert bestimmt.

Um den Infektionsverlauf zu beobachten und die Funktion einzelner Offener Leserahmen (engl. *open reading frame*, ORF) zu bestimmen, wurden ICMV-Chimären erstellt, die anstelle des Kapsidprotein (engl. *coat protein*, *CP*) Gens das *green fluorescence protein* (GFP) Gen exprimierten. Damit konnte das Virus durch Fluoreszenzmikroskopie *in vivo* nachgewiesen und die betroffenen Gewebe bestimmt werden. Die Phloemlimitierung von ICMV konnte dadurch bestätigt werden.

Die Funktion des ORF AV2, der nur in Alte-Welt-Begomoviren auftritt, wurde mit Hilfe von GFP-Fusionsproteinen analysiert. Neben der Expression des Fusionsproteins im viralen Hintergrund wurde ein GFP-AV2-Fusionsprotein unter der Kontrolle des konstitutiven *Cauliflower mosaic virus* (CaMV) 35S Promotors erstellt. Beide Konstrukte wurden in *Nicotiana benthamiana* exprimiert, entweder transient, in isolierten Blättern oder systemisch, in Pflanzen. Fusionsproteine beider Konstrukte ergaben dieselbe zelluläre Verteilung. Mikroskopische Analysen zeigten eine mögliche Assoziation von AV2 mit Plasmodesmata und eine Funktion beim viralen Zell-zu-Zell Transport. Demnach kann AV2 die Funktion eines redundanten Transportproteins haben, obwohl ICMV DNA B noch zusätzliche Transportproteine beisteuert. Möglich ist, dass AV2 ein genetisches Relikt eines monopartiten Begomovirus ist.

Summary

This work was part of the International Scientific Cooperation Project „*Improved screening against selected systemic arboviruses*“ financed by the European Union. The project investigated the growing ecological importance of geminiviruses in South India and Central Africa. Important crops, such as cassava, maize, beet, bean and tomato, are infected by geminiviruses, leading to enormous loss of yield. During the last decades infection rates of geminiviruses raised, either by the increased dissemination of whiteflies (*Bemisia tabaci*, Genn.), which functions as transfer vector for geminiviruses, and fast adaptation of viruses to environment and host plants, by mutation, recombination and pseudorecombination. Cassava production nearly broke down in parts of Africa, affected by cassava mosaic disease (CMD), and recovered only years later.

Investigations on biodiversity of CMD-causing begomoviruses in India are incomplete so far. This gap should be closed by this work, investigating the diversity of cassava-associated begomoviruses in South India. The results demonstrate, that beside the *Indian cassava mosaic virus* (ICMV), the *Sri Lankan cassava mosaic virus* (SLCMV), is widely distributed on the Indian subcontinent. To challenge new CMD-resistant cassava cultivars for infection, cassava-infectious ICMV clones were established. ICMV DNA A and DNA B components were isolated and cloned from an experimentally infected tobacco plant. This clone induces CMD in experimentally used *Nicotiana* spec. as well as in the natural host cassava.

The tissue tropism of ICMV in systemically infected tobacco and cassava plants was determined to be phloem-limited using *in situ* hybridisation.

To monitor the progress of infection and to analyse the function of selected open reading frames (ORF), ICMV-chimeras were generated with the coat protein (*CP*) gene replaced by the *green fluorescence protein* (GFP) gene. Using these chimera, the virus could be detected in affected plant tissues *in vivo* by fluorescence microscopy. The phloem-limitation was confirmed using microscopic analysis of GFP-expressing chimera.

The function of ORF AV2, only present in Old World begomoviruses, was analysed using AV2-GFP fusion proteins. Therefore two constructs were established. One expressing the fusion protein within the viral background, the other construct was driven by the constitutive 35S promoter of *Cauliflower mosaic virus* (CaMV), expressing the fusion protein in the absence of any other viral proteins. Both

constructs were expressed in *Nicotiana benthamiana*, either transiently in detached leaves or systemically in plants. Fusion proteins, expressed form both constructs, were similarly distributed within plant cells. AV2 protein was probably associated with plasmodesmata and cell-to-cell movement. Therefore AV2 may function as a redundant movement protein, though ICMV DNA B provides additional movement proteins. A possible explanation would be that AV2 is a genetic relict from a monopartite begomovirus.

Einleitung

Maniok (*Manihot esculenta* CRANTZ, engl. *cassava*) ist eine der wichtigsten Nutzpflanzen weltweit und wird als einziger Vertreter der Familie *Euphorbiaceae* als Nahrungsmittel verwendet (Fauquet and Fargette, 1990). Die stärkereiche Wurzelknolle des Manioks ist der drittgrößte Kohlenhydratlieferant für die menschliche Ernährung (Fauquet and Fargette, 1990).

Maniok wurde bereits 5.000-7.000 vor Christus im Ursprungsland Brasilien kultiviert (Latharp, 1970). Im 16. Jahrhundert wurde Maniok durch portugiesische Sklavenhändler in Ostafrika eingeführt und verbreitete sich von dort über den gesamten Kontinent. Im späten 18. Jahrhundert gelangte Maniok nach Indien und Sri Lanka und weiter in den gesamten asiatischen Raum (Fauquet and Fargette, 1990). Neben der Verwendung als Grundnahrungsmittel entwickelte sich Maniok in der ersten Hälfte des 20. Jahrhunderts zum Exportprodukt. Heute wird Maniok in tropischen Regionen weltweit angebaut, in Afrika zumeist kleinflächig zur Deckung des Eigenbedarfs, in Indien hingegen großflächiger meist mit anschließender industrieller Verwertung. Die Welt-Jahresproduktion 2003 betrug 187,6 Millionen Tonnen, wovon 7,1 Millionen Tonnen in Indien produziert wurden (FAO, 2004). Maniok wird unter anderem zur Produktion von Alkoholen, modifizierten Stärken und Zuckern sowie Textilien und Klebstoffen eingesetzt (Onwueme, 2002). Die Europäische Union ist der Hauptimporteur asiatischen Manioks, und setzt diesen zumeist als Futtermittel ein (Onwueme, 2002).

Eine der größten Beeinträchtigungen der Maniokproduktion in Afrika und zunehmend auch in Indien ist die Maniok Mosaik Krankheit (engl. *Cassava mosaic disease*; CMD), die durch verschiedene Begomoviren der Familie *Geminiviridae* verursacht wird (Fauquet and Fargette, 1990; Malathi, 1985). CMD wurde 1894 erstmals von Warburg als „Kräuselkrankheit“ beschrieben (Warburg, 1894). 1983 wurde als erster Vertreter das *African cassava mosaic virus* (ACMV) von Stanley and Gay (1983) kloniert und CMD konnte auf eine Virusinfektion zurückgeführt werden. In Afrika wurden seither verschiedene CMD-auslösende Begomoviren beschrieben (zusammengefaßt in Berry and Rey, 2001), während in Indien (Hong *et al.*, 1993) und im benachbarten Sri Lanka (Saunders *et al.*, 2002) jeweils ein maniokinfizierendes Begomovirus dokumentiert ist. In Afrika kam es durch

Rekombination verschiedener Begomoviren in den 90er Jahren des 20. Jahrhunderts zu Epidemien mit verheerenden Auswirkungen auf die lokale Landwirtschaft (Pita *et al.*, 2001; Fondong *et al.*, 2000; Zhou *et al.*, 1997). Ernteeinbußen zwischen 20 und 95%, treten in Abhängigkeit von Manioksorten und Land auf (Fauquet and Fargette, 1990). Die Ausbreitung von Begomoviren hängt eng mit dem Übertragungsvektor, der Weißen Fliege (*Bemisia tabaci*, Genn.), zusammen, wobei eine Koevolution zwischen Virus und Vektor stattfindet. Einzelne Genotypen der Weißen Fliege können mit unterschiedlicher Effektivität verschiedene Begomoviren übertragen (Legg *et al.*, 2002; Maruthi *et al.*, 2002). Die Kontrolle der Weißen Fliege mittels Insektiziden ist schwierig, da sich bereits pestizidresistente Populationen entwickelt haben (Brown and Bird, 1992).

Neben der wirtschaftlichen Bedeutung stellen Geminiviren wichtige wissenschaftliche Untersuchungsobjekte dar. Die Replikation des viralen Genoms ist abhängig von der zellulären Replikationsmaschinerie. Geminiviren können in differenzierten Pflanzenzellen die DNA-Synthesephase einleiten (Nagar *et al.*, 2002) und erlauben somit Einblick in die Regulation des Zellzyklus höherer Pflanzen. Durch verschiedene experimentelle Ansätze wurden für Gemini- und andere Pflanzenviren intrazelluläre und interzelluläre Transportmechanismen untersucht (Zhang *et al.*, 2001a) und Interaktionen viraler Proteine mit Komponenten des Cytoskeletts (Heinlein *et al.*, 1995), der Replikationsmaschinerie (Castillo *et al.*, 2003; Kong *et al.*, 2000; Nagar *et al.*, 1995) und der Signaltransduktion aufgezeigt (Castillo *et al.*, 2004; Mariano *et al.*, 2004; Hao *et al.*, 2003; Wang *et al.*, 2003; Kong and Hanley-Bowdoin, 2002). *Posttranscriptional gene silencing* (PTGS) als möglicher pflanzeneigener Abwehrmechanismus gegen Viren (Mlotshwa *et al.*, 2002; Vance and Vaucheret, 2001; Voinnet, 2001) zeigte, dass RNA als Signalmolekül in der Zell-zu-Zell Kommunikation dienen könnte. Virale Proteine konnten identifiziert werden, die PTGS unterdrücken und die Ausbreitung eines Virus in der Pflanze erleichtern (Reed *et al.*, 2003; Matzke *et al.*, 2001; Vance and Vaucheret, 2001; Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998). Endogene micro (mi) RNAs, die in den selben oder einen verwandten Signalübertragungsweg wie das PTGS münden, sind möglicherweise an der Gewebedifferenzierung beteiligt (Bartel, 2004). Erste Ergebnisse deuten darauf hin, dass einige pflanzen- und humanpathogene Viren sowohl indirekt als auch direkt über den miRNA Signalwege die eigene

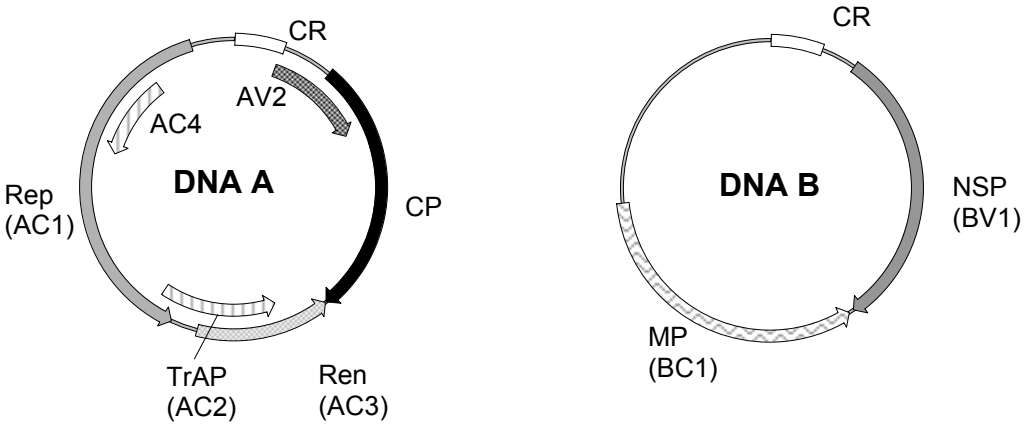

Abb. 1. Schematische Genomorganisation eines bipartiten Begomoviruses. DNA A kodiert sechs *open reading frames* (ORF), zwei in viraler (AV1, AV2) und vier in komplementärer Orientierung (AC1, AC2, AC3 und AC4). DNA B kodiert je ein ORF in jeder Orientierung.

Genexpression und die des Wirtes regulieren können (Pfeffer *et al.*, 2004; Kasschau *et al.*, 2003).

Geminiviren sind Pflanzenpathogene mit einem zirkulär einzelsträngigen DNA-Genom, das in Zwillingspartikeln aus zwei unvollständigen Ikosaedern verpackt ist (Böttcher *et al.*, 2004; Zhang *et al.*, 2001b). Von dieser einzigartigen Kapsidstruktur leitet sich der Name Geminivirus (lat. *gemini*: Zwilling) ab. Die Familie *Geminiviridae* umfasst vier Gattungen, die aufgrund von Genomstruktur, Wirtspflanze und Übertragungsvektor unterschieden werden (Rybicki *et al.*, 2000). Viren der Gattung *Mastrevirus* (Typus *Maize streak virus*) haben ein monopartites Genom und werden durch Zikaden (*Cicadellidae*) auf monokotyledone Wirtspflanzen übertragen. Dabei stellt Mais die ökonomisch wichtigste Wirtspflanze dar. Die Genomorganisation der Mastreviren unterscheidet sich durch das Auftreten einer *long intergenic region* (LIR) und einer *short intergenic region* (SIR) von der aller anderen Geminiviren. Mitglieder der Gattung *Curtovirus* (Typus *Beet curly top virus*) haben ein monopartites Genom und werden durch Zikaden (*Cicadellidae*) übertragen, unterscheiden sich jedoch von den Mastreviren durch Infektion dikotyledoner Pflanzen und die Genomorganisation. Die Gattung *Topocuvirus* umfasst als einzigen Vertreter und gleichzeitig Typus das *Tomato pseudo-curly top virus* (TPCTV). Die Genomorganisation ähnelt in Teilen der von Curto- und Begomoviren (Briddon *et al.*, 1996). TPCTV wird durch *Micrutalis malleifera* (*Membracidae*; Zikade) übertragen, einen bis dahin für die Übertragung von Geminiviren nicht bekannten Vektor. Die Gattung *Begomovirus* (Typus *Bean*

golden mosaic virus) ist die Gruppe mit der größten wirtschaftlichen Relevanz, da sie viele wichtige Nutzpflanzen (Bohnen, Maniok, Baumwolle, Tomate u.a.) infizieren. Die Mehrzahl der Begomoviren hat ein bipartites Genom, es treten jedoch auch Viren mit monopartitem Genom auf. Übertragen werden Begomoviren durch die Weiße Fliege.

Die Komponenten des zweigeteilten Genoms der Begomoviren werden als DNA A und DNA B bezeichnet (Abb. 1). Die DNA A kodiert fünf bis sechs Offene Leserahmen (ORF), ein oder zwei in viraler (AV1 - 2) und vier in komplementärer (AC1 - 4) Orientierung. AV2 tritt nur in Alte-Welt-Begomoviren (Afrika, Asien, Australien und Europa) auf und hat kein entsprechendes Homolog in Neue-Welt-Viren (Amerika) (Rybicki *et al.*, 2000). Der ORF AV1 kodiert das Kapsidprotein (CP: *coat protein* , AV1). Die ORFs *replication associated protein* (Rep, AC1), *transcriptional activator protein* (Trap, AC2) und *replication enhancer protein* (Ren, AC3) sind für die Replikation und Regulation der Transkription notwendig (Hanley-Bowdoin *et al.*, 2000). Die Funktion des ORF AV2 ist nicht eindeutig geklärt (Padidam *et al.*, 1996; Etessami *et al.*, 1989). Auf der DNA B ist jeweils ein ORF in viraler (BV1) und komplementärer (BC1) Orientierung kodiert. Die DNA B Komponenten sind für die Zell-zu-Zell Ausbreitung (*movement protein* [MP], BC1) bzw. für den intrazellulären Transport (*nuclear shuttle protein* [NSP], BV1) notwendig (Zhang *et al.*, 2001a; Ingham *et al.*, 1995; Etessami *et al.*, 1988). Die Nukleotidsequenzen von DNA A und DNA B eines Virus haben bis auf eine etwa 200 Nukleotid große Region, die sog. *common region* (CR) keine Homologie untereinander. Innerhalb der CR befinden sich Promotorelemente, eine Bindungsstelle für das Rep-Protein sowie der Replikationsursprung in einer hochkonservierten Haarnadelstruktur (Hanley-Bowdoin *et al.*, 2000). Da Geminiviren keine eigene Polymerase kodieren, ist die virale Replikation von der zellulären Replikationsmaschinerie abhängig. Durch eine Infektion kann der Zellzyklus aus der Ruhephase (G1-Phase) in die DNA-Synthesephase (S-Phase) geleitet werden (Nagar *et al.*, 2002). Die Replikation des viralen Genoms mittels *rolling circle replication* (RDR) bzw. *recombination dependent replication* (RDR) (Jeske *et al.*, 2001) findet im Zellkern statt. Die Bildung von chimären Genomen durch Rekombination verschiedener Gattungen (Briddon *et al.*, 1996; Stanley and Townsend, 1986) und Familien (Saunders *et al.*, 2002; Saunders and Stanley, 1999)

in mischinfizierten Pflanzen kann zu einer schnellen evolutionären Anpassung an Wirt und Umweltbedingungen führen. Diese hohe Variabilität macht eine Kontrolle der Viren schwierig und geeignete Pflanzenschutzmaßnahmen problematisch.

Neue Forschungsergebnisse haben gezeigt, dass Satelliten-DNA mit Begomoviren assoziiert sein kann (Dry *et al.*, 1997). Zur vollständigen phänotypischen Ausbildung der Krankheit in Baumwolle (Briddon *et al.*, 2001) und Ageratum (Stanley *et al.*, 1997) bedarf es einer sog. DNA β, einer etwa 1600 Nukleotide großen DNA Komponente, deren DNA-Sequenz bis auf den Replikationsursprung in einer typischen Haarnadelstruktur keine Homologie zu Begomoviren aufweist. Die Replikation und Verbreitung der DNA β ist von Helferviren abhängig. Bisher wurde eine Assoziation von DNA β ausschließlich mit monopartiten Begomviren gezeigt (Briddon *et al.*, 2003).

Ziel dieser Arbeit war es, die Biodiversität Maniok-assoziierter Begomoviren in Indien zu untersuchen. Die aktuelle Darstellung der Verbreitung und des Auftretens verschiedener Viren kann als Grundlage für Prognosen des weiteren Verlaufs von Infektionen oder möglichen Epidemien dienen. Neben der Beschreibung der Situation in Indien sollten Maniok-infektiöse Klone verschiedener Begomoviren erstellt werden, die in Züchtungsprogrammen zur Testung neuer Manioksorten eingesetzt werden sollten. Funktionsanalysen einzelner viraler Gene, sowie die Gewebelokalisation des Virus in infizierten Pflanzen sollen Einblicke in den Infektionsverlauf geben.

Als Grundlage dieser Arbeit und für spätere Resistenztestungen wurde *Indian cassava mosaic virus* (ICMV) DNA A und DNA B aus einer durch Pflanzensaft infizierten Tabakpflanze (*Nicotiana tabacum*) isoliert und kloniert. Die Inokulation von gesunden Tabak- bzw. Maniokpflanzen mit der klonierten viralen DNA führte zu einer systemischen und symptomatischen Infektion. Die Infektion konnte in beiden Fällen mittels *Southern blot* Analyse nachgewiesen werden. Somit wurden die Koch´schen Postulate erfüllt und die Vermutung, dass Begomoviren auch in Indien für das Auftreten der CMD verantwortlich sind, bestätigt. (**Reintroduction of cloned Indian cassava mosaic virus (ICMV) DNA into cassava proves its aetiology**, Seite 26).

Die große Biodiversität innerhalb der Geminiviren wurde auf die verstärkte Evolution durch Rekombination zwischen Familien und Gattungen zurückgeführt (Padidam *et al.*, 1999). Durch diese forcierte Evolution konnten sich Viren an Wirte

anpassen und neue Virusstämme entwickelten sich, die verheerende Epidemien auslösten. Um die momentane Situation der Biodiversität von Maniok-infizierenden Begomoviren in Südindien zu erhalten, wurden Proben (Stecklinge) von phänotypisch kranken Maniokpflanzen gesammelt und auf die darin enthaltenen Viren untersucht. Die gesammelten Stecklinge wurden regeneriert und virale DNA mittels Polymerase-Kettenreaktion (PCR) aus Gesamt-DNA-Isolationen amplifiziert. Durch Restriktions-Fragmentlängen-Polymorphismus (RFLP) Analysen konnten verschiede Gruppen von Begomoviren charakterisiert werden. Zur genaueren Untersuchungen der Biodiversität wurden komplette DNA A und DNA B Moleküle aus Maniok kloniert und sequenziert. Die Sequenzanalysen zeigten das weitverbreitete Auftreten von *Sri Lankan cassava mosaic virus* (SLCMV) in Südindien. (**Biodiversity of cassava-infecting begomoviruses in South India**, Seite 37).

Aus früheren Untersuchungen waren zwei unterschiedliche Gewebelokalisierungen von Geminiviren bekannt: eine strikte Beschränkung des Virus auf Leitgewebe bzw. die Verbreitung in allen Gewebearten (z.B. Wege *et al.*, 2001). Bipartite Geminiviren können in beiden Lokalisationsmustern auftreten. Dabei lassen sich keine Rückschlüsse von der phänotypischen Symptomausprägung auf die Gewebe-lokalisation ziehen. In unterschiedlichen Experimenten mit verschiedenen Viren wurde einerseits ein Einfluss der Wirtspflanze auf die Gewebelokalisation festgestellt, andererseits war bei vielen Begomoviren die Gewebelokalisation unabhängig vom Wirt. Aufgrund dieser Kenntnisse wurde die Gewebeverteilung des ICMV im natürlichen Wirt (Maniok) als auch im experimentellen Wirt (Tabak) durch *in-situ* Hybridisierungsstudien bestimmt. (**Tissue tropism of *Indian cassava mosaic virus* (ICMV) in natural and experimental plants**, Seite 54)

Um einen besseren Einblick in den Infektionsverlauf zu erhalten, sollten nicht nur die Gewebelokalisation des ICMV bestimmt, sondern auch die Zell-zu-Zell Ausbreitung untersucht werden. Da bisherige Ergebnisse zur Charakterisierung der Funktion des ORF AV2 widersprüchlich waren, wurde dessen Funktion mit Hilfe von *green fluorescence protein* (GFP)-Fusionsproteinen in systemisch infizierten und transient exprimierenden *N. benthamiana* Pflanzen bzw. Blättern untersucht. Es war bekannt, dass eine systemische Ausbreitung von CP-mutierten Geminiviren in Pflanzen möglich ist. Chimäre Viren, die entweder einen Austausch des *CP* Gens gegen das GFP-Gen enthielten oder ein AV2-GFP Fusionsprotein in CP-deletierten

Viren exprimierten wurden zur Untersuchung eingesetzt. Zusätzlich wurde ein GFP-AV2 Fusionsprotein unter der Kontrolle des *Cauliflower mosaic virus* (CaMV) 35S Promotor (pGFP-AV2) erstellt. Durch dieses transient exprimierende Plasmid konnte die Funktion des ORF AV2 ohne viralen Hintergrund untersucht werden. Durch Partikelbeschuss von *N. benthamiana* Blättern mit pGFP-AV2 konnte für den ORF AV2 eine Transportfunktion gezeigt werden, die möglicherweise die evolutionäre Entwicklung bipartiter Begomoviren widerspiegelt. Zusätzlich zu der AV2 Funktionsbestimmung konnte die Phloemlimitierung in systemisch infizierten Pflanzen bestätigt werden. (**GFP-labelled AV2 protein of *Indian cassava mosaic virus* moves cell-to-cell**, Seite 68).

Ergebnisse und Diskussion

In dieser Arbeit wurden Untersuchungen an Maniok-infizierenden Begomoviren, die in Südindien auftreten, durchgeführt. Das *Indian cassava mosaic virus* (ICMV) und das *Sri Lankan cassava mosaic virus* (SLCMV) aus unterschiedlichen Herkünften wurden als Untersuchungsobjekte eingeführt. Verschiedene molekularbiologische Methoden zur Charakterisierung der Biodiversität von Begomoviren, der Erstellung Maniok-infizierender Klone sowie der Bestimmung der Gewebelokalisation in systemisch infizierten Pflanzen wurden angewendet.

Klonierung eines Maniok-infizierenden ICM-Virus

Aus einer ICMV-infizierten Tabakpflanze (von P. Markham, John Innes Centre, Großbritannien, freundlicherweise zu Verfügung gestellt) wurde virale DNA über eine CsCl-Dichtegradienten-Ultrazentrifugation isoliert. Beide DNA Komponenten des bipartiten ICMV wurden über singuläre Restriktionsschnittstellen kloniert und ihre Nukleotidsequenz bestimmt (Reintroduction of cloned *Indian cassava mosaic virus* (ICVM) DNA into cassava proves its aetiology, Seite 26). Durch mindestens dreifache Sequenzierung der Inserts konnten Fehler in der Sequenz ausgeschlossen werden. Die Sequenz der DNA A umfasste danach 2733 Nukleotide (nt), die der DNA B 2643 nt. Die Genomorganisation war der von anderen Begomoviren ähnlich. DNA A enthielt sechs Open Reading Frames (ORF), zwei in viraler und vier in komplementärer Orientierung. ICMV DNA B kodierte für je einen ORF in jeder Orientierung. Sequenzhomologie zwischen DNA A und DNA B trat nur in der *common region* (CR) auf (DNA A 2601-82 nt, DNA B 2511-82 nt). Die CR enthält Promotorelemente, eine Rep-Bindungsstelle sowie eine potentielle Haarnadelstruktur. Innerhalb dieser Haarnadelstruktur liegt das in allen Geminiviren auftretende, hochkonservierte Nonanukleotid (TAATATT_AC), das den *rolling-circle* Replikationsstartpunkt enthält. Der Datenbank-Sequenzvergleich erbrachte die größte Homologie zu ICMV-[Mah], einem ICMV-Isolat aus Westindien (Accession Nummer AJ314737/AJ314738) (Saunders *et al.*, 2002). Die Homologie beträgt 98,5% zur DNA A bzw. 95,1% zur DNA B. Während nur einzelne Nukleotidaustausche innerhalb der DNA A Sequenz auftraten, wurden in der DNA B zwei Sequenzdeletionen festgestellt (77-138 nt und 2043-2052 nt). Direkte Sequenzwiederholungen in der CR, wie von Hong *et. al* (1993) beschrieben, traten weder in diesem Isolat noch in ICMV-[Mah] auf. Die Funktion dieser

Sequenzwiederholungen ist unklar, da beide Isolate infektiös sind und diese Sequenzwiederholungen keine wesentliche Rolle im Infektionsverlauf zu spielen scheinen.

Um die biologische Funktionalität des ICMV Klons zu testen, wurden *Nicotiana* spec. durch Aufreiben von DNA oder durch Partikelbeschuss mit DNA-beschichteten Goldpartikeln inokuliert. *N. benthamiana* Pflanzen konnten mit beiden Methoden, Tabakpflanzen hingegen nur durch Partikelbeschuss infiziert werden. Erste Symptome traten nach 10 Tagen auf. Die Infektiosität des Klons lag bei durchschnittlich 20%. Infizierte Pflanzen zeigten die gleichen Blattdeformationen wie das Ausgangsmaterial. Die Infektion wurde mittels PCR und Southern blot Analyse bestätigt. Virale DNA konnte nur in phänotypisch erkrankten, nicht aber in mock-inokulierten Pflanzen nachgewiesen werden. Somit wurden die Koch´schen Postulate für diesen Klon erfüllt. Eine Infektion von Maniok-Stecklingen im drei- bis fünf-Blatt-Stadium konnte in der Partikel-Beschuss-Kammer nicht durchgeführt werden, da kein Steckling die Evakuierung und den Beschuss überlebte. Maniokpflanzen in einem älteren Wachstumsstadium wurden von R. Briddon am John Innes Centre, Norwich, Großbritannien mittels einer Hand-Partikelpistole inokuliert. Zwei Monate nach der Inokulation konnten erste Symptome festgestellt werden. Durch *Southern blot* Analysen mit DNA A- und DNA B- spezifischen Sonden wurde die Infektion bestätigt. Damit konnte zum ersten mal gezeigt werden, dass ICMV für die Ausbildung der Maniok Mosaik Krankheit (engl. *Cassava mosaic disease*, CMD) in Indien ursächlich ist. Die Annahme, dass wiederholtes Passagieren von Viruspopulationen in experimentellen Wirten zu einer selektierten Population führt, die den natürlichen Wirt nicht mehr infizieren kann (Liu *et al.*, 1997), traf für dieses Isolat nicht zu. Ursachen dafür, dass die bisher beschriebenen ICMV Klone (Saunders *et al.*, 2002; Hong *et al.*, 1993) in Maniok nicht infektiös sind, konnten aus der Nukleotidsequenz nicht abgeleitet werden.

Der Austausch von DNA Komponenten unter Bildung lebensfähiger Viren wurde als Pseudorekombination beschrieben und ist im Allgemeinen auf nahverwandte Viren beschränkt (Polston and Anderson, 1997; Sung and Coutts, 1995; Frischmuth *et al.*, 1993; von Arnim and Stanley, 1992b; von Arnim and Stanley, 1992a; Lazarowitz, 1991; Stanley *et al.*, 1985). Die Beschränkung der Bildung lebensfähiger Pseudorekombinanten zwischen Virusisolaten wurde aufgrund der virusspezifischen Funktionsweise des Rep-Proteins angenommen (Orozco *et al.*, 1998; Fontes *et al.*,

1994a; Fontes *et al.*, 1994b). Eine Pseudorekombination zwischen ICMV und ACMV, konnte nicht beobachtet werden. Unterschiede in der Iteronsequenz, die für eine effiziente Bindung des Rep-Proteins notwendig ist und damit die Replikation einleitet (Arguello-Astorga *et al.*, 1994; Fontes *et al.*, 1994a), könnten die Inkompatibilität der ICMV und ACMV Komponenten zueinander erklären.

Für einige bipartite Begomoviren wurde eine autonome Ausbreitung der DNA A Komponente beschrieben (Evans and Jeske, 1993; Klinkenberg and Stanley, 1990). Für das *Tomato yellow leaf curl virus* (TYLCV) konnten monopartite (Kheyr-Pour *et al.*, 1991; Navot *et al.*, 1991) und bipartite (Rochester *et al.*, 1990) Vertreter gefunden werden, jedoch wird die DNA B der bipartiten TYLC-Viren für eine systemische Infektion nicht benötigt (Rochester *et al.*, 1990). Das bipartite SLCMV kann *Nicotiana* spec. durch beide DNA Komponenten oder nur durch DNA A infizieren, wobei sich die Symptome deutlich voneinander unterscheiden (Saunders *et al.*, 2002). Eine ICMV DNA A autonome Ausbreitung in *N. benthamiana*, wie für ACMV DNA A beschrieben (Klinkenberg and Stanley, 1990), konnte nicht gezeigt werden.

Da der, in dieser Arbeit klonierte ICMV Klon eine systemische Infektion in der natürlichen Wirtspflanze auslöst, werden neue Forschungsgebiete möglich. Die Züchtungsdauer ICMV-resistenter Manioksorten kann durch die effiziente Infektion dieser Pflanzen verkürzt werden. Darüber hinaus können nun Untersuchungen des Infektionsverlaufs nicht nur in experimentellen Pflanzen, sondern auch im natürlichen Wirt durchgeführt werden.

Biodiversität von Maniok-assoziierten Begomoviren in Südindien

Untersuchungen in Afrika zeigten, dass das Auftreten verschiedener Virustämme in einer gemeinsamen Region die Bildung von Chimären durch Rekombination begünstigt. Rekombinante Viren aus *African cassava mosaic virus* (ACMV) und *East African cassava mosaic virus* (EACMV) traten in den 90er Jahren des Zwanzigsten Jahrhunderts während verheerender Epidemien in Uganda und Kamerun auf (Pita *et al.*, 2001; Fondong *et al.*, 2000; Zhou *et al.*, 1997). Daten über die biologische Varianz Maniok-infizierender Begomoviren in Indien sind selten und somit sind Vorhersagen über die Weiterentwicklung der Viren und deren Auswirkung auf die Landwirtschaft schwierig. Um die Biodiversität CMD-auslösender Begomoviren in Indien zu untersuchen, wurden Stecklinge phänotypisch infizierter Maniok-Pflanzen in den südindischen Bundesstaaten Tamil Nadu und Kerala gesammelt (Biodiversity

of cassava-infecting begomoviruses in India, Seite 37). Aus den regenerierten Stecklingen wurde Gesamtnukleinsäure isoliert, um einerseits die in allen Pflanzen enthaltenen Viren mittels *Southern blot* Analyse nachzuweisen und andererseits mittels Polymerase-Kettenreaktion (PCR) virale DNA zu amplifizieren. Um die Biodiversität PCR-amplifizierter DNA zu bestimmen, wurde eine Restriktions-Fragmentlängen-Polymorphismus (RFLP) Analyse mit den Restriktionsenzymen *Eco*R I, *Hpa* II und *Sau*3A I durchgeführt. Dadurch wurden sechs verschiedene Begomovirus-Gruppen identifiziert. PCR-basierende RFLP-Analysen wurden zur Bestimmung der biologischen Varianz innerhalb verschiedener Geminivirus-Gattungen verwendet (Jovel *et al.*, 2004; Martin *et al.*, 2001; Willment *et al.*, 2001). Eine Beschränkung einzelner Virusisolate auf bestimmte geographische Bereiche konnte nicht gefunden werden. Durch das Auftreten verschiedener Virusisolate in einer Region kann es zu Mischinfektionen in Wirtspflanzen kommen. Dies wurde im Falle der Pflanze 14 nachgewiesen, in der zwei unterschiedliche Virusisolate auftraten. Mischinfektionen sind eine Voraussetzung für die Bildung neuer Virusstämme durch Rekombination oder Pseudorekombination, den Austausch von Genomkomponenten zwischen zwei verschiedenen Viren. Wegen der spezifischen Interaktion zwischen Rep-Protein und der Rep-Bindungsstelle (Orozco *et al.*, 1998; Fontes *et al.*, 1994a; Fontes *et al.*, 1994b) wurde zunächst angenommen, dass die Bildung lebensfähiger Pseudorekombinationen auf Isolate eines Virus beschränkt ist. Pseudorekombinationen können aber auch zwischen verschiedenen Viren vorkommen (Unseld *et al.*, 2000a; Unseld *et al.*, 2000b; Frischmuth *et al.*, 1997; Höfer *et al.*, 1997; Gilbertson *et al.*, 1993). Hou und Gilbertson (1996) führten die gesteigerte Pathogenität von Pseudorekombinanten aus *Bean dwarf mosaic virus* (BDMV) und *Tomato mottle virus* (ToMoV) auf nachfolgende Rekombinations-ereignisse zurück, durch die die CR einer DNA Komponenten auf die andere, heterologe übertragen wurde. Jovel *et. al*. (2004) konnte ebenfalls die Übertragung der CR zwischen verschidenen *Sida micrantha mosaic* (SimM)-Viren in einer inifzierten Pflanze zeigen. Computerunterstütze Sequenzvergleiche der Rep-Bindungsstelle (Iterons) und von Promotorelementen innerhalb der CR zeigten eine große Übereinstimmung der neu isolierten DNA A Komponenten mit SLCMV aber Unterschiede zu ICMV. Saunders *et al*. (2002) haben gezeigt, dass Pseudo-rekombinanten aus SLCMV und ICMV symptomatische, systemische Infektionen

auslösen können. Ob dabei ein Austausch der CR durch Rekombination zur Bildung von lebensfähigen Pseudorekombinanten führte, wurde nicht bestimmt.

Die Sequenzierung von sechs vollständigen DNA As der Begomovirusisolate aus Südindien ergab eine Genomorganisation wie bei anderen Alte-Welt-Begomoviren. Die Sequenzen hatten die größte Verwandtschaft zu SLCMV und nicht, wie zunächst erwartet, zu ICMV. Damit ist erstmals gezeigt, dass SLCMV auf dem indischen Subkontinent weitverbreitet auftritt und häufiger als ICMV vorkommt. Ähnliche Ergebnisse wurden zeitgleich von den indischen Kooperationspartnern, I. Dasgupta *et al*., erhalten. Dendrogramme wurden mit *neighbour joining* and *bootstrap* (1000 Wiederholungen) in dem Programm Clustal X 1.81 berechnet. Wegen der geringen Sequenzvariabilität der sechs neuen Isolate wurden diese in einer Gruppe zusammengefasst, von der sich nur die Probe C4, die eine größere Homologie zu SLCMV zeigte, absetzte. Die südindische Gruppe der Maniok-infizierenden Begomoviren teilt sich danach in SLCMV-ähnliche und ICMV-ähnliche Viren auf. Die Gruppe der zentralafrikanischen CMD-auslösender Viren war deutlich verschieden.

Die Biodiversitäts-Untersuchungen zeigten mehrere neue Aspekte der CMD-auslösenden Begomoviren in Indien. Neben den bereits beschriebenen ICM-Viren tritt weitverbreiteter das SLCMV in Südindien auf. Eine kontinuierliche Beobachtung der Entwicklung ist für Voraussagen über den weiteren Verlauf der CMD in Indien unabdingbar.

Gewebelokalisation des ICMV in verschiedenen Pflanzenarten

Während einer Infektion können Geminiviren verschiedene Gewebe infizieren. Virale DNA akkumuliert dabei in zwei grundlegend verschiedenen Mustern. Entweder mit einer Beschränkung der Infektion auf das Leitgewebe (Phloemlimitierung), oder mit einer zusätzlichen Ausbreitung in Schwamm-, Palisadenparenchym und Epidermiszellen. Die Gewebelokalisation von ICMV ist im natürlichen (Maniok) und experimentellen Wirt (*N. tabacum*) mittels *in situ* Hybridisierung bestimmt worden (Tissue tropism of *Indian cassava mosaic virus* (ICMV) in natural and experimental plants, Seite 53). Dabei traten Signale häufiger in *N. tabacum* als in Maniok auf. Die Lokalisation des Virus war unabhängig von der Pflanzenart und immer auf das Leitgewebe beschränkt. Diese Ergebnisse stimmen mit früheren Untersuchungen von Roberts (1989) überein, in denen gezeigt wurde, dass infektionsbedingte Änderungen der zellulären Ultrastruktur meist in phloem- und xylemassoziierten

Zellen auftreten und Viren in Zellen außerhalb des Leitgewebes nur selten detektiert werden. Gewebeabdruck-Analysen von infizierten *N. benthamiana* Sprossachsen zeigten, dass sowohl ICMV DNA A als auch DNA B mit dem Leitgewebe assoziiert sind (D. Haible, Studienarbeit, 2004). Das gemeinsame Auftreten von DNA A und DNA B spiegelt die Interaktion der beiden Komponenten bei der Replikation und dem Transport wider. Die Replikation der DNA B ist von DNA A kodierten Proteinen abhängig (Hanley-Bowdoin *et al.*, 2000), während der intra- und interzelluläre Transport durch die auf der DNA B kodierten Proteine BC1 und BV1 vermittelt wird (Lazarowitz and Beachy, 1999; Gilbertson and Lucas, 1996).

Für die meisten Begomoviren, wie z.B. *Abutilon mosaic viurs* (AbMV) (Wege *et al.*, 2001; Horns and Jeske, 1991; Abouzid *et al.*, 1988), *Sida micrantha mosaic virus* (SimMV) (Jovel *et al.*, 2004; Wege *et al.*, 2001), *Tomato yellow leaf curl virus* (TYLCV) (Morilla *et al.*, 2004) und *Bean golden mosaic virus* (BGMV) (Morra and Petty, 2000; Kim *et al.*, 1978) konnte ebenfalls eine wirtsunabhängige Phloemlimitierung gezeigt werden. Seltener können Begomoviren Gewebe außerhalb der Leitbahnen effizient infizieren (Wege *et al.*, 2001; Wang *et al.*, 1996). Dabei war besonders interessant, dass *African cassava mosaic virus* (ACMV), welches ebenfalls Maniok als natürlichen Wirt hat, effizient alle Gewebearten infizieren kann (Wege *et al.*, 2001), während ICMV auf das Leitgewebe beschränkt ist. Untersuchungen mit Hybridviren aus *Bean golden mosaic virus* (BGMV) und den mesophyllinvasiven Begomoviren *Tomato golden mosaic virus* (TGMV) und *Cabbage leaf curl virus* (CabLCV) lassen erkennen, dass die Gewebespezifität genetisch kodiert ist (Qin and Petty, 2001; Morra and Petty, 2000). Neben einer essentiellen nicht-kodierenden Region vor dem ORF BV1 sind entweder die ORFs AV2/3 und BV1/BC1 für eine effiziente Infektion außerhalb des Leitgewebes notwendig (Qin and Petty, 2001; Morra and Petty, 2000). Der Austritt aus dem Phloemgewebe war dabei unabhängig von der DNA Akkumulation und Symptomausprägung (Qin and Petty, 2001).

Eine wirtsabhängige Gewebelokalisation wurde für das *Euphorbia mosaic virus* (EuMV) gezeigt (Kim and Lee, 1992). Eine Interaktion zwischen geminiviralen Proteinen und Wirtsfaktoren wird zudem durch die virale Replikation nahegelegt. Geminiviren kodieren keine Polymerase und sind von der zellulären Replikationsmaschinerie abhängig. Bei einer Infektion muss daher in der Zelle ein Milieu geschaffen werden, das eine Virusvermehrung ermöglicht. TGMV AC1

induziert die Akkumulation des *proliferation cell nuclear antigen* (PCNA) (Nagar *et al.*, 1995), einem Faktor der DNA Polymerase δ. Differenzierte Zellen werden so stimuliert, in die S-Phase einzutreten (Nagar *et al.*, 2002) und ermöglichen dadurch die Replikation der Geminiviren. Eine Beeinträchtigung der Interaktion zwischen TGMV AC1 und pflanzlichen Homologen des Zellzyklusregulatorproteins Retinoblastoma (pRBR) beschränken das Virus auf Phloemgewebe (Kong *et al.*, 2000). Aufgrund dieser Ergebnisse ist zu vermuten, dass Interaktionen von Wirts- und Virusproteinen die Gewebelokalisation mitbestimmen. Gewebespezifische TGMV Promotoraktivierung bzw. -reprimierung von Genen in viraler Orientierung konnte in infizierten Pflanzen nachgewiesen werden (Sunter and Bisaro, 1997). Eine vollständige gewebespezifische Repression des ICMV Kapsidprotein Promoters kann zumindest für *N. benthamiana* Epidermiszellen ausgeschlossen werden. Eine ICMV Mutante, die an Stelle des *CP* Gens das *green fluorescence protein* (*GFP*) Gen enthielt, exprimierte GFP nach Partikelbeschuss in *N. benthamiana* Blättern (GFP-labelled AV2 protein of ICMV moves cell to cell, Seite 67). Dies legt den Schluss nahe, dass die Ausbreitung von ICMV im Übergang zwischen Phloem und Mesophyll blockiert wird oder spezifische Resistenzmechanismen im Mesophyll zum Tragen kommen und eine Ausbreitung unterbinden.

Es wurde vorgeschlagen, dass die Entwicklung von Symptomen phloemlimitierter Viren in Meshophyllzellen durch einen gestörten Kohlenhydratabtransport zustande kommt (Jeske and Werz, 1978). Neuere Untersuchungen zeigten Interaktionen von viralen Proteinen mit Regulatoren des Metabolismus. Sowohl TGMV AC2 als auch BGMV C2 interagieren und inaktivieren eine Adenosinkinase (ADK) (Wang *et al.*, 2003) sowie eine *sucrose nonfermenting 1* (SNF1) Kinase (Hao *et al.*, 2003), ein Hauptregulatorprotein des Kohlenhydrat-Metabolismus (Halford *et al.*, 2003; Hrabak *et al.*, 2003). Eine Störung der Interaktion zwischen SNF1 Kinase und ADK durch virale Proteine könnte daher zu Wachstumsstörungen führen. Weitere Interaktionen wurden mit rezeptorähnlichen Kinasen (Mariano *et al.*, 2004) und mit dem Sumoylations-System in Pflanzen (Castillo *et al.*, 2004) gezeigt. Die Entwicklung der gesamten Pflanze könnte so durch Interferenz von viralen Proteinen mit endogenen Signalwegen beeinträchtigt werden. Ein Verteidigungsmechanismus der Pflanzen gegen Virusinfektionen ist möglicherweise das *posttranscriptional gene silencing* (PTGS) (Voinnet, 2001). Über RNA Signale werden Fremdgene erkannt und deren mRNA in einem konservierten Mechanismus degradiert. Endogene micro (mi) RNAs,

die die Entwicklung regulieren, haben eine ähnliche Struktur, wie die am PTGS beteiligten RNA Signale (Bartel, 2004). Durch Repression der Genexpression bzw. Degradation von mRNA werden entwicklungsspezifische Gene reguliert. Virale Suppressoren des PTGS können sowohl den Abwehrmechanismus blockieren als auch mit miRNAs interagieren. Neben dieser indirekten Auswirkung auf den miRNA-Signalweg, wurde für humane DNA-Viren eine direkte Interaktion mit der Zellentwicklung gezeigt (Pfeffer *et al.*, 2004). Viren kodieren hierbei miRNAs, die sowohl die eigene Genexpression als auch die der Wirtszelle regulieren können. Durch den Eingriff phloemlimitierter Viren in diese zentralen Entwicklungssignale wäre eine Symptombildung außerhalb des Leitgewebes denkbar. Eine Detektion von RNA Signalen war durch die in dieser Arbeit angewandten Untersuchungsmethode der *in situ* Hybridisierung nicht möglich, die eine RNase A Behandlung der Schnitte einschloss. Die Anpassung dieser Methode zur Detektion von RNA könnte neue Einblicke in die Symptommodulation geben.

Ausbreitung und Replikation von ICMV-Chimären

Durch GFP-exprimierende ICMV-Chimären sollte eine Infektion frühzeitig und leicht detektierbar werden. Die Testung neuer Manioksorten auf Resistenz könnte dadurch vereinfacht und die Züchtungsdauer verkürzt werden. Der Verlauf einer ICMV Infektion wurde mit chimären ICMV DNA A-Konstrukten untersucht. Es war bereits bekannt, dass sich verschiedene bipartite Geminiviren ohne *CP* systemisch in Pflanzen ausbreiten können (Sudarshana *et al.*, 1996; Ingham *et al.*, 1995; Padidam *et al.*, 1995). Diesen Umstand ausnutzend, wurde das *CP* Gen gegen das *GFP* Gen ausgetauscht. Hierbei wurde allerdings auch der C-terminale Teil des ORF AV2 deletiert [ICMV DNA A(ΔCPΔAV2-GFP)]. Das Konstrukt exprimierte unfusioniertes GFP unter der Kontrolle des Kapsidproteinpromotors nach Partikelbeschuss in *N. benthamiana* Epidermiszellen, obwohl das Virus systemisch phloemlimitiert blieb. Weitere chimäre ICMV DNA As wurden von der *CP*-deletierten ICMV DNA A ausgehend erstellt: ICMV DNA A(ΔCP-AV2:GFP), das ein Fusionsprotein aus AV2 und GFP exprimierte und ICMV DNA A(ΔCP-AV2stopGFP), das ein Stop-Codon zwischen *AV2* und *GFP* enthielt. Alle chimären ICMV DNA As in Kombination mit ICMV DNA B erzeugten in *N. benthamiana* systemische und symptomatische Infektionen (GFP-labelled AV2 protein of *Indian cassava mosaic virus* moves cell to cell, Seite 67). Die Symptome traten gegenüber einer Wildtyp (wt) ICMV Infektion mit

einer Verzögerung von 5-7 Tagen auf. Durch den Austausch zwischen *CP* und *GFP* wurde in allen Chimären die Gesamtgröße der DNA A weitgehend erhalten. Größenänderungen im Genom von Geminiviren werden im Allgemeinen während der Replikation und des Transports revertiert (Gilbertson *et al.*, 2003; Bisaro, 1994; Etessami *et al.*, 1989). Die stabile Integration des *GFP* Gens in den chimären ICMV DNA As wurde jedoch durch *Southern blot* Analyse systemisch infizierter Blätter bestätigt. Dabei zeigte sich, dass chimäre ICM-Viren geringere Mengen viraler DNA als wt ICMV akkumulierten. Auch bei anderen *CP*-mutierten Geminiviren wurde eine solche Reduktion der viralen DNA beschrieben (Padidam *et al.*, 1996; Rigden *et al.*, 1993; Etessami *et al.*, 1989; Stanley and Townsend, 1986). Während bei einer wt ICMV Infektion größere Mengen einzelsträngiger (ss)DNA synthetisiert wurden, wurden bei Infektionen mit chimären ICM-Viren etwa gleiche Mengen an ssDNA und supercoiled (sc) doppelsträngiger DNA gefunden (Abb. 2, Seite71). Auffallend war, das die Akkumulation von ssDNA in der AV2-deletierten Mutante deutlich erniedrigt war. Das AV2 Protein könnte deshalb einen Einfluss auf den Wechsel zwischen ssDNA und dsDNA Synthese haben. Für BCTV V2 bzw. TYLCV V1 Proteine, deren ORFs an der gleichen Position wie ICMV AV2 liegen, wurde ein ähnlicher Einfluss auf die DNA-Synthese beschrieben (Wartig *et al.*, 1997; Hormuzdi and Bisaro, 1993).

Funktionsanalyse des ORF AV2

Fluoreszenzmikroskopische Untersuchungen der Ausbreitung von ICMV DNA A(ΔCPΔAV2-GFP) und ICMV DNA A(ΔCP-AV2:GFP) in systemisch infizierten *N. benthamiana* zeigten, dass die GFP-Signale ausschließlich mit Leitgeweben assoziiert waren (siehe Seite 67ff.). Dies entspricht der Phloemlimitierung des ICMV in den *in situ* Hybridisierungsstudien (siehe Seite 53ff.). Die Signale der ICMV DNA A(ΔCP-AV2:GFP) unterschieden sich dabei deutlich von denen des unfusionierten GFPs (Abb3. Seite 72). Während ICMV DNA A(ΔCPΔAV2-GFP) ein kontinuierliches GFP Signal entlang der Leitbahnen hat, ist das AV2:GFP Fusionsprotein punktförmig entlang den Leitbahnen lokalisiert. In Querschnitten von Blattstielen traten GFP Signale ausschließlich im Phloemgewebe auf. In Längsschnitten konnten einzelne Phloemparenchymzellen untersucht werden. Neben einem dominierenden GFP-Signal in der Zelle traten an der Zellperipherie punktförmige Signale auf. Um diese Signale genauer zu untersuchen und mögliche Funktionen des ORF AV2 zu bestimmen, wurde auf der Grundlage des Plasmids pGFP (Unseld *et al.*, 2001) ein

GFP:AV2 Fusionsprotein unter der Kontrolle des CaMV 35S Promoters (pGFP:AV2) erstellt. Dieses Konstrukt ermöglichte einerseits die einfache Expression des GFP:AV2 Fusionsproteins in *N. benthamiana* Epidermiszellen und andererseits die von anderen Virusproteinen unabhängige Untersuchung von AV2. Die transiente Expression von pGFP:AV2 in Epidermiszellen von *N. benthamiana* erbrachte die gleiche Signalverteilung wie in ICMV DNA A(ΔCP-AV2:GFP) systemisch infizierten Pflanzen (Abb. 4, Seite 73). Epifluoreszenz Mikrokopie zeigte, dass das Fusionsprotein als Punkte an der Zellperipherie und innerhalb der Zelle als Einschlusskörper (*inclusion body*) akkumuliert. Ähnliche punktförmige Signale an der Zellperipherie wurden für GFP-markiertes AbMV BC1 gezeigt (Zhang *et al.*, 2001a). Elektronenmikroskopische Untersuchungen in *Schizosaccharomyces pombe* zeigten, das BC1 überwiegend an der Plasmamembran lokalisiert ist (Aberle *et al.*, 2002). BC1 in Kooperation mit BV1 transportiert das Virus bzw. dessen viralen Transportform von Zelle zu Zelle (Zhang *et al.*, 2001a). Andere GFP-markierte Transportproteine zeigten eine ähnliche Verteilung an der Zellperipherie (Heinlein *et al.*, 1998; Itaya *et al.*, 1998; Oparka *et al.*, 1997). Mittels DAPI-Färbung konnte ausgeschlossen werden, dass der GFP-Einschlusskörper im Zellkern lokalisiert ist. Aufgrund der Multimerisierung von AV2 Proteinen (Padidam *et al.*, 1996) könnte dessen starkes Signal entstehen. Innerhalb der Zellkerne wurden dem gegenüber meist ein bis zwei kleine GFP Signale detektiert, die möglicherweise eine Assoziation mit den Nukleoli anzeigen. Die Möglichkeit einer Assoziation von Geminiviren mit den Nukleoli, die Zentren der viralen Replikation und/oder Genexpression sein könnten, wurde unter anderem von Rojas *et. al.* (2001) vorgeschlagen. Die Verhinderung einer AV2 Akkumulation im Zellkern könnte somit zur Beeinträchtigung der viralen Replikation oder Genexpression führen. In *Tomato leaf curl virus* (ToLCV) AV2 Mutanten wurde eine eingeschränkte DNA Replikation nachgewiesen (Rojas *et al.*, 2001) und in ICMV DNA A(ΔCPΔAV2-GFP) systemisch infizierten Pflanzen das fast vollständige Fehlen von ssDNA gezeigt. Ob das Verteilungsmuster des Fusionsproteins auf eine mögliche Funktion im viralen Transport schließen lässt, wurde weitergehend untersucht. *N. benthamiana* Blätter wurden mit pGFP:AV2 beschossen und der Zell-zu-Zell Transport des GFP:AV2 Fusionsproteins beobachtet. Dabei wurden jeweils die selben Epidermiszellen, die das Fusionsportein exprimierten, 10, 24 und 48 Stunden nach dem Beschuss mikroskopisch verfolgt (Abb. 4, Seite 73). Nach 10 Stunden war das Fusionsprotein in der Zelle als dominante Akkumulation und in

Punktform an der Zellperipherie zu erkennen. Nach 24 Stunden zeigten die Nachbarzellen erste Fluoreszenz-Signale, die nach 48 Stunden sicher nachgewiesen werden konnten. In Kontrollen, die unfusioniertes GFP exprimierten, wurde kein Transport in die Nachbarzellen beobachtet.

Die Funktion des AV2 als Transportprotein wirft natürlich die Frage auf, warum Begomoviren zusätzliche Transportproteine auf der DNA B kodieren. Daneben hat auch das ACMV CP mögliche Funktionen in der intrazellulären Lokalisierung zum Zellkern bzw. zu einem späteren Zeitpunkt zur Zellperipherie (Unseld *et al.*, 2001). Ähnliche Muster treten für die AbMV DNA B Proteine auf (Zhang *et al.*, 2001a). Sowohl SLCMV als auch TYLCV-Thailand DNA A allein können zu einer systemischen und symptomatischen Infektion in Pflanzen führen (Saunders *et al.*, 2002; Rochester *et al.*, 1990). Im Falle von SLCMV DNA A Infektionen ähneln die Symptome der von monopartiten Begomoviren. Durch die DNA B Komponente wird die Symptomausprägung moduliert und infizierte Pflanzen zeigen Symptome, die auf bipartite Begomoviren hindeuten. Aufgrund von Sequenzanalysen wurde spekuliert, dass SLCMV aus einem ehemaligen monopartiten Vorläufervirus entstand, das durch Übertragung seiner CR eine DNA B Komponente eines anderen Virus akquiriert hat. Hinsichtlich der Bedeutung der DNA B für die Pathogenität und den Wirtsbereich, wurde spekuliert, dass eine DNA B die Ausweitung des Wirtsbereichs und zusätzliche Anpassung an den Wirt bedeuten könnte (Ingham *et al.*, 1995). Die auf der DNA B kodierten Proteine könnten Transportfunktionen übernehmen. Durch den multifunktionellen Charakter viraler Proteine, zumindest im Falle des Kapsidproteins und der daraus resultierenden Konservierung der Nukleotidsequenz, würden redundante Funktionen in den ehemals zuständigen Proteinen erhalten bleiben.

Darstellung der Ergebnisse als Publikationsmanuskripte in englischer Sprache

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Reintroduction of cloned *Indian cassava mosaic virus* (ICMV) DNA into cassava proves its aetiology

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Abstract

Begomoviruses (*Geminiviridae*) are serious plant pathogens infecting dicotyledonous plants among important crop plants. The majority of the members comprises bipartite genomes (DNA A and B), encapsidated in twin particles and transmitted by the whitefly-vector *Bemisia tabaci*. Cassava (*Manihot esculenta*, Crantz) production is restricted by begomovirus inducing Cassava mosaic disease (CMD) in Africa. Two different cassava-associated begomoviruses were isolated from India and Sri Lanka, but none of these clones induced the typical symptoms in cassava.

We report on the complete nucleotide sequence of a new strain of *Indian cassava mosaic virus* (ICMV) from South India. The cloned viral DNA induces CMD into cassava, fulfilling Koch´s postulates and confirm, that begomoviruses are the aetiology of CMD in India.

Introduction

Cassava (*Manihot esculenta* Crantz, *Euphorbiaceae*) is one of the most important crop plants in the world and represents the third largest source of carbohydrates for human consumption (Fauquet and Fargette, 1990). Cassava is grown throughout equatorial regions of America, Africa and Asia with an annual yield in 2003 of 187,6 million tons (FAO, 2004). The world wide demand for cassava is expected to increase up to 291 million tons in 2020 (Scott *et al.*, 2000). Cassava originated in South America, where it was domesticated 5,000-7,000 years B.C. (Latharp, 1970). European explorers introduced cassava to Africa and Asia (Fauquet and Fargette, 1990). Today India is the third largest cassava producer in Asia. In 2003, about 7 million tons were produced from 270,000 ha. Cassava is used for direct consumption or processed to sago (starch), flour or chips, but it is also

applied for animal feed and for industrial purpose (modified starches, adhesives, ethanol, sugar, textiles). Cassava has progressed to become a cash crop, exported mainly to the countries of the European Union (Onwueme, 2002).

One major restriction of cassava production is the cassava mosaic disease (CMD), caused by whitefly-transmitted begomoviruses (*Geminiviridae*) (Fauquet and Fargette, 1990; Malathi, 1985). CMD was first described on the African continent in 1894 as “Kräuselkrankheit” by Warburg (1894). In Africa, the average yield loss is at least 30-40% (Calvert and Thresh, 2002). The extent of CMD distribution is determined primarily by epidemiological constraints imposed on the whitefly vector. Whiteflies are difficult to control with insecticides, moreover they are often resistant to pesticides (Brown and Bird, 1992).

The occurrence of CMD in India was not reported until 1966 although it is known to have been present earlier (Alagianagalingam and Ramakrishnan, 1966). In 1988, incidence of CMD in India ranged from 20 to 30 %, but CMD seems to have become more prevalent in South India in recent years (Calvert and Thresh, 2002, Dasgupta, Briddon and Rothenstein, 2001 and 2002 unpublished observation). In the district of Tamil Nadu, it is not clear, whether the spread of the disease is due to the infection by whiteflies or the use of infected planting material (Calvert and Thresh, 2002). *Indian cassava mosaic virus* (ICMV) was cloned in 1993 (Hong *et al.*, 1993), but no infectivity into cassava has been shown so far.

ICMV belongs to the genus *Begomovirus* (*Geminiviridae*), circular ssDNA viruses. The majority comprises a bipartite genome, consisting of DNA A and DNA B of approximately same size (2.6-2.8 kb) (Stanley, 1983; Stanley and Gay, 1983). Each DNA component is encapsidated in a twinned particle (approximate size of 22 $\times$ 38 nm), consisting of two joined, incomplete icosahedra (Böttcher *et al.*, 2004; Zhang *et al.*, 2001b). DNA A contains six open reading frames (ORF), two in viral (AV1 and AV2) and four in complementary orientation (AC1, AC2, AC3 and AC4). AC1 and AC2 are responsible for replication and regulation of gene expression and AV1 for the encapsidation of viral ssDNA components (Sunter *et al.*, 1987; Rogers *et al.*, 1986; Townsend *et al.*, 1986; Stanley, 1983). The function of AV2 is not clear yet, but it may possesses functions of a movement protein (Rothenstein *et al.*, 2004d). DNA B is essential for symptom

development, cell-to-cell and long-distance movement (Zhang *et al.*, 2002; Ingham *et al.*, 1995; von Arnim *et al.*, 1993; Etessami *et al.*, 1988). Both DNA components possess an identical 200 bp nucleotide stretch, the common region (CR). The CR contains regulatory elements for the transcription and replication. Geminiviruses use a rolling-circle replication mechanism to amplify their ssDNA genomes to dsDNA forms, that serve as replicative and transcriptional templates. A further mechanism of recombination-dependent replication (RDR) was suggested by Jeske *et al*. (2001), explaining the frequent formation of chimeric viral genomes. Recombination between genera (Briddon *et al.*, 1996; Stanley *et al.*, 1986) and families (Saunders *et al.*, 2001; Saunders and Stanley, 1999) was suggested to be the major driving force for the diversity of geminiviruses (Padidam *et al.*, 1999), breaking resistance in crop plants and leading to epidemics.

On the African continent, geographically separated distinct strains of the virus exist. For India and Sri Lanka, less sequence data is published. The reintroduction of ICMV into its natural host, cassava, has not been shown so far, only model plants (*Nicotiana* spec.) were susceptible for cloned viral DNA (Saunders *et al.*, 2002).

In this report we isolated and characterized a bipartite begomovirus associated with CMD in India. The virus is a strain of the already known ICMV. Reintroduction into cassava, resulted in typical symptoms of virus infection, fulfilling Koch`s postulates. This is the first report on an ICMV clone which induces CMD symptoms in its natural host, confirming that the disease is caused by a begomovirus in India.

Results

Infection of N. benthamiana with cloned ICMV DNA

ICMV supercoiled DNA (scDNA) was isolated from sap-infected *Nicotiana tabacum* plants. Full-length copies of ICMV DNA A and DNA B were cloned into pBluescript KS II (+) vector using singly cutting restriction enzymes. The viral DNAs were released from the vector DNA and mechanically inoculated into *N. benthamiana* plants. In three independent experiments 12 out of 60; 7 out of 20 and 2 out of 21 inoculated plants were infected. First symptoms appeared 14 days post inoculation (dpi), the shoots were bent down, leaves curled and developed a

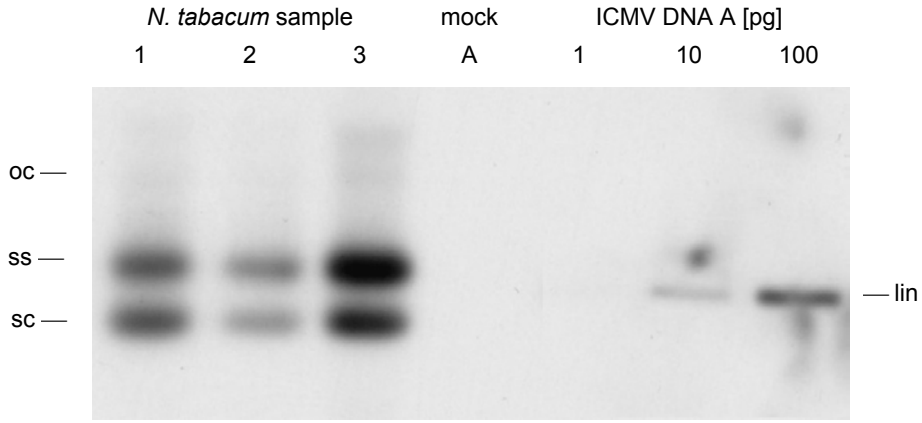

Figure 1. Southern blot analysis of samples extracted from *N. tabacum* plants infected with ICMV by ballistic inoculation (1 – 3). Mock-inoculated *N. tabacum* plants were inoculated with pBluescript DNA (A). 5 µg of total DNA were loaded per lane and the blot was hybridised with a ICMV DNA A-specific probe. The position of open circular (oc), linear (lin), supercoiled (sc) and single-stranded (ss) DNA forms are indicated.

yellow-green mosaic pattern. Systemically infected plants were extremely stunted compared to mock-inoculated plants. Symptoms were severe but plants survived even after 30 days.

As mechanical reintroduction of cloned ICMV components into *N. tabacum* cv. Samsun nn plants failed, plants were inoculated biolistically. *N. tabacum* plants were approximately 30 days old (two- to three-leaf stage) when they were inoculated. The infection rate was about the same as observed for mechanically inoculated *N. benthamiana* plants, 3 out of 15 plants developed a systemic infection. First symptoms appeared 15 days post bombardment (dpb). Symptoms were similar to those of infected *N. tabacum* plants, from which viral DNA was isolated for cloning. The infection was confirmed by Southern blot analysis (Fig. 1), thus fulfilling Koch´s postulates. ICMV clones were also tested in poinsettia plants (*Euphorbia pulcherima*), but no infection could be established (data not shown). Cassava plants were inoculated with a handhold particle gun according to Briddon *et al.* (1998). All plants (3/3 inoculated plants) showed mild symptoms after 2 month (Fig. 2 B). The presence of viral DNA was confirmed by Southern blot analysis with ICMV DNA A- and DNA B-specific probes (Fig. 2 A). Therefore these ICMV clones are the first clones which induced CMD in its natural host cassava, confirming that CMD in South India is caused by a begomovirus. ICMV and ACMV, the later causing CMD in Africa, had been tested

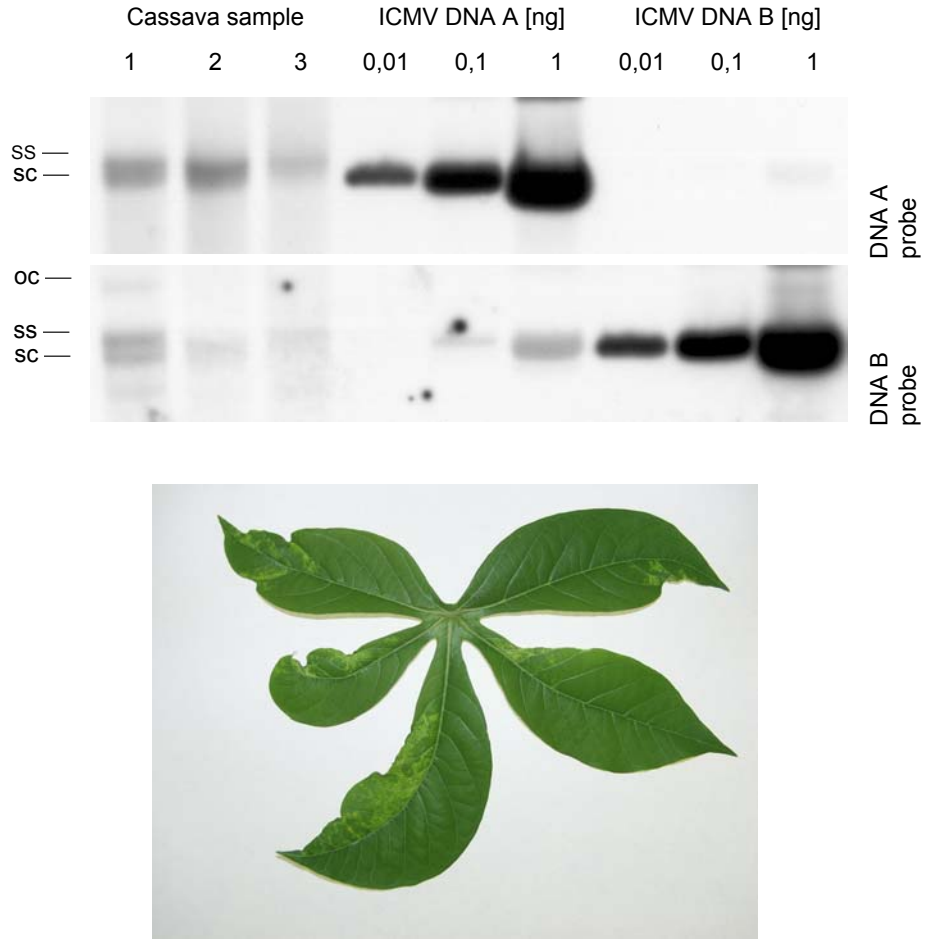

Figure 2. Southern blot analysis of cassava plants (A), infected with cloned viral ICMV DNAs A and B. Three cassava plants were inoculated twice by particle bombardment in an interval of one month. Symptoms, characteristic for ICMV-infection, developed in all three plants four to six weeks after inoculation. Southern blot were hybridised with a ICMV DNA A-specific probe (upper blot), stripped and rehybridised with a ICMV DNA B-specific probe (lower blot). The position of open circular (oc), supercoiled (sc) and single-stranded (ss) DNA forms are indicated. (B) Symptomatic leaf of an ICMV-infected cassava plant biolistically inoculated. Symptoms developed four to six weeks post inoculation.

whether these viruses can form viable pseudorecombinats. Co-inoculation of ICMV DNA A in combination with ACMV DNA B nor the reverse combination did produce any symptoms in *N. benthamiana* plants when mechanically inoculated. Systemic spread of ICMV DNA A alone did not occur either.

Determination of ICMV nucleotide sequence

The complete nucleotide sequences of the infectious ICMV DNA A and DNA B clones were determined. DNA A comprises 2733 and DNA B 2643 nucleotides (nt). The genome organization was similar to that of other bipartite begomoviruses. DNA A contains six ORFs, two in viral sense

orientation AV1 and AV2 and four ORFs in complementary sense orientation (AC1, AC2, AC3 and AC4). DNA B encodes two ORFs, one in each direction (BV1 and BC1). The intergenic region, in between the viral sense and complementary sense orientated genes, was identical for DNA A and DNA B (DNA A: 2601-82 and DNA B: 2511-82) including the conserved TAATATT_AC nonanucleotide, the start of rolling-circle replication (Stanley, 1995). The sequence is most closely related to ICMV-[Mah] (Accession numbers AJ314737/ AJ314738) (Saunders et al., 2002), 98,5% homology for DNA A and 95,1% for DNA B, respectively. The sequence comparison between ICMV-[Mah] DNA A and our ICMV DNA A, showed only single nucleotide exchanges, in contrast to DNA B where two distinct deletions, an extended 60 nucleotide long deletion (77-138) and a short deletion at position 2043-2052 occurred. The three 41 nt long sequence repeats within the CR, subsequent to the stem loop reported by Hong et al. (Hong *et al.*, 1993) did not occur in this ICMV clone. The iteron sequence, necessary for the binding of AC1 (Hanley-Bowdoin *et al.*, 2000; Eagle *et al.*, 1994) was identical to ICMV-[Mah]: GGTACTCA separated by a 22-bp stretch.

The predicted amino acid sequences of the ORFs were highly conserved, the homology ranged from 99,6 % for the CP to 92,2% for AC2. The predicted amino acid sequence of the CP, mediating nuclear im- and export in infected cells, had only two changes (58 I→M and 129 M→K), not affecting any nuclear localization sequence (NLS) (Unseld *et al.*, 2001). Thus, it is not possible to make any prediction, with regard to the CP, why this clone, in comparison to ICMV-[Mah] is infectious into cassava.

Discussion

A bipartite ICMV strain (Accession number ICMV-[Mah2] DNA A: AY730035, DNA B: AY730036) was isolated and characterised. The genome organisation was similar to that of other CMD-causing begomoviruses in India and Africa. On the basis of nucleotide sequence comparison it was closely related to a previously described virus form Maharastra state in India (ICMV-[Mah]) (Saunders *et al.*, 2002). None of the isolated viruses, associated with CMD in India which have been investigated

so far, were able to induce symptoms in the natural host cassava (Saunders *et al.*, 2002; Hong *et al.*, 1993). In contrast, our ICMV isolate was able to induce systemic infection in cassava plants, fulfilling Koch´s postulates. This confirms the assumption, that begomoviruses are the CMD-causing agents in India. It is the first report of a cassava-infecting ICMV clone.

The sequence homology between our ICMV-isolate and ICMV-[Mah] was about 95%. The coat protein (CP) sequence, directing the virus within cells (Unseld *et al.*, 2001), and the iteron sequences, facilitating the binding of AC1 were investigated in detail. The putative CP amino acid sequence had only few exchanges, but no nuclear transport signals were reported to be located at these sites (Unseld *et al.*, 2001). Therefore it is most presumably, that other viral proteins and/or nucleotide sequences are responsible for its infectivity. The iteron sequence of both isolates were identical, even the length of the gapping stretch. Probably the AC2 protein, which is thought to be an antagonist of post transcriptional gene silencing (PTGS), a defence strategy of plants, has some influence on infection. Supported by the most divergent sequence, compared with ICMV-[Mah] *AV2*.

Although this virus was isolated from *N. tabacum* plants, the assertion, that repeated passaging through model plants before cloning may select a virus population that can no longer infect cassava (Liu *et al.*, 1997), could not be confirmed in our case.

This isolate opens the field for investigation on Indian CMD in its natural host cassava and for artificial infection of existing and newly bred cassava cultivars, to challenge their resistance to mosaic disease.

Material and methods

Construction of infective clones

N. tabacum plants, which were ICMV sap-infected were obtained from P. Markham, John Innes Centre, Norwich, UK. The plants showed leaf deformation and mosaic pattern. Infected plants were propagated vegetatively and maintained at 24°C with a 16 hour photoperiod.

Nucleic acids were isolated from systemically infected *N. tabacum* (cv. Samsun nn) leaf tissues. Viral scDNA was purified by CsCl gradient centrifugation in the presence of ethidium bromide (Schildkraut *et al.*, 1962; Meselson *et al.*, 1957). Full-

length viral DNA was cut using singly cutting restriction enzymes, *Pst* I for DNA A and *Kpn* I for DNA B and inserted into pBlueskript II KS + (Stratagene).

The sequence of both DNA strands was determined at least three times using Thermo Sequenase Primer Cycle Sequencing kit (Amersham Pharmacia biotech) with universal IRD800 labeled primers (forward primer 5`-AGGGTTTTCCCAGTCAC-GACGTT-3`, reverse primer 5´-GAGCGGATAACAATTTCACACAGG-3´). For each clone, several deletion mutants were made to determine the whole sequences. Sequence reactions were analyzed on a LI-COR sequencer. Sequence alignment was done with Vector NTI program.

Inoculation of plants

N. benthamiana plants were mechanically inoculated with mono-meric ICMV DNA A and DNA B, which were released from the vector DNA using the cloning restriction sites. For each component 1µg DNA in a final volume of 10µl were inoculated.

N. tabacum plants were inoculated biolistically with a particle gun in an evacuated chamber (PDS1000/HE; Bio-Rad). Gold particles (1 µm) were coated with vector-released viral DNA as described by Zhang *et al.* (2001a). Plants in the two-leaf stage were bombarded with either 450 psi or 900 psi rupture discs under a vacuum pressure of 27 inch. Hg.

Cassava plants were inoculated with a handhold particle gun as described by Briddon *et al.* (1998).

Analysis of viral DNAs

Total cellular nucleic acids were extracted from systemically infected plant tissues with extraction buffer (100mM Tris-HCl pH 7.0, 100mM NaCl, 10mM EDTA, 1% SDS). Proteins were extracted by phenol-chloroform (1:1) and nucleic acids were ethanol precipitated. DNA was dissolved in 100 µl sterile water. RNAs were digested by adding 1µl of RNase A (1µg/µl). DNA concentrations were measured using the Gene Quant II (Pharmacia). From each plant 5µg total nucleic acids were separated by agarose gel electrophoresis using 0,5 × TBE buffer and transferred to Hybond NX membranes (Amersham).

Viral DNAs were detected by digoxigenin-labeled probes, a Hind III (2435 nt)- Nco I (1119 nt) fragment of DNA A and a EcoR I fragment (1792-2718 nt) of DNA B, according to manufacturer's recommendation (Boehringer Mannheim).

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Biodiversity of cassava-infecting begomoviruses in India

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Abstract

Cassava mosaic disease (CMD) is caused by begomoviruses (*Geminiviridae*) and leads to enormous yield losses in Africa and India. Genomic variation resulting from recombination of different strains and genera seems to be the major driving force to generate new virus strains with enhanced biological fitness. Recent CMD epidemics in Africa were associated with a recombinant virus of two cassava-infecting begomoviruses. Here we report a survey on the biodiversity of begomoviruses, infecting cassava in India. Cassava stem cuttings were collected in South India (states Tamil Nadu and Kerala) from plants displaying CMD symptoms. Investigations on the biodiversity of begomoviruses, associated with these plants were performed by PCR-based RFLP analysis and DNA A nucleotide sequence comparisons. Homology analysis showed that the majority of begomoviruses infecting cassava in India are most closely related to *Sri Lankan cassava mosaic virus* (SLCMV) and not to *Indian cassava mosaic virus* (ICMV).

Introduction

Geminiviruses are serious plant pathogens, infecting a range of important crop plants in tropical, subtropical and to a minor extend in temperate regions. The family *Geminiviridae* is divided into four genera on the basis of genome organisation and biological properties (Rybicki *et al.*, 2000). They posses circular single-stranded (ss) DNA genomes encapsidated in twinned (geminate) particles of approximately 20 × 30 nm (Böttcher *et al.*, 2004; Zhang *et al.*, 2001). *Mastreviruses* have a monopartite genome and are transmitted by leafhoppers to monocotyledonous plants. *Curto-viruses*, also possess a monopartite genome but genome organization differs from those of mastreviruses. They are transmitted by leafhoppers to dicotyledonous plants. The genus

Topocuvirus has only one member, which is transmitted by a treehopper to dicotyledonous plants. The economically most important genus, comprising more than 100 members is *Begomovirus*. Most members possess a bipartite genome, consisting of DNA A and DNA B, although there are monopartite begomoviruses from the Old World. Begomoviruses are transmitted by the whitefly *Bemisia tabaci* Genn. to dicotyledonous plants.

Geminiviruses can adapt quickly to environmental conditions and host plants. Different types of genomic variation were responsible for this forced evolution. Mutations distributed throughout the genome of *Maize streak virus* (MSV) led to closely related isolates (Isnard *et al.*, 1998). Point mutations within the genome of begomoviruses can affect symptom modulation and severity (Stanley *et al.*, 1985), host range (Lazarowitz, 1991) and whitefly transmission (Höhnle *et al.*, 2001; Noris *et al.*, 1998). Pseudorecombination, by reassortment of DNA A and DNA B components, was supposed to be limited to strains of a virus (Frischmuth *et al.*, 1993; von Arnim and Stanley, 1992a; Lazarowitz and Lazdins, 1991), but it also occurs between different viruses (Unseld *et al.*, 2000a; Unseld *et al.*, 2000b; Frischmuth *et al.*, 1997; Höfer *et al.*, 1997; Gilbertson *et al.*, 1993). Recombination of geminiviruses was suggested to be the driving force for biodiversity (Padidam *et al.*, 1999) and may also has led to new genera, as supposed for *Topocovirus* (Briddon *et al.*, 1996) and *Curtovirus* (Rybicki, 1994; Stanley *et al.*, 1986). Acquisition of new genomic components by donating the origin of replication (Roberts and Stanley, 1994) has probably contributed to the evolution of bipartite begomoviruses from monopartite ancestors (Saunders *et al.*, 2002).

In the last decades, incidence and severity of disease caused by begomoviruses has increased in many parts of the world (Polston and Anderson, 1997; Brown and Bird, 1992). One reason for the increased pathogenesis was the formation of new virus strains due to recombination. This was reported for viruses associated with cassava in Africa (Pita *et al.*, 2001; Fondong *et al.*, 2000; Zhou *et al.*, 1997) and cotton in Pakistan (Zhou *et al.*, 1998).

Additionally, the whitefly vector has become more prevalent, and often resistant to pesticides (Brown and Bird, 1992). Distinct whitefly biotypes have evolved, which were accounted to

contribute for successful dissemination of geminiviruses, e.g. in Brazil (Ribeiro *et al.*, 2003; Ambrozevicius *et al.*, 2002).

The objective of this work was to give a detailed report on the genetic biodiversity of begomoviruses infecting cassava in India.

Results

Sixteen plants were regenerated from stem cuttings independently collected from fields in the states Tamil Nadu and Kerala (India) in 2001 and 2002. All plants showed typical CMD symptoms, including distortion and mosaic pattern of leaf blades.

According to their symptom severity plants were grouped as mild, intermediate and severe symptomatic, plants 1, 3, 10, 15, 16 and C5 showed only mild symptoms, plants 11, 14, 17, 18, C3 and C4 displayed intermediate symptoms (leaf mosaic and distortion) and plants 2, 5, 6 and 7 exhibited severe symptoms, reduction of leaf blades in combination with yellow-green mosaic pattern. Nucleic acids of each plant were isolated from leaf tissues. In all regenerated plants, viral DNA was detected by Southern blot analysis (Fig.1). Viral DNA content and

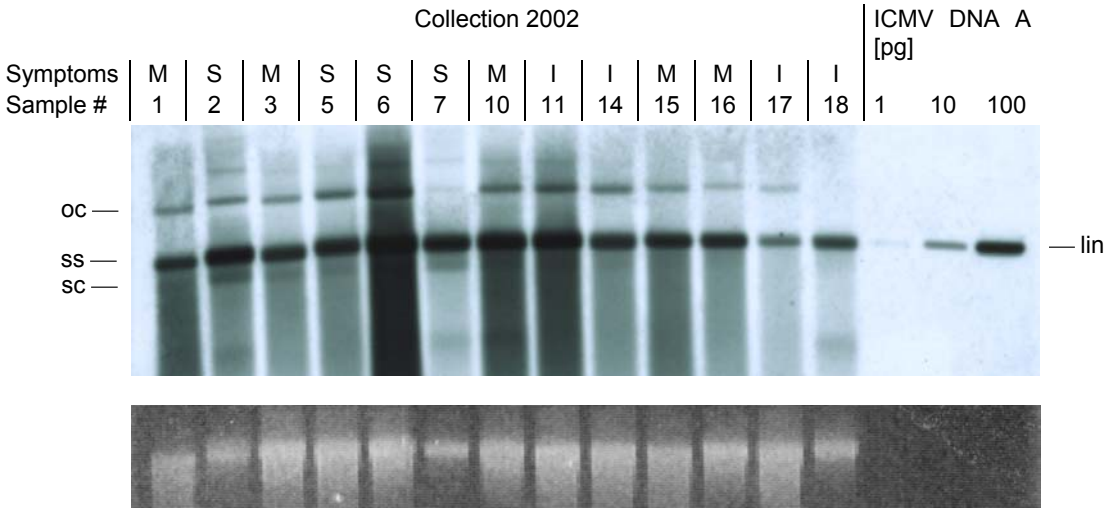

Figure 1. Southern blot analysis of CMD affected cassava plants. Stem cuttings of CMD-displaying cassava plants form India (Tamil Nadu samples number 1 – 7 and Kerala samples number 10 –18) were regenerated and total nucleic acids were extracted. Similar DNA amounts (loading control below Southern blot) were separated on an 1% agarose gel and transferred to a nylon membrane. Begomoviral DNA was detected with an ICMV DNA A-specific probe. In all plants, viral DNA was detectable, however, the amounts of detected DNA did not correlate with symptom severity, scored as M: mild, I: intermediate and S: severe symptoms.

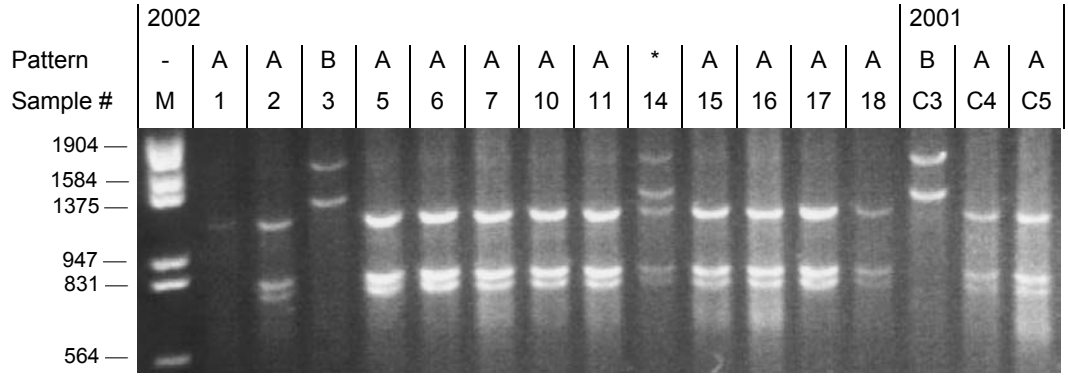

Figure 2. PCR-RFLP analysis using *Eco*R I restriction enzyme. PCR-amplified begomoviral DNA A was *Eco*R I-digested, separated on a 1,5% agarose gel, and stained by ethidium bromide solution. Restriction patterns segregate into two different groups (A and B), majority of which belongs to group A. The summarized fragment sizes of viral DNA for group A and B was approximately 2880 nt and 3000 nt, respectively. Sample #14 (indicated by asterisk) showed a mixed infection with viruses from both groups.

symptom severity were not directly correlated.

Full-length viral DNA A components were PCR amplified using primers # 1 and 2 (Table 2). The primers were located within the highly conserved CP gene of begomoviruses (Harrison *et al.*, 2002). Viral DNA was amplified from each sample, no amplification product was obtained using DNA from a non-infected control plant. To investigate biodiversity of begomo-viruses, restriction fragment length polymorphism (RFLP) analysis was performed. Each viral component was restricted with three different restriction enzymes (*Eco*R I, *Hpa* II or *Sau*3A I). RFLP using *Eco*R I showed two different groups of restriction patterns (Fig. 2). A prevalent three-bands pattern of 1100 nt, 850 nt and 800 nt for 13 samples and with a two-bands

pattern consisting of 1500 nt and 1200 nt for samples # 3 and C3. A doubly infected plant (#14), containing viruses of both groups was identified (Fig 2, asterisk). RFLP analysis using *Hpa* II showed a more complex pattern, where samples split into four distinct groups (Fig. 3). The majority of samples (10 out of 16) had a pattern of five bands with an approximate total size of 3040 nt. The mixed infection of plant 14 was confirmed by *Hpa* II restriction. Only samples 3 and C3 had a similar restriction pattern to a formerly cloned ICMV DNA A component (Rothenstein *et al.*, 2004a). The third RFLP analysis was performed using *Sau*3A I (Fig. 4). The

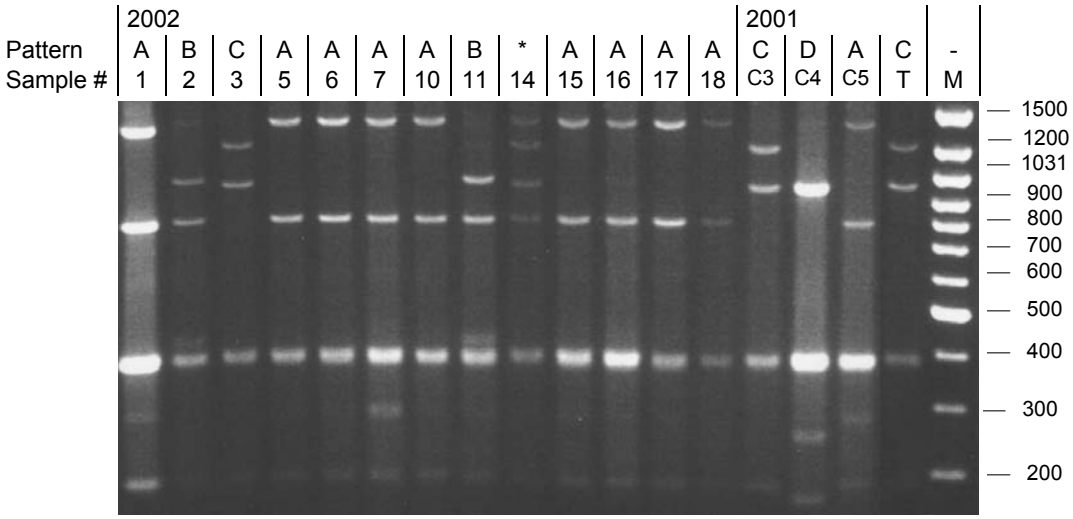

Figure 3. RFLP analysis of PCR-amplified DNA As using *Hpa* II. DNA was extracted from CMD-displaying cassava plants, collected in South India (Kerala and Tamil Nadu) in 2001 (samples C3, C4 and C5) and 2002 (samples 1, 2, 3, 5, 6, 7, 10, 11, 14, 15, 16, 17, and 18). Equal amounts of PCR-amplified DNA As were digested with *Hpa* II, separated on a 1,5% agarose gel and stained by ethidium bromide. Four restriction patterns (A to D) of DNA As were detected. The predominant group A included 10 out of 16 samples. Sample number 14 (marked with an asterisk) showed a mixed infection of viruses from group A and C. ICMV DNA A, amplified from sap-infected *N. tabacum* and digested with *Hpa* II were separated on lane "T". M: 100 bp Gene Ruler TM (Fermentas).

restriction of DNA A components revealed the most complex pattern of all restriction enzymes used in this report. Five different groups of distinguishable patterns were obtained. The double infection of sample 14 was confirmed.

In summary, six different groups of distinct begomovirus strains have been distinguished on the basis of RFLP analysis (Table 1). All samples sum up to a total DNA size in between 2820 to 3040 nt, which corresponds to the normal size of a begomoviral DNA A. Seven samples clustered in one group, whereas the remaining eight samples split up into 5 different restriction patterns. Some of the samples had a unique restriction pattern (# 6 and C4). Remarkably, only two samples had a restriction pattern similar to the formerly cloned ICMV DNA A. RFLP patterns of viral DNA from samples collected in 2001 and 2002 match to each other. Thus, isolates seem to be stably maintained in natural environment. Restriction patterns of plant #14 confirmed mixed infection. The summary given in table 1 shows that the combination of three restriction enzymes provides a detailed picture of biodiversity among CMD-causing

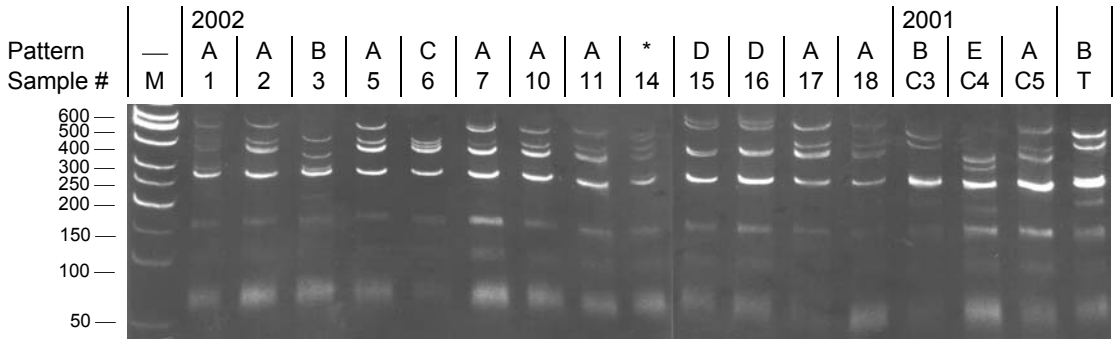

Figure 4. RFLP analysis of PCR-amplified ICMV DNA A using *Sau*3A I. 16 independent samples split into five different groups. Equal amounts of PCR-amplified DNA As were digested with *Sau*3A I and separated on a 12% polyacrylamide gel, stained by ethidium bromide. Samples indicated with "C" remained from the stem cutting collection in 2001, all other samples were collected in 2002. The sample indicated with "T", originated from an ICMV-infected *N. tabacum* plant, from which a cassava-infectious clone was isolated (Rothenstein et al., 2004a). M:50 bp ladder plus (Fermentas), band sizes are indicated.

begomoviruses in South India. For preliminary overview, the restriction with either *Eco*R I or *Hpa* II would provide a sufficient hint on diversity.

The main purpose of this project was to clone different cassava-infecting begomoviruses, and to use them in challenge infection of new cassava cultivars to test for ICMV resistance. To this aim, unique restriction sites of a formerly sequenced ICMV DNA A and DNA B (Rothenstein *et al.*, 2004a) were used to design abutting primers (including the unique restriction site).

For DNA A the primers harboured a *Pst* I recognition sequence (Table 2, # 3 and 4) and a *Kpn* I site for DNA B (Table 2, # 5 and 6). Using these

Table 1. Summary of RFLP analysis. PCR amplified begomoviral DNAs were grouped into six different restriction patterns (Group I-VI). The geographical origin of selected stem cuttings did not correlate with grouping. Sample #14 contain two distinct virus isolates. The restriction pattern of samples # 3 and # C3 were similar to a formerly cloned ICMV DNA A. MI: mixed infected.

Sample #	1	2	3	5	6	7	10	11	14	15	16	17	18	C3	C4	C5	ICMVA
RFLP-EcoR I	A	A	B	A	A	A	A	A	A+B	A	A	A	A	B	A	A	-
RFLP-Hpa II	A	B	C	A	A	A	A	B	A+C	A	A	A	A	C	D	A	C
RFLP-Sau3A I	A	A	B	A	C	A	A	A	A+B	D	D	A	A	B	E	A	B
Pattern	I	II	III	I	IV	I	I	II	MI	V	V	I	I	III	VI	I	III

primers, full-length DNA As and DNA Bs were amplified. The amplified genomes were inserted into pCR-XL-TOPO vector. Since the primers contained unique restriction site it was possible to release the inserted viral DNA for infectivity studies. Full length clones were obtained from 12 different cassava plants for DNA A and from six different cassava plants for DNA B. From each group, defined by RFLP analysis, one DNA A clone was completely sequenced (Cassava 2, 6, 7, 15, 17 and C4). Sequences were in between 2668 to 2751 nt in length. The genome organization of all DNA A clones were similar to other begomoviruses (Fig. 5 A). DNA A contains six ORFs, two in viral sense orientation (AV1 and AV2) and four ORFs in complementary sense orientation (AC1, AC2, AC3 and AC4). All sequences were most closely related to *Sri Lankan cassava mosaic virus* (SLCMV; accession number AF314738). The nucleotide sequences were compared to those of CMD-causing begomoviruses from Africa, India and Sri Lanka, using the software Clustal X 1.81 (Fig. 5 B). The dendrogram was calculated using neighbour-joining and bootstrap (1000 replications). *Maize streak virus*, a distantly related geminivirus was chosen as outgroup. Two main clades were calculated, reflecting the geographical origin of DNA sequences. The biodiversity of the six new DNA As was relatively low, except for # C4, which was closer to SLCMV. The organization of the CR of all sequenced DNA As was similar to SLCMV. Within the CR direct nucleotide repeats, so called iterons, were responsible for effective binding of the Rep-protein. Only molecules with identical iteron sequences could be efficiently replicated. The iteron sequences (Saunders *et al.*, 2002) including the gapping nucleotides in between the direct repeats of the new DNA As and SLCMV were identical and differed form ICMV iteron sequences.

Discussion

Extended studies on ACMV biodiversity have been carried out (e.g. Briddon *et al.*, 2003; Bull *et al.*, 2003; Berrie *et al.*, 2001). In comparison little information on Indian begomoviruses, infecting cassava was available. To discriminate begomovirus field isolates, we used a PCR-RFLP based method in combination with complete sequencing and homology analysis of six new DNA As. Similar approaches have been proven useful for other

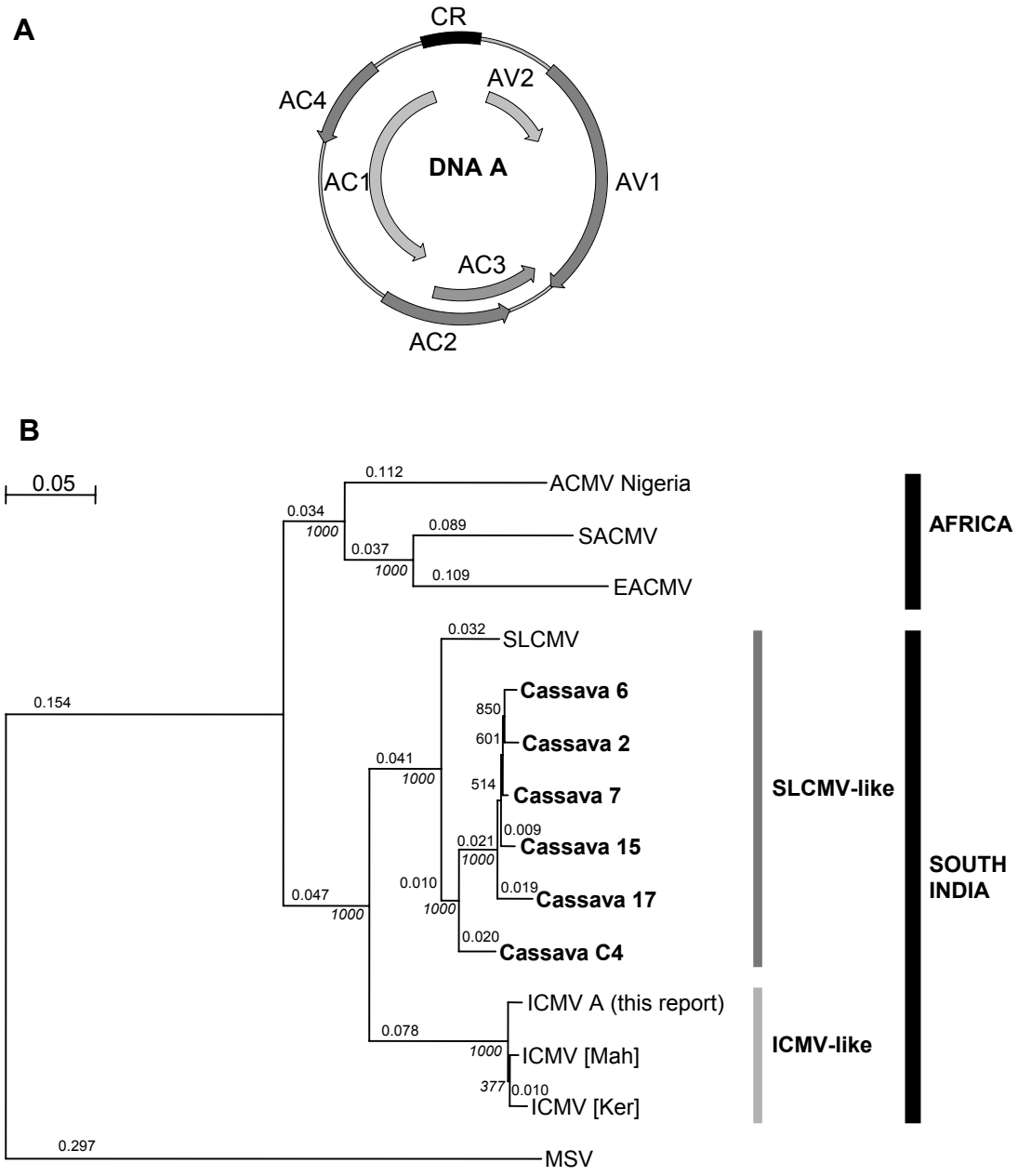

Figure 5. (A) Genome organization of begomovirus DNA A. DNA A contains six ORFs, two in viral sense orientation (AV1 and AV2) and four ORFs in complementary sense orientation (AC1, AC2, AC3 and AC4). (B) Phylogenetic tree obtained from the alignment of full-length DNA A nucleotide sequences of *Maize streak virus* (MSV; NC001346), ACMV Nigeria (X17095), SACMV (AF155807), EACMV (AF126804), SLCMV (AF314738), ICMV [Maha] (AJ314740), ICMV [Ker] (Z24758), ICMV A (Rothenstein *et. al*., 2004a) and the sequences from this report. The dendrogram was calculated using neighbour-joining and bootstrap (1000 replications) of Clustal X 1.81. The tree was rooted on the DNA sequence of MSV, a distantly related geminivirus. Vertical branches are arbitrary, horizontal branches are proportional to calculated mutation distances. Scale bar indicate the horizontal distances equivalent to 0.05 replacements per position. Numbers above branches indicate branch length. Numbers in italic at the nodes indicate bootstrap confidence values.

geminiviruses (Jovel *et al.*, 2004; Martin *et al.*, 2001; Willment *et al.*, 2001).

Sixteen viral DNA A components were grouped into six different clusters. Only two RFLP patterns derived from the new isolates resembled to the restriction pattern of a formerly cloned ICMV isolate (Rothenstein *et al.*, 2004a). Sequence analysis of six DNA A components from different groupings showed high homology among these isolates. All isolates were most closely related to SLCMV and not to ICMV. Comparison of DNA A nucleotide sequences of cassava-associated begomoviruses showed two distinct clades, reflecting the geographically separated African- and Indian/Sri Lankan group. SLCMV-like DNA A sequences also occur in India. Whether SLCMV originated in India and was transported to Sri Lanka or vice versa can not be determined. ICMV-like and SLCMV-like DNA As, based on RFLP analysis were found in India in 2001 and 2002. Therefore, we assume that these are distinct begomovirus isolates associated with cassava in India, which have achieved genetic stability in natural conditions.

Geographical separation of virus isolates have been reported for a range of geminiviruses. A direct relation of geographical origin of viral DNA and RFLP patterns or DNA sequences, respectively, could not be drawn in case of Indian begomoviruses. Isolates, originating from Tamil Nadu (South-eastern India) and Kerala (South-west India) had similar restriction patterns. Thus, distinct virus strains occur in the same geographical regions in India. This implies the possibility of recombination and pseudorecombination for the evolution of begomoviruses as well as synergistic effects during infection. Mixed infected plants are a suitable environment for producing new strains with altered properties. Enhanced pathogenicity of an isolate, originated by pseudorecombination and intermolecular recombination has been reported (Hou and Gilbertson, 1996).

Two distinct viral DNA As were detected in a naturally infected cassava plant. A prerequisite for recombination of viruses in nature are mixed infected plants, where different strains or genera can interact. The severe epidemic of CMD in Eastern Africa was associated with a recombinant virus of ACMV and EACMV (Pita *et al.*, 2001; Fondong *et al.*, 2000; Zhou *et al.*, 1997). Recombination may be the driving force of evolution in the *Geminviridae*

family. It has been assumed that curtoviruses may have evolved by recombination of mastre- and begomoviruses. The BCTV CP is similar to that of mastreviruses, whereas other genomic components resemble more to those of the genus *Begomoviridae* (Rybicki, 1994; Stanley *et al.*, 1986). A recombinant origin was also suggested for the *Tomato pseudo-curly top virus* (TPCTV), which genomic components resembles *Mastre-* and *Begomoviridae*, respectively (Briddon *et al.*, 1996). In the case of ICMV and SLCMV, however, no obvious footprints of recombination were detectable.

Beside recombination, reassortment of DNA components, known as pseudorecombination, can lead to diversity among viruses. Pseudorecombination was assumed to be generally limited to closely related strains or viruses (Polston and Anderson, 1997; Sung and Coutts, 1995; Frischmuth *et al.*, 1993; von Arnim and Stanley, 1992b; von Arnim and Stanley, 1992a; Lazarowitz, 1991; Stanley *et al.*, 1985). The limitation to form viable pseudorecombinants to strains of a virus is ascribed to the virus specific action of Rep (AC1) in DNA replication (Orozco *et al.*, 1998; Fontes *et al.*, 1994a; Fontes *et al.*, 1994b). The Rep binding specificity has been associated with direct repeats (iterons) in the CR, which are identical in cognate DNA components (Hanley-Bowdoin *et al.*, 2000). Furthermore, DNA B-facilitated transport of heterogenomic DNA A might be impaired in pseudorecombinants, originating from different geographical regions (Frischmuth *et al.*, 1993). The iteron sequences of the six new cassava-associated begomoviruses from this study were different to that of ICMV, suggesting, that at least two distinct viruses were present in South India. The close relationship of the new begomoviruses to SLCMV, revealed by sequence comparison was emphasised by identical iteron sequences. Thus ICMV and SLCMV having different iteron sequences, pseudorecombination can not occur. However, ICMV DNA A in combination with SLCMV DNA B as well as the reverse combination lead to systemic symptomatic infection in *N. benthamiana* (Saunders *et al.*, 2002). Formation of viable pseudorecombinants of distinct viruses also have been shown for other geminiviruses (Unseld *et al.*, 2000a; Frischmuth *et al.*, 1997; Höfer *et al.*, 1997; Gilbertson *et al.*, 1993). Viable pseudorecombination of *Bean dwarf*

mosaic virus (BDMV) and *Tomato mottle virus* (ToMoV) has been reported. Detailed investigations found the increased pathogenicity of pseudo-recombinants to donating the CR from one DNA to the heterologous other DNA (Hou and Gilbertson, 1996). Whether such an intermolecular recombination occurred in case of ICMV and SLCMV to produce viable pseudorecombination was not determined.

How the situation for ICMV- and SLCMV-like begomoviruses in India will develop, should be aimed on further investigations. Therefore we developed a simple method to investigate diversity of cassava-associated begomoviruses. The restriction pattern using *Eco*R I and/or *Hpa* II lead to a overview, and discrimination between ICMV and SLCMV. More detailed analysis on begomovirus strains were obtained using *Sau*3A I restriction enzyme.

Material and Methods

Virus source and maintenance

Stem cuttings from CMD-affected cassava samples grown in Tamil Nadu and Kerala state were collected in 2001 and 2002. The cuttings were regenerated and maintained in a glasshouse at Stuttgart University at 24°C with supplementary lightning to give a 16-h photoperiod. Plants were grouped according to their visible symptom severity of leaf distortion and mosaic pattern.

DNA extraction and amplification

Nucleic acids were isolated form 3 g of cassava leaf tissues using the modified procedure from Rogers and Bendich (1985). Leaf tissues were ground in liquid nitrogen, mixed with 22.5 ml extraction buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA; 1,4 M NaCl; 3,5 % cetyltrimethylammonium bromide (CTAB); 0,2 % 2-mercapto-ethanol) and incubated for 30 min at 60°C. The suspension was mixed with one volume of chloroform/isoamyl-alcohol (24:1) and centrifuged 10 min at 3700 × g. Nucleic acids in the supernatant were precipitated with isopropanol, pelleted by centrifugation (20 min, 12.000 × g) and resuspended in 1 ml 10 mM Tris-HCl pH 8.0. RNase A was added to a concentration of 10 µg/ml and incubated for 30 min at 37°C. DNA was ethanol precipitated and washed with 70 % ethanol. 5 µg DNA were fractionated by 1% agarose gel electrophoresis in 0.5 × TBE buffer (8.9 mM Tris-HCl, 8.9 mM boric acid, 2 mM EDTA) and transferred to Hybond-

Table 2
Primers used for amplification, cloning and sequencing of viral DNA A and DNA B components

No.	Primer	Sequence [a]	Location [b]
1	ICMV for	5´-TGC ATC TCT GAT GTC ACT CGT GGA ATT G -3´	565 - 592 ICMV A
2	ICMV rev	5´-CAT GAC CTT ACC TAT ATG GAC CAC ATC G -3´	537 - 564 ICMV A
3	ICMV Pst for	5´-AGC TGC AGT GAT GAG TTC CCC TGT GCG TGA -3´	1431 – 1460 ICMV A
4	ICMV Pst rev	5´-CAC TGC AGC TCA GGC GAT GAA TGG CGT C -3´	1413 – 1440 ICMV A
5	ICMV Kpn up	5´-ATG GGT ACC TGG TTT CTA TAC TCT AGG ACG -3´	1754 – 1783 ICMV B
6	ICMV Kpn down	5´-CCA GGT ACC CAT TAA CGC CAC AGG GCA T -3´	1738 – 1765 ICMV B
8	T7 [c]	5´-TAA TAC GAC TCA CTA TAG GG -3´	407 – 424 pTOPO
9	M13 Reverse –20 [c]	5´-CAG GAA ACA GCT ATG ACC ATG -3´	205 – 221 pTOPO
10	ICMV A intern 2200 [c]	5´-TGT TAA GTG CTG CGG CGT AA -3´	2189 – 2308ICMV A
11	ICMV A intern730 [c]	5´-CTT ATC AAC AGG CCT ACG AT -3´	726 – 745 ICMV A

[a] Restriction sites within PCR primers are underlined
[b] ICMV DNA A and DNA B: numbering according to Rothenstein *et al*. (2004a), pTOPO (pCR-XL-TOPO) according Invitrogen.
[c] Sequencing primer, DY-781 or IDR-800 labelled.

N membrane (Amersham Life Science). Viral DNAs were detected by hybridisation with digoxigenin-labelled (Boehringer Mannheim) ICMV DNA A probes.

Abutting primers (Table 2, # 1 and 2), located within the *CP* gene were designed to amplify full-length viral DNA As for RFLP-analysis. Viral DNA was amplified from 0.5 to 1 µg DNA in the presence of 100 pmol of each primer, 20 mM of each dNTP, 1.5 mM $MgCl_2$,10mM Tris-HCl (pH 7,5), 5% Q-solution and 1.5 units Taq polymerase (Qiagen, Germany) in a final volume of 50 µl. The amplification program 1 × 95°C, 5 min; 55°C, 1 min; 72°C, 1.5 min; 30 × 95°C, 1 min; 55°C, 1 min; 72°C, 1.5 min; 1 × 95°C, 5 min; 55°C, 1 min; 72°C, 10 min was used. For further purposes, PCR products were ethanol precipitated and stored at –20°C.

For cloning DNA A and DNA B components, overlapping primers were designed. For DNA A, primers # 3 and 4 (Table 2) were located at the unique *Pst* I (1434 nt) restriction site according to Rothenstein *et al.* (2004a). For DNA B, primers # 5 and 6 (Table 2) bound at the unique *Kpn* I restriction site (1755 nt, Rothenstein *et al.*, 2004a). PCR amplification was performed as above. The amplified viral DNA was cloned into pCR-XL-TOPO (Promega) vector according to the manufacturer´s recommendations. Clones were analysed by digestion with either *Pst* I

(DNA A) or *Kpn* I (DNA B), respectively. Selected clones were sequenced on a LI-COR system according to the manufacturer´s instructions with IRD-800 labelled primers (Table 2; Biomers.net and Thermo Hybaid) using the Thermosequenase kit (Amersham).

RFLP analysis

PCR amplified DNA A was digested with 5 units of either *Eco*R I, *Hpa* II or *Sau*3A I for 3 hrs at 37°C and separated by polyacrylamide or agarose gel electrophoresis. *Eco*R I- and *Hpa* II- restrictions were analysed on 1.5 % agarose gels containing 0.5 × TBE in the absence of ethidium bromide and stained after the run in a 0.5 mg/l ethidium bromide solution. *Sau*3A I samples were fractionated by 12 % polyacrylamide gel electro-phoresis (acrylamid/bisacrylamid 30:0.8, 1 × TBE, 0.14 % ammonium peroxodisulfate and 0.014 % tetramethylethylen diamine) in 1 × TBE buffer. Gels were stained after the run by ethidium bromide.

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Tissue tropism of Indian cassava mosaic virus (ICMV) in natural and experimental plants

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Abstract

Cassava mosaic disease (CMD) occurs in Asia and Africa, wherever cassava (*Manihot esculenta*, Crantz) is grown. Geminiviruses are the causal agents for CMD, they comprise a bipartite single-stranded (ss) DNA genome encapsidated in a twin particle. The tissue tropism of ICMV has been determined. Viral DNA was associated with the vascular system in its natural host cassava as well as in the experimental hosts *Nicotiana tabacum* and *N. benthamiana*. In contrast, ACMV DNA can invade almost all cell types in *N. benthamiana* plants. Though ICMV and ACMV both infect cassava and induce similar symptoms, there is no direct relationship between symptom development and tissue tropism.

Introduction

Geminiviruses are plant viruses with circular single-stranded (ss)DNA genomes encapsidized in twin (geminate) particles. They infect important crop plants, such as tomato, maize, beet and cassava, thus having an enormous impact on agriculture and economy. The family *Geminiviridae* comprises four genera: *Mastrevirus*, *Curtovirus*, *Begomovirus* and *Topocuvirus*, divided according to their genome organisation and transmission vector (Rybicki *et al.*, 2000).

Begomoviruses are serious plant pathogens, transmitted by the whitefly vector *Bemisia tabaci*. Most members of this group possess a bipartite genome, consisting of DNAs A and B, each approximately 2.7 kb in size (Rybicki *et al.*, 2000). Beside bipartite viruses, monopartite begomoviruses comprising a single DNA component exist (Dry *et al.*, 1993; Kheyr-Pour *et al.*, 1991; Navot *et al.*, 1991). Infected plants usually show severe developmental distortions: mainly stunting, leaf deformation and

chlorosis. Some viruses, however, induce mild symptoms like mosaic pattern of the leaf, and do not affect the overall development of the plant (Fauquet and Fargette, 1990).

Plant virus movement has been studied extensively (Gafni and Epel, 2002; for review see Lazarowitz, 1999; Leisner, 1999) and components required for systemic spread have been determined. Generally, systemic spread of bipartite geminiviruses requires the DNA B component. However, DNA A of some gemini-viruses, including *Abutilon mosaic virus* (AbMV) (Evans and Jeske, 1993) and *African cassava mosaic virus* (ACMV) (Klinkenberg and Stanley, 1990) can spread autonomously in plants. *Sri Lankan cassava mosaic virus* (SLCMV) (Saunders *et al.*, 2002) DNA A can infect plants, but in combination with DNA B, symptoms were modulated. The coat protein (CP) of monopartite geminiviruses is essential for viral spread (Liu *et al.*, 1998; Rigden *et al.*, 1993), however some CP-deleted bipartite viruses can spread within plants (Sudarshana *et al.*, 1998; Pooma *et al.*, 1996; Padidam *et al.*, 1995).

The tissue tropism of mono- and bipartite geminiviruses has been investigated. Monopartite viruses, e.g. *Beet curly top virus* (BCTV) (Latham *et al.*, 1997), is restricted to the vascular system, whereas bipartite viruses can spread into non-vascular tissues (Wege *et al.*, 2001). DNA B was assumed to mediate escape of phloem and efficient viral transport into mesophyll tissues (Sanderfoot and Lazarowitz, 1995; Noueiry *et al.*, 1994). However, a range of bipartite geminiviruses, e.g. *Abutilon mosaic virus* (AbMV) (Wege *et al.*, 2001; Horns and Jeske, 1991; Abouzid *et al.*, 1988), *Bean golden mosaic virus* (BGMV) (Morra and Petty, 2000) and *Squash leaf curl virus* (SLCV) are restricted to the phloem.Tissue tropism seems to be genetically determined (Morra and Petty, 2000). In addition the plant species and its developmental state are important factors for tissue invasion (Wege *et al.*, 2001; Wang *et al.*, 1996; Kim and Lee, 1992).

Indian cassava mosaic virus (ICMV), a member of the begomoviruses, is a widespread causal agent for Cassava mosaic disease (CMD) in India (Rothenstein *et al.*, 2004a), and similar geminiviruses occur in India (ICMV, Hong *et al.*, 1993), Sri Lanka (SLCMV) (Saunders *et al.*, 2002) and Africa (ACMV) (Stanley and Gay, 1983). These viruses are the most important constraint to cassava production in

Africa and Asia. Infected plants display severe developmental distortions and reduced tuber formation (Calvert and Thresh, 2002; Fauquet and Fargette, 1990). In order to combat these epidemics, it is highly desirable to establish reliable diagnostic tools for quarantine measures as well as breeding purposes. To this aim, we investigated the tissue tropism of ICMV DNA in the natural host *Manihot esculenta* (cassava) and the experimental hosts *Nicotiana tabacum* by microscopic *in situ* hybridisation and *N. benthamiana* by tissue blot analysis.

Results

The tissue tropism of ICMV in naturally infected cassava and experimentally infected tobacco was compared on light microscopical level by *in situ* hybridisation. The specimens were hybridised with biotin-labelled ICMV DNA A-specific probes, resulting in precipitation of a blue stain (Fig. 1 and 2). In general, signals were more abundant and pronounced in tobacco then in cassava. No viral DNA was detected in healthy control plants hybridised with the same probe (data not shown). In both plants, its natural host and the experimental plant, ICMV was generally phloem-limited (Fig. 1 with few rare exceptions, Fig. 1F).

Infected cells were found adjacent to xylem elements in the main vein of the leaf (Fig. 1 A), within the petiol (Fig. 1 B) and to close proximity to tracheids in the leaf blades (Fig. 1 C to E). In spite of severe symptoms, only few infected nuclei were detected within the samples. Single infected cells were mainly surrounded by cells without any signals. Only in one case in 200 sections, hybridisation signals were found outside of the vascular bundle within spongy parenchyma cells (Fig. 1 F).

In tobacco, ICMV was exclusively phloem-limited (Fig. 2). Viral DNA was associated with phloem tissue of minor veins of the leaf blade (Fig. 2 A and B) and localised around the central xylem elements of the main vein (Fig. 2 C and D). Although infected cells were more abundant than in cassava, no “chain” of infected cells, indicating a lateral cell-to-cell movement of the virus was observed. In order to an easy-manageable tool for detection of ICMV under field conditions, tissue blot analysis of infected cassava and *N. benthamiana* plants was performed. Tissue blots were hybridised with

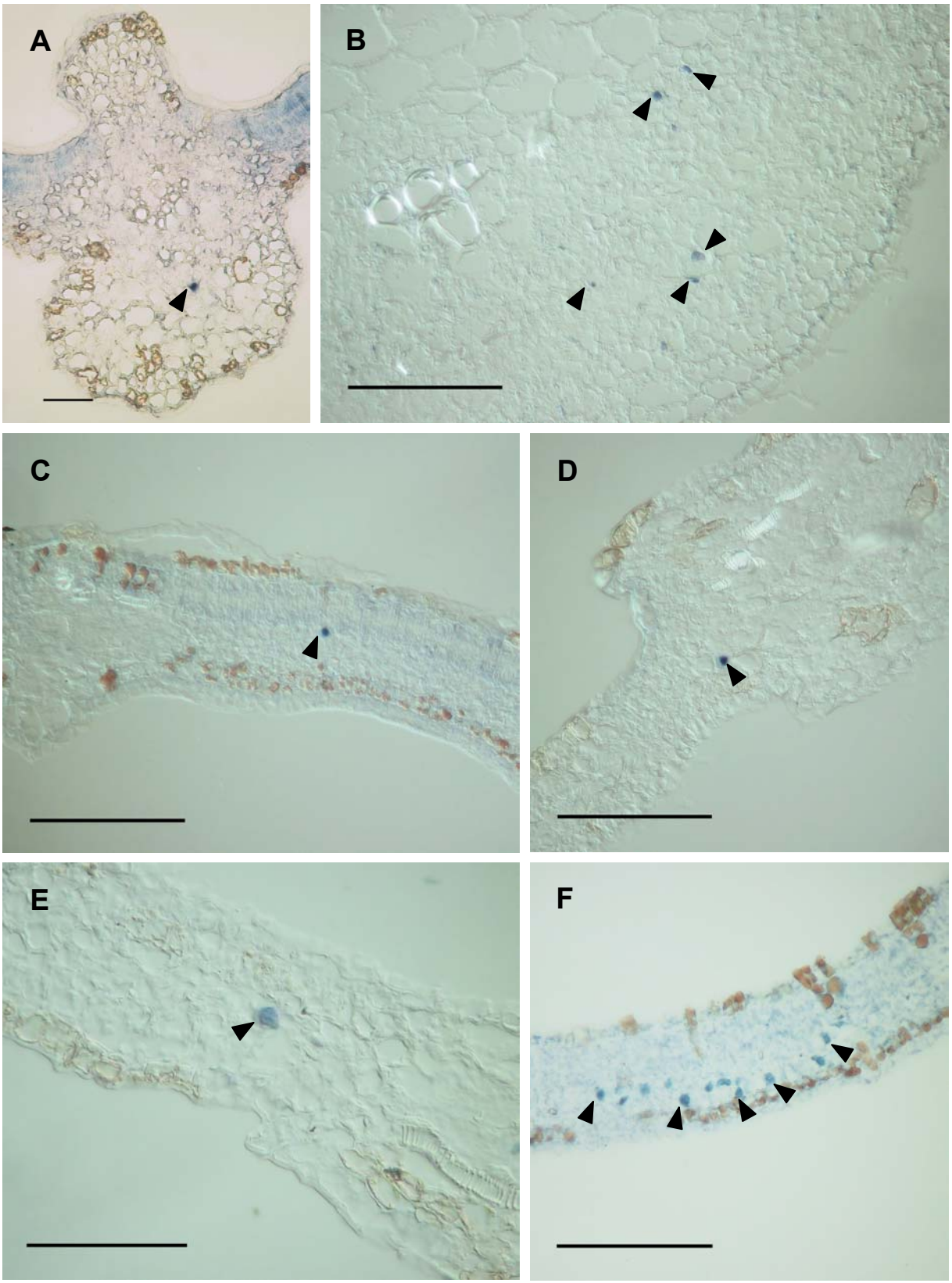

Figure 1. *In situ* hybridisation of ICMV DNA in naturally infected cassava plants using biotin-labelled DNA A-specific probes. Nuclei of infected cells were stained blue by TMB substrate

(continued next page).

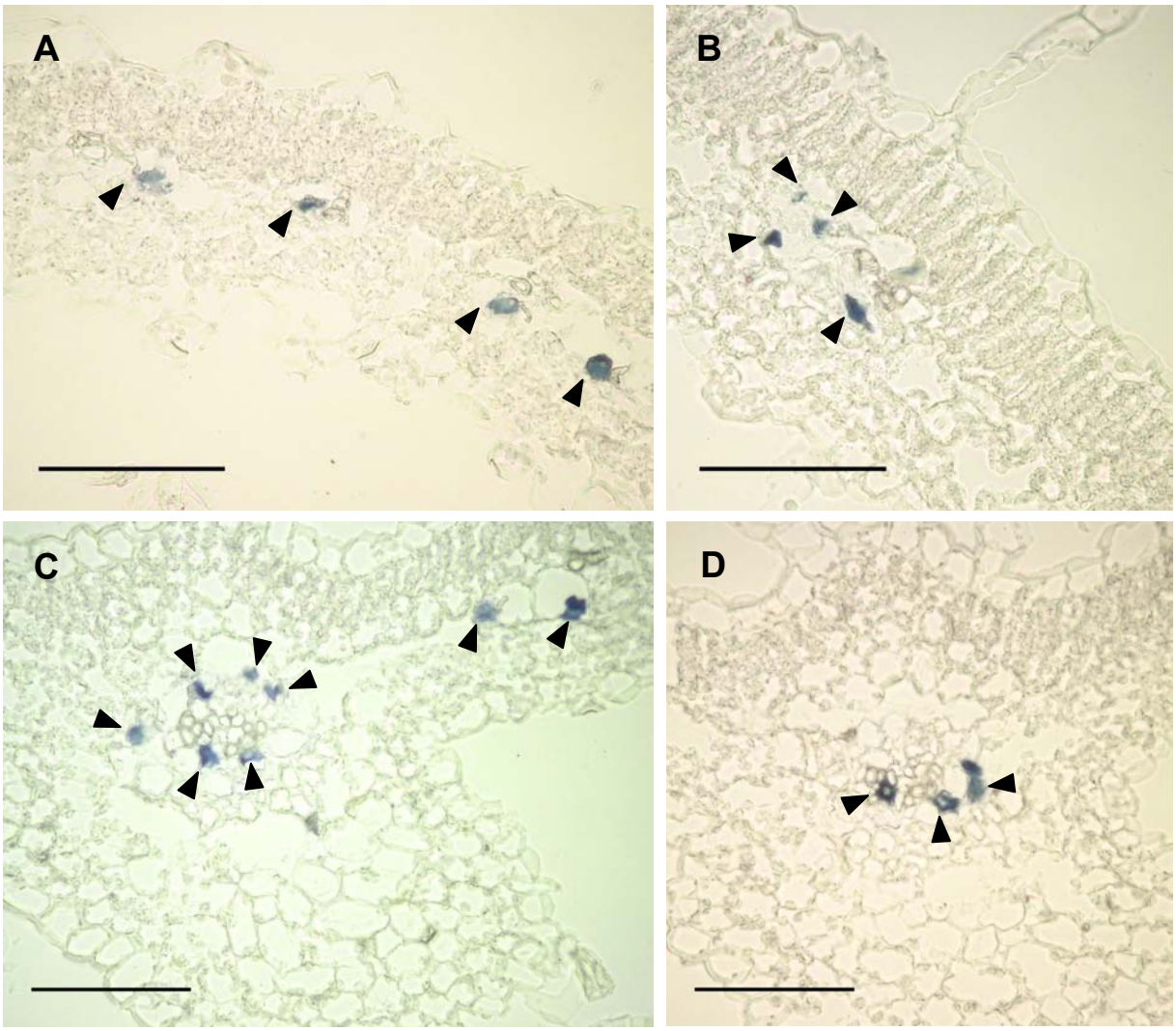

Figure 2. *In situ* hybridisation of ICMV DNA in tobacco plants using a biotin-labelled DNA A-specific probe. In the leaf blade (A and B), signals were below the palisade parenchyma in association to xylem tracheids of minor veins. In cross sections, the virus was localized around the xylem in the main vein of leaves (C and D). Bars represent 100 µm.

(continue **Figure 1.**) Cross section of the main vein of a leaf (A) showed the distortion of normal development as a thickening of the upper and, more pronounced lower parts of the vein In cross sections of the petiole (B) signals were more abundant, but no clusters of infected cells were detected. Signals within the leaf blade (C and D) were beneath the palisade parenchyma within the phloem, associated xylem tracheids are in evidence. (E) Viral DNA associated with the vasculature, xylem tracheids are clearly distinguishable. (F) In one of 200 sections, virus was located in spongy parenchyma. In this case the nuclei of several adjacent cells were labelled. Bars in A to D and F represent 100µm, in E 50 µm.

biotin- labelled ICMV probes. Due to its latex, tissue blots of cassava plants were not interpretable. In *N. benthamiana* viral DNA A (Fig. 3 A) as well as DNA B (Fig. 3 C) were exclusively associated with the vascular bundle of stems. Singular signals, outside of the phloem, were ascribed to vascular traces of branching leaves within the stem. In mock-inoculated plants, no signals were detectable (Fig. 3 B and D).

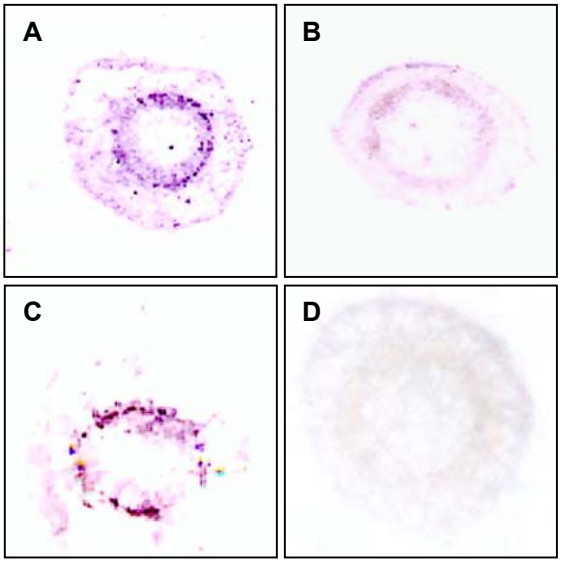

Figure 3. Tissue blot of ICMV-infected *N. benthamiana* plants. Petioles of systemically infected (A and C) and mock-inoculated (B and D) plants were blotted onto a nylon membrane and probed with ICMV DNA A- (A and B) and DNA B- (C and D) specific biotin-labeled probes. Infected cells were detected with NBT/BCIP. The viral DNA was associated with the vascular system of infected plants, as shown into cassava and tobacco plants. No signals were detectable in negative control plants.

Discussion

The tissue tropism of the bipartite geminivirus ICMV in its natural host cassava and in experimental plants (*Nicotiana* spec.) was determined by *in situ* hybridisation and tissue blot analysis. The virus was phloem-restricted in all plants investigated. Thus, the tissue tropism was independent from the host. These results are consistent with earlier investigations on tissue tropism of ICMV in *N. benthamiana* plants (Roberts, 1989), where changes of the ultrastructure were found mostly in phloem- and in some xylem-associated cells. Consistent with our results, where ICMV DNA was detected in vascular cells (Fig. 1).

Different tissue tropisms of various begomoviruses have been reported. Phloem-limitation of bipartite Gemini-viruses in different host plants, e.g. AbMV (Wege *et al.*, 2001; Horns and Jeske, 1991; Abouzid *et al.*, 1988) and *Sida micrantha mosaic virus* (SimMV) (Jovel, 2004; Wege *et al.*, 2001), and on the other hand efficient invasion of non-phloem tissues. ACMV DNA, TGMV DNA and *Bean dwarf mosaic virus* (BDMV) was detected in phloem as well as in palisade, spongy parenchyma and epidermal cells (Wege *et al.*, 2001; Wang *et al.*, 1996).

It has been proposed, that the tissue tropism of bipartite geminiviruses is genetically determined (Morra and Petty, 2000). *Bean golden mosaic virus* (BGMV) is phloem-limited whereas TGMV is mesophyll-invasive in their common host *N. benthamiana*. BGMV based hybrid viruses, containing TGMV elements, revealed that coding and non-coding regions are required for efficient invasion of non-phloem tissues (Qin and Petty, 2001; Morra and Petty, 2000). The efficient mesophyll invasion of BGMV hybrid viruses were determined by the TGMV BRi non-coding region upstream of the BV1 gene in combination with either TGMV AV2/3 or BV1/BC1 (Qin and Petty, 2001; Morra and Petty, 2000). Invasion of the mesophyll could be genetically separated from viral DNA accumulation and symptom severity (Qin and Petty, 2001). Sequence comparisons of ICMV (phloem-limited) and ACMV (mesophyll-invasive) with TGMV BRi, did not show similar regions in one of the viruses and sequences upstream the BV1 differ largely.

The nuclear shuttle protein (BV1) and movement protein (BC1), which influence tissue tropism (Morra and Petty, 2000), may function differently and/or adapt variably to host plants. Complementation experiments have demonstrated differences in DNA B specificities (Frischmuth *et al.*, 1993).

In contrast to strict maintenance of tissue tropism in different plant species, *Euphorbia mosaic virus* (EuMV) has different tissue tropisms in its natural and experimental host (Kim and Lee, 1992). Thus, host factors are involved in the invasion of various tissues. Interaction of geminivirus proteins with plant factors has been reported. As geminiviruses do not encode for a polymerase, they rely on the host cellular replication machinery. Geminiviruses can dedifferentiate plant cells. TGMV AC1 stimulates cells to enter S-phase (Nagar *et al.*, 2002) and induces accumulation of proliferation cell nuclear antigen (PCNA) (Nagar *et al.*, 1995), the processivity factor of DNA polymerase δ. Impaired interaction of TGMV AC1 with a plant retinoblastoma (Rb) homologue (pRBR), a cell cycle regulator, leads to phloem-restriction of TGMV (Kong *et al.*, 2000). Thus, host adaptation may have an impact on replication and tissue tropism. Promoter elements function in a tissue-specific activation and derepression of virion-sense gene expression (Sunter and Bisaro, 1997). Insufficient replication of ICMV in non-phloem cells into *N. benthamiana*,

whether through perturbed interaction of viral and host proteins or repression of promoter activity, may not be the main reason for phloem-limitation. Chimeras in which the *CP* of ICMV DNA A was replaced by *GFP*, sufficiently expressed GFP in epidermal cells (Rothenstein *et al.*, 2004d). This suggests, that the virus is impaired in the spread between phloem and other tissues.

Wang *et al*. (1996) have shown that the tissue tropism of BDMV is dependent on the developmental stage of the infected plant suggesting that either a coordinate interaction between virus and plant factor(s) or structural changes during maturation, e.g. of plasmodesmata, are involved in viral spread. Potato virus X (PVX, potyvirus) can invade parenchyma cells only before the sink-source transition of the leaf (Roberts *et al.*, 1997). During transition, plasmodesmata undergo structural alterations, from simple (sink) to branched (source) type. Investigations aimed on AbMV movement protein BC1, have shown that it localised predominantly at plasma membranes of *Schizo-saccharomyces pombe* (Aberle *et al.*, 2002) and at the cell periphery in plant cells (Zhang *et al.*, 2001). BC1 and BV1 were co-transported only in sink, but not in source tissues (Zhang *et al.*, 2001).

Generally, viral DNA was rarely detectable, probably reflecting the limitation of the method: detection of viral genomes beneath a certain threshold within a cell is not possible. Horns and Jeske (1991) suggested that a signal-gain is only possible in cells harbouring several tens copies of the viral genome. Jeske and Werz (1978) ascribed symptom development in mesophyll tissues of a phloem-limited virus to impaired carbohydrate transport, which may influence surrounding tissues. Interaction of viral components with plant metabolism components and signalling pathways have been reported recently. TGMV AC2 and BCTV C2 interact with an adenosine kinase (ADK) (Wang *et al.*, 2003) and a SNF1 (sucrose nonfermenting1) kinase (Hao *et al.*, 2003), which has a major role in regulation of metabolism (for review see Halford *et al.*, 2003; Hrabak *et al.*, 2003; Gibson, 2000; Halford and Hardie, 1998). TGMV BV1 interacts with a novel receptor-like protein kinase (NIK), which may be involved in resistance and/or developmental signal transduction pathway (Marianao *et al.*, 2004), and TGMV AC1 interacts with

the sumoylation system in *N. benthamiana* (Castillo *et al.*, 2004).

RNA silencing functions as antiviral defence in plants (Voinnet, 2001). Such specific defence mechanisms could act efficiently in mesophyll cells and prevent the spread of viruses into non-phloem tissues. Moreover, viruses have evolved counter defence strategies. Viral suppressors of RNA silencing have been shown to interfere with plant development and micro (mi) RNAs (Kasschau *et al.*, 2003). Endogenous miRNAs regulate plant development (for review see Bartel, 2004; Juarez *et al.*, 2004; Kidner and Martienssen, 2004). Beside indirect interaction of virus encoded proteins and miRNAs, it has been shown that a human DNA virus encode miRNAs (Pfeffer *et al.*, 2004). Thus, viruses share miRNA signal pathway for host and viral gene regulation. Further investigations may reveal whether a RNA signal is liable for tissue distortion during virus infection.

Material and Methods

Plants and viruses

Stem cutting from symptomatic ICMV-infected cassava plants (*Manihot esculenta* spec.) were collected in South India in the states Tamil Nadu and Kerala in 2001 and 2002. Regenerated cuttings were grown in an insect-free glasshouse at 24°C with supplementary lighting. ICMV infection was verified by Southern blot analysis using ICMV DNA A-specific probes.

N. tabacum (L.) cv. Samsun nn plants in the two to three leaf stage were inoculated with ICMV clones (Rothenstein *et al.*, 2004a) by particle bombardment as described (Zhang *et al.*, 2001). Infection was established 14 to 17 days post inoculation (dpi) and confirmed by Southern blot analysis.

N. benthamiana plants were sap-inoculated. Systemically infected cassava leaves were ground in a mortar in 1 ml water. 10 µl were mechanically inoculated on carbo-rundum-dusted leaves. Infection was established 15-18 dpi and confirmed by Southern blot analysis.

Preparation of tissue sections

Leaf- and petiol-sections, 3 to 4 mm in diameter, from ICMV-infected cassava and tobacco plants were paraffin-infiltrated and embedded as described by (Wege *et al.*, 2001)

In situ hybridisation and detection

A virus-specific fragment, covering the *CP* gene was amplified by polymerase chain reaction (PCR) from cloned ICMV DNA A (Rothenstein *et al.*, 2004a). For PCR reactions, degenerated primers ICMV-CP for (5´-

ATGTCGAAGCGACCAGSAGATATW-AT-3´) and ICMV-CP rev (5´-TTAATT-KSTCACTGMATCATAGAARTA-3´) were used. Probes were biotin-labelled by nick-translation according to the manufacturer´s recommendations (Gibco-BRL, Germany). Unincorporated nucleotides were removed (QIAquick nuceliotide removal kit; Qiagen).

All hybridisation steps were performed in a thermocycler (Omnislide, Thermo Hybaid). Samples were incubated in pre-hybridisation solution (600mM NaCl; 1 mg/ml denatured herring sperm; 1 mM EDTA; 10 mM Tris-HCl (pH 7.5); 50 % deionised formamide; 1 × Denhardt´s solution) at 42°C for three hours. Hybridisation was carried out in pre-hybridisation solution supplemented with 5 % dextran sulphate and 0.8 µg/ml biotin-labelled DNA probe. The hybridisation solution was denatured at 95°C for 5 minutes, 50 µl were placed on each slide and covered with a Repel-silane coated cover glass and incubated for 19 h at 42°C.

Subsequently, the samples were washed twice for 30 minutes at 42°C in W1 (2 × SSC; 10 mM EDTA, pH 7.0), once in W2 (1 × SSC; 10 mM EDTA, pH 7.0) for 15 minutes at 65 °C and twice in W1 for 30 minutes at 42°C. Finally, the samples were rinsed briefly with deionised water.

The detection of biotin-labelled probes with tetramethylbenzidine (TMB), which resulted in a blue precipitate, was carried out according to the manufacturer´s recommendations (Vector Laboratories, USA). Development of signals was monitored under a light microscope and stopped, when the chromogen-derived background increased, usually after 5-15 minutes. Specimens were analysed using either differential interference contrast (DIC) or bright field microscopy (Zeiss Axioskop; Carl Zeiss, Jena, Germany). Photographs were taken with a Canon Power Shot G1 digital camera.

Tissue blot

Cross sections of ICMV-infected cassava and *N. benthamiana* stems and petiols were pressed on Hybond NX membranes (Amersham Bioscience, UK) and UV-cross linked. Membranes were incubated for three hours in pre-hybridisation solution according to Wege *et al.* (2001) at 42°C. Hybridisation was performed in pre-hybridisation solution supplemented with 2 % dextran sulphate and 0,5 ng/ml digoxigenin (DIG)-labelled probe overnight at 42°C. The detection

of digoxigenin with anti-DIG-AP Fab fragments (Roche, Germany) and nitro blue tetrazolium (NBT) / 5-brome-4-chloro-3-indolyl-phospate (BCIP) (Applichem, Germany) was performed according to manufacturer´s recommendations.

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GFP-labelled AV2 protein of Indian cassava mosaic virus moves cell to cell

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Abstract

Begomoviruses, including *Indian cassava mosaic virus* (ICMV) are small single-stranded (ss) DNA viruses, which infect a number of important dicotyledonous crop plants. In order to monitor the spread of ICMV within the plant, its coat protein (CP) gene was replaced by the *green fluorescence protein* (GFP) gene, either to obtain a translational fusion with the precoat protein AV2 or to express unfused GFP. Viral movement was studied in systemically infected *N. benthamiana* plants as well as in biolistically inoculated leaves. AV2:GFP fusion protein was restricted to the vasculature of systemically infected plants. In inoculated leaves, cell-to-cell movement was observed. We conclude, that ICMV AV2 may function in facilitating cell-to-cell movement. It might be an ancestral movement protein (MP) of a formerly monopartite begomovirus, this monopartite ancestral may has acquired a second DNA molecule (DNA B) for improved spread within plants.

Introduction

Indian cassava mosaic virus (ICMV) is a begomovirus belonging to the family *Geminiviridae*, which are single-stranded (ss) DNA plant viruses with a circular genome packed in geminate particles (Rybicki *et al.*, 2000). Begomoviruses infect dicotyledonous plants, are whitefly-transmitted and most members possess a bipartite genome of DNA A and DNA B. DNA A encodes the coat protein (CP / AV1), proteins for the regulation of replication, a replication-associated protein (Rep / AC1) and a replication enhancer protein (REn / AC3), and transcription, transcriptional activator protein (TrAP / AC2). DNA B genes, movement protein (MP / BC1) and nuclear shuttle protein (NSP / BV1), are involved in intra- and intercellular movement, symptom development and host range determination (Gafni and Epel, 2002; Lazarowitz and Beachy, 1999).

In Old World begomoviruses, *AV2* (“precoat”) proceeds the coat protein

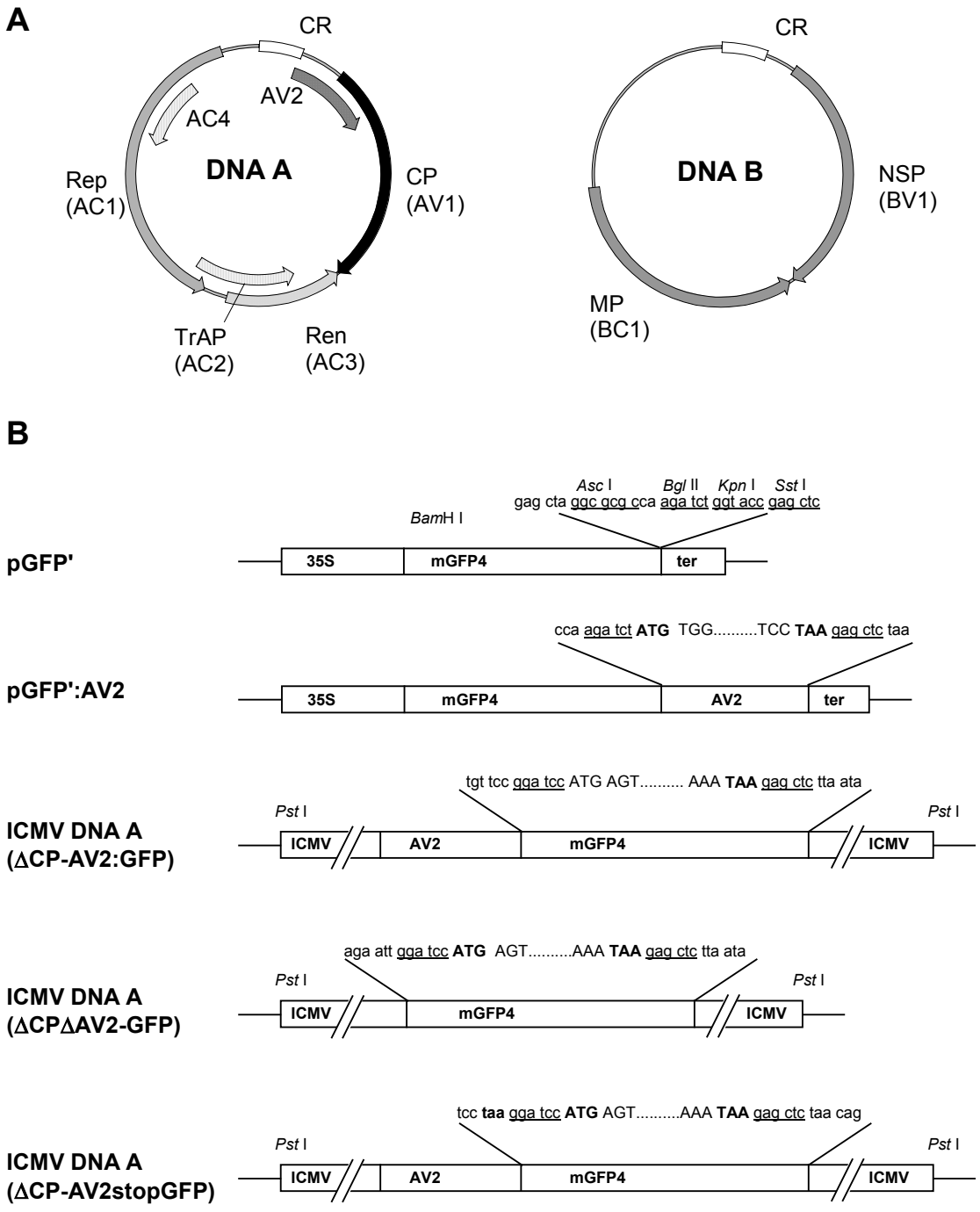


Figure 1. (A) Genome organization of *Indian cassava mosaic virus* (ICMV). The circles represent DNAs A and B of the bipartite virus. Open reading frames are indicated. Common region (CR; white box) contains promoter elements and a highly conserved hairpin-loop with the origin of replication. (B) Genomic maps of GFP expression vector, GFP:AV2 fusion in the expression vector and chimeric ICMV DNA As. Note that ICMV DNA A(ΔCPΔAV2 -GFP) has a truncated ORF AV2, while ICMV DNA A(ΔCP-GFP) and ICMV DNA A(ΔCP-AV2:GFP) contain the entire ORF AV2. 35S indicates *Cauliflower mosaic virus* (CaMV) 35S promoter, ter indicates nopaline synthase terminator. Recognition sequences of *Bam*H I, *Bgl* II and *Sst* I cloning sites introduced using PCR are underlined. *Pst* I site for cloning in pBluescript vector is indicated.

gene (Fig.1 A). For historical reasons, similarly located genes are inconsistently called V1 or V2, for monopartite mastre- or begomoviruses. To circumvent this conflict AV2, V2 and V1 gene products will here referred to as precoat proteins (PCP).

To establish a systemic infection in plants, viruses have to spread from infected to healthy tissues generally including two steps: localised cell-to-cell spread and long-distance movement via the phloem (Leisner, 1999; Nelson and van Bel, 1998). Viral movement proteins (MPs) can interact with plasmodesmata (Heinlein *et al.*, 1998) and host factors, e.g. tubulin (Heinlein *et al.*, 1998) to transport viral nucleic acids. Two different mechanisms have been discovered, e.g. *Tobacco mosaic virus* (TMV) forms MP-nucleoprotein complexes capable to pass into adjacent cells and the MP increases the size exclusion limit (SEL) of plasmodesmata. The other mechanism is dependent on tubule formation and alteration of the plasmodesmata structure (Lazarowitz and Beachy, 1999).

For geminiviruses, two modes of cell-to-cell transport have been discovered. CP-dependent spread of monopartite geminiviruses of all genera (Liu *et al.*, 1998; Rigden *et al.*, 1993; Boulton *et al.*, 1989; Briddon *et al.*, 1989) including the monopartite begomovirus *Tomato yellow leaf curl virus* (TYLCV) (Rigden *et al.*, 1993) and CP-independent movement of bipartite begomoviruses in plants (Sudarshana *et al.*, 1998; Ingham *et al.*, 1995; Padidam *et al.*, 1995; Gardiner *et al.*, 1988; Stanley and Townsend, 1986). The dispensability of CP was used to substitute *CP* with *green fluorescent protein* (GFP) gene to investigate viral distribution and spread within plants. The exchange of *CP* with *GFP* generally conserves the size of DNA A, maintaining the *GFP* during replication and movement (Gilbertson *et al.*, 2003; Bisaro, 1994; Etessami *et al.*, 1989).

Some DNA As of bipartite begomoviruses, e.g. *African cassava mosaic virus* (ACMV) (Klinkenberg and Stanley, 1990) and *Abutilon mosaic virus* (AbMV) (Evans and Jeske, 1993) can spread systemically in the absence of DNA B but lacking symptom development. *Sri Lankan cassava mosaic virus* (SLCMV) DNA A can establish a systemic symptomatic infection when inoculated into *N. benthamiana*, but symptoms were modulated when DNA B was co-inoculated (Saunders *et al.*, 2002).

Mutation experiments aimed at PCP, encoded on the DNA A, led to contradictory results for different viruses. Monopartite geminiviruses, including the subgroups *Mastrevirus* and *Begomovirus*, require the PCP for efficient spread in plants (Rojas *et al.*, 2001; Kotlizky *et al.*, 2000; Boulton *et al.*, 1993; Rigden *et al.*, 1993). In contrast, in the monopartite *Beet curly top virus* (BCTV) PCP is not essential for cell-to-cell movement. In bipartite begomoviruses the function of PCP is ambiguous. While ACMV PCP has no function in infection (Etessami *et al.*, 1989), *Tomato leaf curl virus* India (ToLCV-Ind) PCP may be involved in viral spread (Padidam *et al.*, 1996).

In this report we provide first direct evidence that ICMV PCP may function as MP, like PCP in mastreviruses.

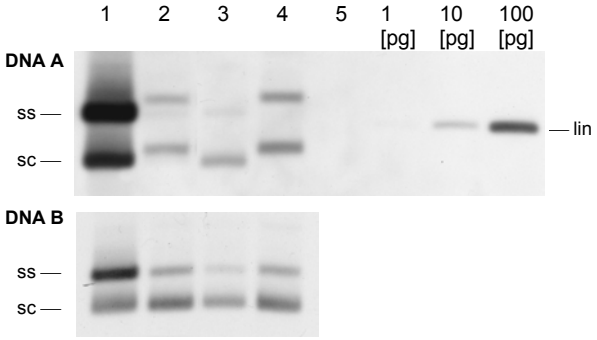


Figure 2. Southern blot analysis. Replication of ICMV-GFP chimera in systemically infected *N. benthamiana* plants. Plants were inoculated with wild-type ICMV (lane 1), ICMV DNA A(ΔCP-AV2:GFP) (lane 2), ICMV DNA A (ΔCPΔAV2-GFP) (lane 3), ICMV DNA A (ΔCP-AV2stopGFP) (lane 4) or mock-inoculated (lane 5). DNAs were extracted 20 dpi, three µg of each sample were separated on a 1% agarose gel and blotted. Hybridisation standards of linear (lin) ICMV DNA A (1; 10; 100 pg) are separated on the upper blot. The upper blot was hybridised with an ICMV DNA A- the lower with a DNA B-specific probe. The positions of single-stranded (ss) and supercoiled (sc) double-stranded DNA forms are indicated.

Results

Infectivity and replication of chimeric ICMV DNA As

N. benthamiana plants were successfully infected with either ICMV DNA A(ΔCPΔAV2 -GFP), ICMV DNA A(ΔCP-AV2stopGFP) or ICMV DNA A(ΔCP-AV2:GFP) and wild-type (wt) DNA B. Symptoms developed 19 to 22 days post inoculation (dpi), 5-7 days delayed in comparison to wild type (wt) ICMV. Plants displayed less pronounced symptoms, exhibited crinkling and faint leaf yellowing and were of slightly larger size than wt ICMV-infected plants.

Replication of chimeric ICMV was analysed by Southern blot hybridisation. The amounts of viral DNA were significantly less in all GFP-chimeras (Fig. 2, lanes 2-4), than in wt ICMV (Fig. 2, lane 1). Wild-type virus produced stronger bands for single-stranded (ss)DNA than for supercoiled (sc) double-stranded DNA. GFP

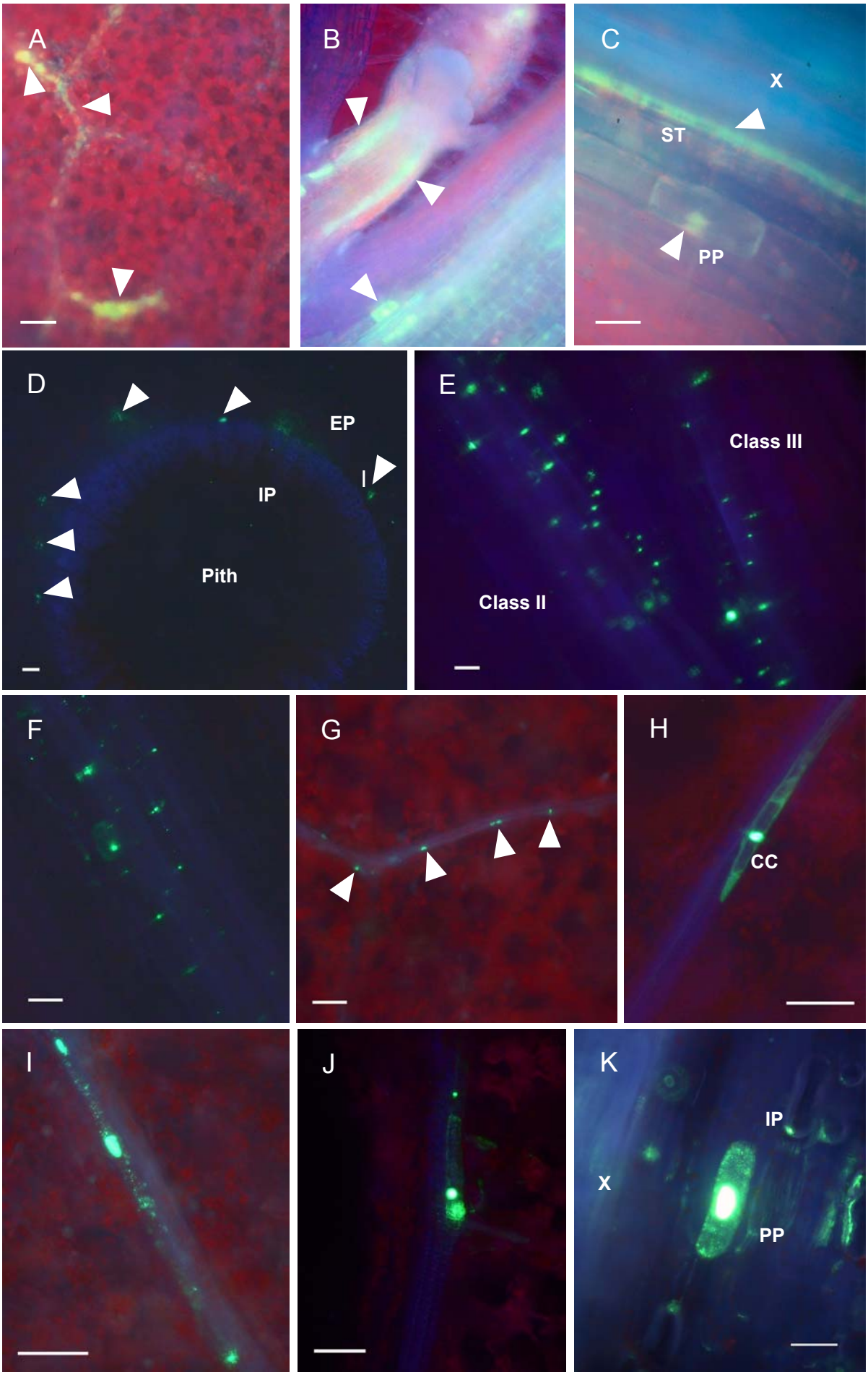
chimeras with a complete ORF AV2 had approximately equal amounts of ss- and dsDNA (Fig. 2 lanes 2 and 4), whereas the AV2-mutated chimera had markedly reduced proportions of ssDNA (Fig. 2 lane 3). The alterations in sizes of chimeric ICMV DNA As were retained during the infection process. *GFP* was maintained stably, thus the constructs were within the size-limitations that are tolerated without producing size reversion (Gilbertson *et al.*, 2003; Bisaro, 1994; Etessami *et al.*, 1989).

GFP detection

Leaves and stems of systemically infected *N. benthamiana* plants were investigated by fluorescence microscopy. ICMV DNA A(ΔCPΔAV2 -GFP) and DNA B showed GFP fluorescence in association with the vascular system of leaves (Fig. 3A). In cross sections of petiols, companion cells (Fig. 3B) and phloem parenchyma cells (Fig. 3C) were infected. Cell types apart from the vascular tissues did not express GFP suggesting a phloem-limitation of ICMV.

ICMV DNA A(ΔCP-AV2:GFP) induced fluorescence was associated with the vascular system of stems and predominantly with sink portions of leaves. In cross-sections of stems, infected cells were exclusively located within the phloem (Fig. 3 D). GFP accumulated in class II, III veins (Fig. 3 E), and with high abundance in main

Figure 3. (next page) Cellular localisation of ICMV A(ΔCPΔAV2-GFP) or A(ΔCP-AV2:GFP) in the presence of DNA B in systemically infected *N. benthamiana* plants. (A) GFP fluorescence within the vascular system of the leaf lamina. The signals are evenly distributed along the vasculature. (B) Transversal section of a shoot and side shoot expressing GFP within the vasculature. (C) Stem with infected phloem parenchyma cells and sieve tube-elements. Cellular localisation of AV2:GFP fusion protein in systemically infected *N. benthamiana* plants. Fluorescence micrographs of stem sections (D and H to K) and leaf (E to G). (D) Cross section of a stem. Expression of AV2:GFP in the external phloem cells (arrowheads). Transversal sections through a leaf with GFP fluorescence in class II and class III veins (E) and mid vein (F). Signals appear in discrete spots at high abundance within the basal (sink) part. (G) Minor vein (class III) within the leaf lamina. (H, I and J) The companion cell in stems frequently showed fluorescence. (K) Phloem parenchyma cell. Punctated fluorescence at the cell periphery may reveal plasmodesmata association of the fusion protein. X, xylem; IP, internal phloem; EX, external phloem; PP, phloem parenchyma; CC companion cell; ST, sieve tube. Bars represent 5 µm.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veins of the leaf base (Fig. 3 F). Signals were detected as discrete spots along the vasculature of leaves (Fig. 3 G). Cross-sections of petiols

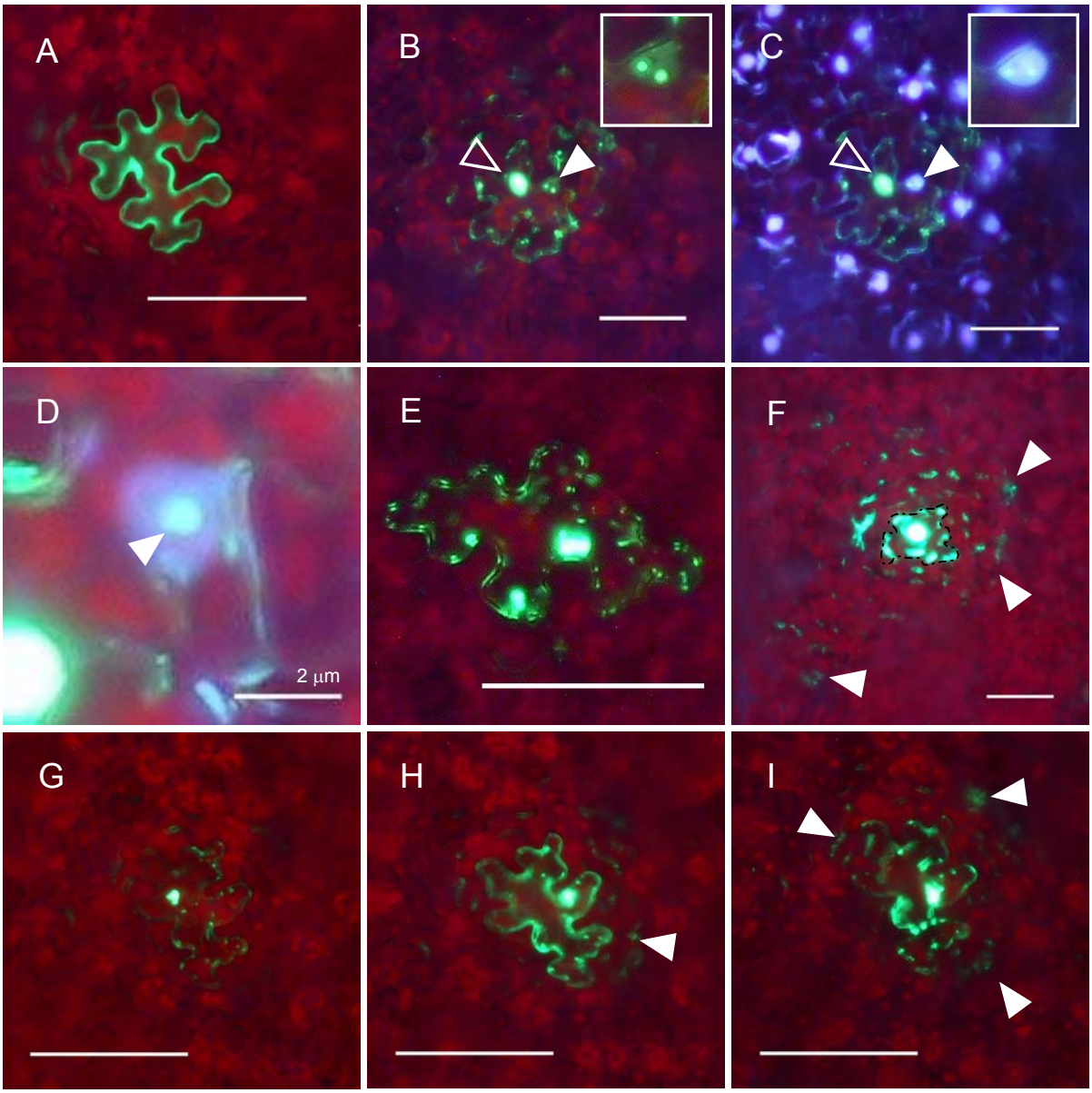

Figure 4. Subcellular localisation of transiently expressed GFP:AV2 in epidermal cells of *N. benthamiana* sink leaves bombarded with pGFP:AV2. (A) As a control, unfused GFP did not show movement to neighbouring cells 48 hours post bombardment (hpb) under the same conditions. (B, C) GFP or GFP / DAPI fluorescence, respectively, of the same cell 18 hpb: GFP mainly accumulated (open white arrowheads) outside of the nucleus (filled white arrowheads). Within the nucleus two discrete GFP spots were regularly detected (B and C insets). (D) Nucleus with a single GFP spot. (E) Discrete punctated spots were associated with the cell periphery indicating a possible plasmodesmata association of the fusion protein. (F) GFP signals have moved from the initially bombarded cell (dotted black line) into adjacent cells 72 hpb. (G to I) pGFP:AV2 expressed in the same cell, 10 hpb, GFP:AV2 signals were confined to a single cell (G), 24 hpb (H), and 48 hpb (I) GFP:AV2 moved to adjacent cells (white arrowheads). Bars represent 5 µm for A-C and E-I, 2 µm for D.

showed infection of companion cells (Fig. 3 H to J). The punctated shape of the signals in phloem parenchyma cells (Fig. 3 K) may indicate plasmodesmata association, like reported for MPs (Blackman *et al.*, 1998; Heinlein *et al.*, 1998; Itaya *et al.*, 1998; Ryabov *et al.*, 1998; Oparka *et al.*, 1997; Padgett *et al.*, 1996).

To study the spread of transiently expressed AV2 from the initially infected cell, *N. benthamiana* leaves of various sizes were inoculated with pGFP:AV2 by particle bombardment. In small leaves (1 to 3 cm in length, sink leaves) and leaves undergoing the sink to source transition (3 to 4 cm in length of leaf base to leaf tip) (Oparka *et al.*, 1999) GFP fluorescence appeared 8 to 10 hours post bombardment (hpb) and was restricted to single cells. The subcellular distribution of pGFP:AV2 was different from that of unfused GFP (Fig. 4 A). The majority of cells (~90 %, 990 out of 1100 GFP expressing cells) usually showed one bright fleck of GFP (Fig. 4 B, open arrowhead) and several discrete spots and stretches of fluorescence along the cell periphery (Fig. 4 B, C and E). Although the bright fleck looks like a nucleus, counter-staining with DAPI (Fig. 4 C, white arrowhead) clearly showed that it lies outside the nucleus, presumably within the cytoplasm (Fig. 4 B, C, open arrowhead). Within the nucleus one or two bright GFP spots were regularly located, which may be associated with nucleoli (Fig. 4 A and B insets, and 4 D). In approximately 20 % of the cells (~200 out of 1100 GFP expressing cells), only punctated GFP signals were detected at the cell periphery (Fig. 4 E).

48 hpb, fluorescence was detected in neighbouring cells of approximately 60 % of the initially GFP-expressing cells. The signal moved from the initially bombarded cell to four neighbouring cells (Fig. 4 F). In the case of unfused GFP (data not shown), only weak fluorescence appeared in rare cases in neighbouring cells, probably due to leakage.

To exclude the possibility of co-bombardment adjacent cells, GFP:AV2 distribution was monitored in the same cell over 48 hours. At 10 hpb, fluorescence accumulated in the cytoplasm and in punctate spots at the cell periphery (Fig. 4G), 24 hpb fluorescence was enhanced in the initially expressing cells and weak fluorescent signals appeared in neighbouring cells (Fig. 4 H), which intensified to give bright fluorescence 48 hpb (Fig. 4 I). In initially transformed

cells, AV2:GFP fusion protein moved within the cytoplasm. Cells transformed with unfused GFP did not show a detectable fluorescence in neighbouring cells even after 48 hours (Fig. 4 A).

The construct ICMV DNA A(ΔCP-AV2stopGFP) was infectious in *N. benthamiana*, but fluorescence was not detectable in particle bombarded leaves.

Discussion

Here we report on cell-to-cell movement and distribution of ICMV PCP in the experimental host *N. benthamiana*. Basis of these studies was the dispensability of CP for systemic movement and replication in this host. CP dispensability has been reported for a range of other bipartite geminiviruses (Sudarshana *et al.*, 1996; Ingham *et al.*, 1995; Padidam *et al.*, 1995; Azzam *et al.*, 1994). In place of the *CP*, the modified *green fluorescent protein* (GFP) gene (*mGFP4*, referred here as to GFP) was inserted, thereby conserving the approximate size of DNA A. The maintenance of size is essential for stable integration of transgenes into the viral genome. The normal genome size of enlarged components will be recovered due to recombination (Etessami *et al.*, 1989). The expression of GFP in *N. benthamiana* plants systemically infected with chimeric ICMV confirmed that it was efficiently expressed from the CP promoter and stably maintained within the genome. As reported for other *CP*-mutated begomoviruses, the expression levels of viral DNA were decreased (Padidam *et al.*, 1996; Rigden *et al.*, 1993; Etessami *et al.*, 1989; Stanley and Townsend, 1986). The ratio of ss- and ds-DNA was shifted to equal amounts, except for the CP/PCP mutated ICMV [ICMV DNA A(ΔAV2ΔCP-GFP)], which was impaired in the accumulation of viral ssDNA. Thus, PCP might be involved in the switch from dsDNA to ssDNA synthesis, as suggested for *Tomato yellow leaf curl virus* (TYLCV, Wartig *et al.*, 1997) and *Beet curly top virus* (BCTV, Hormuzdi and Bisaro, 1993).

Both examined GFP-expressing chimeras, ICMV DNA AΔ(ΔAV2CP-GFP) and ICMV DNA A(ΔCP-AV2:GFP), were restricted to vascular tissue of systemically infected plants. This tissue tropism was not caused by lack of CP, since *in situ* hybridisation studies of wt ICMV confirmed its phloem-limitation (Rothenstein *et al.*, 2004c).

Using a transient expression system, we demonstrated cell-to-cell movement of PCP-GFP fusion protein in the absence of other viral proteins in *N. benthamiana* leaf tissue. ORFs at similar positions to AV2 are found in all subgroups of geminiviruses, except of New World begomoviruses (Rybicki *et al.*, 2000). PCP ORFs are not highly conserved between different subgroups, and mutation analysis suggested a complex interaction in viral movement and replication. The PCP of the monopartite mastrevirus *Maize streak virus* (MSV) was involved in viral spread (Kotlizky *et al.*, 2000; Boulton *et al.*, 1993) and localized in secondary plasmodesmata (Dickinson *et al.*, 1996). *Beet curly top virus* (BCTV), mutated in the analogous ORF V2, altered the ratio of viral ss- and dsDNA implicating a role in viral synthesis (Hormuzdi and Bisaro, 1993; Stanley *et al.*, 1992). In the monopartite begomoviruses TYLCV and ToLCV, mutations in the corresponding ORF V1 led to symptomless infection and markedly reduced viral DNA amounts in plants. Since mutants replicate efficiently in protoplasts, it was suggested that PCP functions in spread (Wartig *et al.*, 1997; Rigden *et al.*, 1993). For bipartite geminiviruses, results of mutation experiments for PCP are ambiguous. ACMV AV2 mutations had no effect on infection (Etessami *et al.*, 1989), whereas ToLCV-Ind requires PCP for efficient viral movement and viral replication was impaired by some mutations in *AV2* (Padidam *et al.*, 1996).

GFP:AV2 fusion protein was localized in punctate spots at the cell periphery in epidermal cells and systemically infected plants. Similar distribution pattern of GFP-tagged AbMV BC1 in *N. tabacum* has been shown (Zhang *et al.*, 2002; Zhang *et al.*, 2001). In freeze-fracture immuno-labelling experiments, AbMV BC1 was detected predominantly at plasma membranes (Aberle *et al.*, 2002). The BC1 protein acts as a movement protein (MP) and may facilitate the transport of BV1-ssDNA complexes from the nuclear envelope to plasmodesmata (Pd) and to adjacent cells (Zhang *et al.*, 2001). It has been shown for other viruses that GFP-tagged MPs had similar localization patterns and a plasmodesmata association has been suggested (Heinlein *et al.*, 1998; Itaya *et al.*, 1998; Oparka *et al.*, 1997; Padgett *et al.*, 1996).

GFP:AV2 fusion protein, expressed under the CaMV 35S promoter

Table 1. Primers used for cloning and verification of ICMV / GFP constructs

#	Primer	Sequence [a]	Location [b]
1	AV2-ICMV rev	5´-TAA TGG ATC CGG AAC ATC TGG GCT TCT GTA -3´	ICMV A nt 469-489
2	AV2-ICMV for	5´-CTT CTA TGA TTC AGT GAG CTC TAA TAA ATA-3´	ICMV A nt 1041-1071
3	GFP *Bam*H I	5´-ATA GGA TCC ATG AGT AAA GGA GAA GAA C -3´	*mgfp4* nt 1-19
4	GFP *Sst* I	5´-CGT GAG CTC TTA TTT GTA TAG TTC ATC C -3´	*mgfp4* nt 719-737
5	ΔAV1/AV2 rev	5´-AGC GGA TCC AAT TCT TCG CCC TAA TAA CAG -3´	ICMV A nt 274 - 300
6	ΔAV1stop rev	5´-CCT TCA GGA TCC TTA GGA ACA TCT GGG CTT -3´	ICMV A nt 474 - 503
7	AV2-for *Bgl* II	5´-GTT GCC AGA TCT ATG TGG GAC CCT TTA CTA -3´	ICMV A nt 123-157
8	AV2-rev *Sst* I	5´-CCT TCA GAG CTC TTA GGA ACA TCT GGG CTT -3´	ICMV A nt 474-503
9	GFP-rev [c]	5´-GTA TGT TGC AGC ACC TTC ACC C-3´	*mgfp4* nt 116-137
10	GFP-for [c]	5´-ATT ACC TGT CCA CAC AAT CTG-3´	*mgfp4* nt 596-616

[a] Restriction sites introduced by PCR are underlined
[b] *gfp*: numbering starts at start codon; ICMV A: numbering according to Rothenstein *et al*., in press
[c] Sequencing primer

(pGFP:AV2) led to a predominant GFP-inclusion body. This inclusion body has been shown to localize outside of the nucleus. In systemically infected plants expressing AV2:GFP fusion protein under the CP promoter, similar accumulations were detected. This accumulation could be due to aggregation of PCP in plant cells and reduced levels of (ss) DNA in ICMV DNA AΔ(ΔAV2CP-GFP)-infected plants. A similar situation of impaired DNA replication was observed in ToLCV-Ind AV2 mutants (Padidam *et al.*, 1996).

In all epidermal cells expressing GFP:AV2 fusion protein, one or two bright spots of GFP were detected within the nucleus, probably reflecting the nucleolus. Rojas *et al*. (2001) speculated that the nucleolus may serve as the site of geminiviral replication and/or gene expression.

ICMV PCP may function as MP in *N. benthamiana* epidermial cells though ICMV DNA B provides additional MPs. ACMV CP has distinct localisation sequences for intracellular movement (Unseld *et al.*, 2001), and *Squash leaf curl virus* (SqLCV) CP can functionally substitute for mutations in the nuclear shuttle protein (NSP) of DNA B (Qin *et al.*, 1998). The question arises why bipartite geminiviruses posses additional MPs encoded on the DNA B. PCP and CP of monopartite begomoviruses function in viral movement (Rojas *et al.*, 2001; Wartig *et al.*, 1997; Rigden *et al.*, 1993). The DNA B of the bipartite begomoviruses TYLCV-Tailand (Rochester *et al.*, 1990) and SLCMV (Saunders *et al.*, 2002)

modulate symptom development, but DNA B is not essential for viral movement and infectivity. It was assumed that SLCMV evolved from a monopartite progenitor virus by acquisition of a DNA B component from ICMV (Saunders *et al.*, 2002). Due to the multifunctional character of viral proteins, features may be retained in spite of newly recruited components.

Material and Methods

Cloning of viral constructs

Recombinant DNA techniques were performed as described by Sambrook *et al.* (2001). Restriction endo-nucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

Cloning of monomeric ICMV DNA A in pBluescript vector has been described (Rothenstein *et al.*, 2004a). To construct chimeric ICMV DNA As, restriction sites *Bam*H I and *Sst* I within the multiple cloning site of the vector were removed by digestion with these enzymes and mung bean nuclease treatment followed by religation. For ICMV DNA A(ΔCP-AV2:GFP), the ICMV DNA A sequence 489 to 1062 nt (numbering according to Rothenstein *et al.*, 2004a), including the *AV2* stop codon and the *CP* was removed and *Bam*H I and *Sst* I sites were introduced using polymerase chain reaction (PCR) with primers #1 and #2 (Table 1). The PCR product was digested with *Bam*H I and *Sst* I (pICMVΔCP). The *GFP* gene was amplified from the construct *mgfp*4 (Haselhoff *et al*., 1997) using primers #3 adding a *Bam*H I site and #4 adding a *Sst* I site (Table 1). Fragments were digested with *Bam*H I and *Sst* I and introduced into pICMVΔCP plasmid giving ICMV DNA A(ΔCP-AV2:GFP) (Fig. 1). This construct was only slightly larger in size (2902 nt) than wild-type DNA A (2733 nt). The same strategy was used for ICMV DNA A(ΔCPΔAV2-GFP) (Fig. 1), deleting sequence 395 - 1062 nt of ICMV DNA A using primers #5 and #2 (Table 1) and ICMV DNA A (ΔCP-AV2stopGFP) (Fig. 1), deleting sequence 491 – 1062 nt, thereby conserving the stop codon of AV2 using primers #6 and #2. Both PCR products were digested with *Bam*H I and *Sst* I and GFP was inserted. The sizes of both constructs, 2808 nt for ICMV DNA A(ΔCPΔAV2-GFP) and 2905 nt for ICMV DNA A (ΔCP-AV2stopGFP) were slightly larger than wt.

To study AV2 in the absence of other viral genes, pGFP:AV2 (Fig. 1) was constructed: The *AV2* gene was

amplified by PCR with primers #7 and #8 (Table 1) adding a *Bgl* II site at the 5´-end and a *Sst* I sites at the 3´-end. The resulting fragment was digested with *Bgl* II and *Sst* I and inserted between *Bgl* II and *Sst* I sites of pGFP' (Unseld *et al.*, 2001) (Fig.1).

The sequence integrities of all four constructs were confirmed by automatic sequence analysis (Li-Cor system) using IRD 800 labelled primers (MWG AG) and the Thermosequenase kit (Amersham) according to the manufacturer´s instructions.

Inoculation of plants

Nicotiana benthamiana (Domin) plants in the two leaf stage were inoculated with DNA using particle bombardment at 450 psi (Biolistic PDS-1000/He System, BioRad). The ICMV DNA A(ΔCP-AV2:GFP) construct was released from the vector by *Pst* I digestion and mixed with equal amounts of ICMV DNA B released by *Kpn* I (Rothenstein *et al.*, 2004a). Coated gold particle suspension was prepared as described (Unseld *et al.*, 2001). For transient expression of pGFP:AV2, undigested plasmid DNA and, as a control, p35S:GFP were inoculated onto leaves of various sizes. GFP production was monitored using epifluorescence microscope with filter 02 (BP 450-490; FT 510; LP 520; Zeiss). Nuclei were counterstained with 4´, 6-diamidino-2-phenylindole (DAPI).

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