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CARACTERIZAÇÃO MOLECULAR DO *Sugarcane yellow leaf virus*, DESENVOLVIMENTO DE UM MÉTODO DE DIAGNÓSTICO ALTAMENTE SENSÍVEL E ASPECTOS MOLECULARES DA INTERAÇÃO LUTEOVIRUS/VETOR

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RESUMO

O vírus do amarelecimento foliar da cana-de-açúcar, *Sugarcane yellow leaf virus* (ScYLV) constitui atualmente um grande problema nos principais países produtores de cana-de-açúcar. Este vírus possui diversas características físicas e biológicas, como tamanho e morfologia da partícula, reações serológicas, alterações morfológicas no hospedeiro e aspectos de transmissão, comuns aos membros da família *Luteoviridae*. As informações da sequência genômica do isolado brasileiro obtidas nesse trabalho, referentes às regiões codificantes para capa protéica do vírus (CP), proteína de movimento (17 kDa ou MP) e parte da RNA polimerase dependente de RNA (RdRp), mostram um alto grau de identidade e similaridade de aminoácidos com sequências de luteovirus conhecidos. Isso permite estabelecer o ScYLV como um membro definitivo da família *Luteoviridae*. Análises filogenéticas empregando as sequências deduzidas de aminoácidos da CP e da região C-terminal da RdRp do ScYLV sugerem diferentes afinidades taxonômicas desse vírus dentro da família *Luteoviridae*, de maneira similar ao que acontece com o *Soybean dwarf virus* (SDV). Análises comparativas de seqüência entre o isolado brasileiro e um isolado norte-americano revelaram diferenças de apenas dois nucleotídeos nas posições 4201(G por T) e 4232 (A por C) do genoma do ScYLV, correspondentes respectivamente à região codificadora da proteína P17 e CP. Os dados de seqüência obtidos neste trabalho foram usados no desenvolvimento de um método sensível de diagnóstico baseado na combinação da técnica de amplificação isotérmica de ácidos nucléicos NASBA (nucleic acid sequence based amplification) e "molecular beacons", denominado AmpliDet RNA. Esse sistema de detecção foi

altamente específico e ofereceu uma sensibilidade de aproximadamente 100 fg de vírus purificado, permitindo a detecção em plantas com baixos níveis de infecção viral e em um único pulgão virulífero.

Os membros da família *Luteoviridae* são transmitidos por afídeos de uma maneira circulativa e não-propagativa. Após a aquisição, o vírus permanece no corpo do inseto por várias semanas, graças a associação com a proteína GroEL, produzida por um endossimbionte primário do pulgão. As partículas de vírus purificadas apresentam dois tipos de proteína, a proteína principal da capa protéica de 22 kDa e menores quantidades de outro componente do capsídeo, a proteína de transleitura ou "readthrough" (RTD) de 54 kDa. A protéina RTD contém determinantes responsáveis pela transmissão e acúmulo do vírus em plantas agroinfectadas. O *Potato leafroll vírus* (PLRV) é uma espécie do gênero *Polerovirus* dentro da família *Luteoviridae*. Clones infectivos de cDNA do PLRV e um mutante deletério da proteína RTD foram usados para estudar as interações moleculares entre esse luteovirus e seu afídeo vetor *Myzus persicae*. O mutante do PLRV, no qual a proteína RTD estava inteiramente ausente, não foi transmissível por *M. persicae* e não se ligou a proteína GroEL. Adicionalmente, esse mutante mostrou-se significativamente menos persistente na hemolinfa do afídeo do que as partículas não modificadas do vírus.

SUMMARY

Sugarcane yellow leaf virus (ScYLV) is widely distributed in Brazil and other sugarcane producing countries causing significant yield losses. This virus shares biological features typical of the luteovirids. Comparisons of the coat protein (CP), 17 kDa protein and C-terminus of the RNA-dependent RNA polymerase coding regions showed that the deduced amino acid sequences of the Brazilian isolate share a considerable degree of identity and similarity with corresponding sequences of known luteovirids, thus clearly establishing ScYLV as a member of the family *Luteoviridae*. Phylogenetic analyses also suggest that the 5' and 3' coding blocks of the ScYLV genome possess different taxonomic affinities within the *Luteoviridae* family, as for the genome of *Soybean dwarf virus* (SDV). Our results were published simultaneously as the sequence of a North American strain of ScYLV. Comparative analyses between the deduced peptides of Brazilian and North American strains revealed two nucleotide substitutions at positions 4201 (G→T) and 4232 (A→C) of the ScYLV genome, corresponding to P17 and CP proteins coding regions, respectively. Our sequence data were used to develop a highly sensitive detection method based on the combination of isothermic nucleic acid sequence based amplification (NASBA) and molecular beacons, named AmpliDet RNA. This system offered a sensitivity of about 100 fg of purified virus and could detect ScYLV in plant samples with low virus titer and in one single aphid.

Members in the *Luteoviridae* are transmitted by aphids in a circulative, nonreplicative manner. After acquisiton, luteovirus particles persist in the aphid's hemolymph for several weeks in association with a GroEL homolog, produced by the primary endosymbiont of the aphid. Luteovirus purified particles contain two types of

proteins; a major 22 kDa coat protein (CP) and the minor capsid component of 54 kDa, the readthrough protein (RTD). The RTD contains determinants responsible for virus transmission and accumulation in agroinfected plants. Potato leafroll virus (PLRV) is a member of the genus *Polerovirus* in the family *Luteoviridae*. An infectious cDNA full length clone of PLRV and a mutant devoid of the RTD were used to study and better understanding the molecular interactions between this luteovirus and its aphid vector *Myzus persicae*. The PLRV mutant lacking the entire RTD protein was not transmissible by *M. persicae* and did not bind to *Buchnera* GroEL. Furthermore, the mutant was significantly less persistent in the aphid's hemolymph than the wild type virus. These data corroborate previous observations with *Beet western yellow virus* (BWYV) and *Barley yellow dwarf virus* (BYDV) that the RTD domain is involved in luteovirus transmission and persistence in the aphid's body.

1. INTRODUÇÃO

1.1. O vírus do amarelecimento foliar da cana-de-açúcar, *Sugarcane yellow leaf virus* (ScYLV).

O amarelecimento foliar da cana-de-açúcar (Saccharum sp.) começou a se tornar um problema mundial para a cultura canavieira a partir do início da década de 90 (Borth *et al.*, 1994; Vega; 1994; Schenk *et al.*, 1997; Vega *et al.*, 1997; Comstock *et al.*, 1998). No Brasil, algumas variedades, como a SP71-6163, altamente produtiva e uma das mais plantadas até o aparecimento da doença, foi a primeira a apresentar os sintomas da moléstia, atingindo perdas de até 50%. As plantas afetadas apresentam amarelecimento da nervura central das folhas na face abaxial, seguido do limbo foliar. Folhas mais velhas, sexta ou sétima a partir do ápice, apresentam uma coloração vermelha na face adaxial da nervura central e, posteriormente, uma perda de pigmentação distribui-se pelo limbo foliar, progredindo do ápice para a base, sendo eventualmente seguida pela necrose do tecido. As raízes e colmos mostram crescimento reduzido e, consequentemente a produção é significativamente prejudicada.

Estudos realizados por Vega *et al.* (1997) em plantas com sintomas mostraram a presença de alterações no floema que sugeriram o envolvimento de um vírus associado a esse tecido condutor. Testes de microscopia eletrônica de imunoadsorção (ISEM) denotaram uma fraca reação serológica desse vírus com um anti-soro para o *Barley yellow dwarf virus* (BYDV), espécie tipo do gênero *Luteovirus*. Imunolocalização em membranas de nitrocelulose por "Tissue-Printing" e testes de PTA-ELISA (Plate Trapped Antigen Enzyme-Linked Immunosorbent Assay) com o mesmo anti-soro para o BYDV, também indicaram relação serológica entre esse

luteovirus e o ScYLV. Purificações do vírus a partir de folhas infectadas possibilitaram a obtenção de elevadas concentrações de partículas isométricas de *ca.* 25nm de diâmetro, claramente visíveis em contraste negativo ao microscópio eletrônico (Gonçalves & Vega, 1997; Scagliusi & Lockart, 2000). Um anti-soro policlonal produzido em coelhos a partir de partículas purificadas é utilizado para detectar o vírus em amostras infectadas (Scagliusi *et al.*, 1997, Scagliusi *et al.*, 2000). Partículas purificadas apresentam uma proteína principal de 27 kDa em SDS-PAGE e proteínas secundárias de 17, 27 e 58 kDa em análise por Western blot (Scagliusi *et al.*, 2000). Dados preliminares de sequenciamento indicaram uma homologia de 54% entre a seqüência genômica da proteína do capsídeo desse vírus com a do BYDV- PAV (Maia *et al.*, 1998).

O vírus foi transmitido pelas espécies de afídeos *Sipha flava* e *Melanaphys sacchari*, provocando a expressão de sintomas em plantas de cana-de-açúcar inicialmente sadias. Testes serológicos (DAS-ELISA) realizados com um antisoro policlonal contra o ScYLV confirmaram a presença do vírus nos tecidos foliares dessas plantas e possibilitaram a constatação de infecção latente em plantas assintomáticas (Lopes *et al.*, 1997). Além dessas duas espécies de afídeos o vírus também foi transmitido por *Rophalosiphum maidis* (Scagliusi & Lockhart, 2000). O vírus não foi transmitido em testes de transmissão mecânica (Lopes *et al.*, 1997; Scagliusi & Lockhart, 2000).

1.2. Biologia molecular dos luteovirus

As informações apresentadas indicam que o patógeno em questão é um membro da família *Luteoviridae*, uma das mais importantes entre os fitovirus. Os membros dessa família são transmitidos exclusivamente por afídeos, de maneira

circulativa e não propagativa e permanecem restritos ao floema da planta hospedeira (Kitajima *et al.*, 1997). O genoma desses vírus é constituído por uma molécula de RNA fita simples de polaridade positiva, com tamanho variando entre 5.7 a 6 kb (Mayo & Ziegler-Graff, 1996; Miller & Rasochová, 1997). Este RNA destaca-se pela ausência de uma cauda poli-A em sua extremidade 3'. Seis diferentes "open reading frames" (ORF; Figura 1) estão presentes no RNA genômico, sendo estas separadas por regiões intergênicas de aproximadamente 100 a 200 nucleotídeos. As três últimas ORFs da extremidade 3' do genoma são expressas a partir de um RNA subgenômico. Com relação à funcionalidade, os produtos das ORFs 1 e 2 são necessários à replicação do RNA viral. As proteínas derivadas das ORFs 3 e 5 são parte integrante da partícula viral e conferem as propriedades necessárias à transmissão e ao movimento viral. Da mesma maneira, a ORF 4 codifica uma proteína possivelmente implicada no movimento viral. Por outro lado, a proteína produzida pela ORF 0 é essencial para o acúmulo de vírus na planta (Sadowy *et al.*, 2001), enquanto que o produto da ORF 6 tem função indefinida.

Com base na organização do genoma, os luteovirus eram reagrupados em dois diferentes subgrupos (revistos em Mayo & Ziegler-Graff, 1996). Resumidamente, os membros do subgrupo II apresentam uma ORF na extremidade 5' do RNA genômico (ORF 0; Fig. 1) que está ausente no subgrupo I, cujos membros, por sua vez, possuem uma ORF na extremidade 3' do genoma (ORF 6; Fig. 1) ausente no subgrupo II. A existência de um terceiro subgrupo foi proposta com base no genoma do *Soybean dwarf virus* (SDV) (Rathjen *et al.*, 1994). Os membros dos subgrupos I e II diferem na organização do seu genoma e possuem diferentes tipos de polimerases (RNA-dependent RNA polimerases). Enquanto as polimerases dos membros do subgrupo II estão mais estreitamente relacionadas às dos sobemovirus, as do

subgrupos I estão mais próximas às dos dianto e carmovirus. Os membros do subgrupo II apresentam na região 5' do seu genoma, anteriormente à ORF 1, uma pequena proteína ligada covalentemente, viral genome-linked protein (VPg), a qual está ausente do genoma dos membros do subgrupo I (van der Wilk *et al.*, 1997). Homologias de seqüência entre tais subgrupos existem sobretudo na região codificadora da proteína do capsídeo (ORF 3; Vincent *et al.*, 1990), fato que pode ser explorado na confecção de "primers" e sondas universais para efeito de diagnose. Os luteovirus destacam-se pelas diferentes e marcantes estratégias que empregam durante a expressão de seu genoma (revisto em Maia *et al.*, 1996), o que também os torna interessantes do ponto de vista da pesquisa básica em expressão gênica.

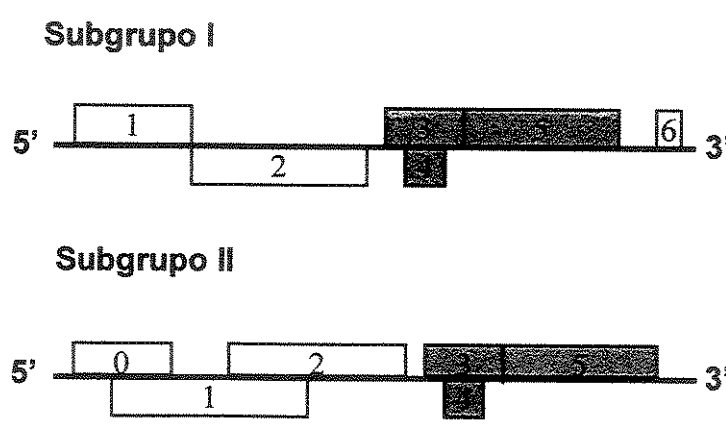

Figura 1. Organização genômica dos subgrupos do gênero *Luteovirus.* Os retângulos numerados representam as diferentes "open reading frames" (ORFs). A região 3' do genoma sombreada representa as ORFs com alta porcentagem de homologia de aminoacidos entre os subgrupos I e II.

Algumas alterações na classificação dos luteovirus foram propostas pelo Comitê Internacional de Taxonomia de Vírus - ICTV (D'arcy & Mayo, 1997; Pringle, 1998; van Regenmortel*et al.*, 2000). Segundo a nova classificação, os luteovirus são englobados dentro da família *Luteoviridae*, a qual compreende os gêneros

Luteovirus, Polerovirus e *Enamovirus*. Os dois primeiros gêneros correspondem, respectivamente, aos subgrupos I e II, enquanto que o gênero *Enamovirus* apresenta como espécie tipo o *Pea enation mosaic virus*, o qual possui por sua vez um genoma bipartido.

É importante salientar que embora o ScYLV seja serologicamente relacionado aos membros do subgrupo I, ou gênero *Luteovirus,* e mais especificamente ao isolado *Barley yellow dwarf virus* -PAV (Vega *et al.*, 1997), pouco se conhece sobre seus aspectos moleculares (Maia *et al*, 1998). No entanto, as informações relatadas no capítulo 1 desta tese indicam que o mesmo também possui homologia de sequência de aminoácidos com membros do gênero *Polerovirus* (formalmente Subgrupo II), em regiões genômicas outras que a proteína do capsídeo. Durante o desenvolvimento desse trabalho, houve um grande aumento no interesse e estudo da organização genômica do ScYLV. As informações geradas por outros autores nesse período serão abordadas oportunamente.

1.3. Aspectos Moleculares da Transmissão de Luteovirus por insetos vetores.

Os membros família *Luteoviridae* são limitados às células do floema das suas plantas hospedeiras e são obrigatoriamente transmitidos por insetos vetores (*Homoptera, Aphididae*). O modo de transmissão desses vírus é conhecido como persistente e circulativo. A rota dos virions no corpo dos insetos vetores inclui, sucessivamente, a ingestão das partículas com a seiva do floema durante a alimentação do afídeo numa planta infectada, transporte através da parede do intestino e então do hemocoel para as glândulas salivares acessórias, accessory salivary glands (ASG). As partículas são liberadas no canal salivar de onde são

injetadas com saliva numa nova planta durante uma alimentação subseqüente (Gildow, 1999).

A "simbionina" (GroEL) é uma proteína liberada na hemolinfa de afídeos por uma bactéria endosimbionte primária, *Buchnera* sp.. A GroEL de *Buchnera* sp., presente em diferentes espécies de afídeos divide extensa homologia de seqüência de aminoácidos em regiões importantes, do ponto de vista estrutural e funcional, com a GroEL de *Escherichia coli*, um membro da família 60 das chaperonas (Filichkin *et al.*, 1997; van den Heuvel *et al.*, 1994). Essas proteínas são essenciais à viabilidade celular, uma vez que elas ligam e estabilizam agregados de polipeptídeos recém-traduzidos/translocados e mediam o seu enovelamento e montagem funcionais, num processo dependente de ATP (Ellis & van der Vies, 1991; Hartl, 1996). A GroEL de *Buchnera* sp. de *Myzus persicae* (MpB GroEL) é encontrada liberada na hemolinfa do afídeo, provavelmente como resultado da lise da bactéria endosinbionte e parece ser essencial para transmissão de luteovirus (van den Heuvel *et al.*, 1994).

Estudos recentes mostraram que espécies dos gêneros *Luteovirus* e *Polerovirus* têm uma afinidade específica, porém diferencial, por homólogos da GroEL de espécies de afídeos vetores e não-vetores (Filichkin *et al.*, 1997; van den Heuvel *et al.*, 1994). A capacidade básica desses vírus em ligarem-se a GroEL de *Buchnera* sp reside na região N-terminal do domínio de transleitura, readthrough domain (RTD) de uma das proteínas associadas ao capsídeo viral (van den Heuvel *et al.* 1997). Duas proteínas estão presentes no capsídeo luteoviral, a proteína principal, coat protein (CP), a qual é codificada pela ORF 3 (Figura 1), e um polipeptídeo secundário (RTD), o qual é expresso como o resultado de uma transleitura e tradução do códon de terminação da ORF 3 extendendo-se na direção da ORF 5 vizinha (Dinesh-Kumar *et al.*, 1992; Reutenauer et al, 1993). Em extratos

de plantas ou protoplastos infectados a proteína de fusão ORF3-ORF5 é prontamente detectada, mas em preparações de vírus purificadas, as quais são altamente transmissíveis pelo afídeo, essa proteína (RTD) está presente numa forma truncada sem a região C-terminal, não-conservada entre os luteovirus (Filichkin *et al.*, 1997; Martin *et al.*, 1990). A região N-terminal da RTD, conservada entre os luteovirus, contém determinantes necessários para transmissão e provavelmente interage com componentes do vetor e da planta hospedeira, uma vez que ela encontra-se exposta na superfície da partícula viral (Brault *et al.*, 1995; Chay *et al.*, 1996; Filichkin *et al.*, 1997).

A interação entre Luteovirus e a GroEL de *Buchnera* sp., permitindo a manutenção da estabilidade da partícula viral na hemolinfa do afídeo vetor, encontra fundamentos em duas linhas de pesquisa independentes. A primeira delas provém da redução drástica dos níveis da MpB GroEL na hemolinfa de larvas de *M. persicae* pelo tratamento com antibiótico, o que conduz à inibição da transmissão do *Potato leafroll virus* (PLRV) e perda da integridade do capsídeo (van den Heuvel *et al.*, 1994). A segunda aborda a injeção direta de mutantes deletérios para a proteína RTD do *Beet western yellow virus* (BWYV). Esses mutantes são incapazes de ligarem-se a GroEL e, conseqüentemente, são menos persistentes na hemolinfa do afídeo do que virions contendo a RTD (van den Heuvel *et al.*, 1997).

Hogenhout *et al.* (1998) localizaram o sítio de ligação do PLRV no domínio equatorial da molécula de MpB GroEL. Mutantes dessa proteína que não possuem o denominado domínio equatorial, ou partes dele, perdem a habilidade de ligarem-se ao PLRV. O domínio equatorial é constituído de duas regiões localizadas no N e C terminal da proteína que não são contíguas na seqüência de aminoácidos, mas estão

em proximidade espacial depois do enovelamento do polipeptídeo de GroEL. As duas regiões, N e C terminal, estão envolvidas na ligação com o vírus.

1.4. "Molecular beacons" e "NASBA"

Uma nova técnica de detecção de ácidos nucléicos baseada em um sinal fluorescente, denominada "molecular beacons" (Tiagi & Kramer, 1996), foi desenvolvida recentemente e vem ganhando espaço na detecção de fitovírus (Eun and Wong, 2000). Molecular beacons são sondas oligonucleotídicas que podem evidenciar a presença de ácidos nucléicos específicos em soluções homogêneas. Elas são particularmente úteis em situações onde não é possível ou desejável isolar os híbridos formados entre a sonda e a seqüência alvo, de um excesso de sondas de hibridização, tal como em monitoramento em tempo real de reações em cadeia da polimerase (PCR) em tubos selados, ou na detecção de RNAs dentro de células vivas.

Essas sondas moleculares consistem de uma molécula de DNA de fita simples (ssDNA) com uma estrutura em forma de presilha ou grampo, "stem-loop" ou "hairpin" (Figura 2). Essa conformação permite que as extremidades 5', marcada com um fluoróforo (FAM, fluoresceína), e a extremidade 3', marcada com um extintor de fluorescência ou "quencher" (DABCYL) estejam, a temperatura aproximada de 41^{0}C, na qual ocorre a reação de amplificação, espacialmente próximas o suficiente para que a fluorescência emitida seja extinta pelo quencher. Após a hibridização da sonda com a sequência alvo, formando uma rígida estrutura de dupla hélice, a sonda assume uma conformação tal que o fluoróforo encontra-se distante o suficiente do quencher, de maneira que ocorra emissão de fluorescência (Figura 2).

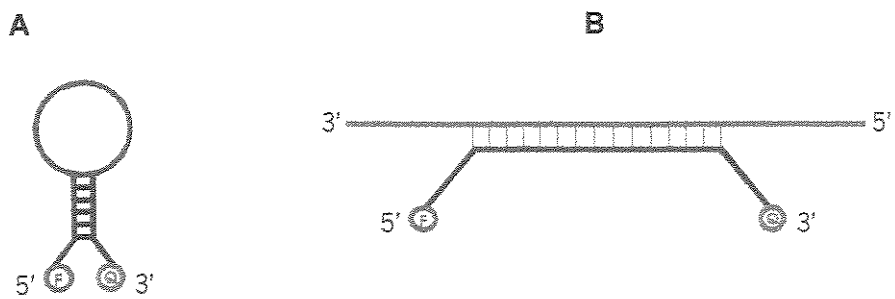

Figura 2. Esquema de funcionamento de uma "molecular beacon". Na sua conformação original (A) essas moléculas não são fluorescentes, pois a estrutura em forma de grampo mantém o fluorófero (F) próximo ao "quencher" (Q). Quando a seqüência da sonda, no anel vermelho, hibridiza-se com a sequência alvo, forma-se uma rígida estrutura de dupla hélice, a qual separa o quencher do fluoróforo, restaurando a fluorescência (B).

Essas sondas oferecem ainda a possibilidade de serem aplicadas para detectar alvos múltiplos, utilizando-se para tal uma gama de fluoróforos com diferentes cores (Tyagy, *et al.*, 1998). DABYCYL, um cromóforo não fluorescente, serve como um extintor universal para qualquer fluoróforo em molecular beacons. Devido à sua forma de presilha, o reconhecimento dos alvos por essas sondas é tão específico que diferenças de apenas um simples nucleotídeo podem ser prontamente detectadas.

A amplificação isotérmica de ácidos nucléicos NASBA (Nucleic Acid Sequence Based Amplification) é uma tecnologia com amplo potencial para aplicação na amplificação e detecção de RNA (Kievits *et al.*, 1991; Leone *et al.*, 1997, Leone *et al.*, 1998). Em comparação com outros sistemas de amplificação de ácidos nucléicos, tal como PCR, esse método, além de ser isotérmico, estende sua

aplicação do diagnóstico viral à indicação de atividades biológicas, tal como expressão gênica e viabilidade celular (Leone *et al.*, 1998). O principal produto de amplificação é um RNA de fita simples (ssRNA), embora pequenas quantidades de híbridos RNA:DNA e DNA dupla fita (dsDNA) também possam ser sintetizados (Kievits *et al.*, 1991). A reação em NASBA é baseada no uso de primers para amplificação específica de RNA com a atividade simultânea das enzimas transcriptase reversa do AMV (AMV-RT), RNase H e T7 RNA polimerase. A reação ocorre à temperatura constante de 41°C e sem a necessidade da adição de reagentes intermediários. Um dos primers específicos utilizados contém a sequência terminal 3' complementar a sequência do RNA alvo a ser amplificado e a sequência terminal 5' de um promotor que é reconhecido pela T7 RNA polimerase. Por sua vez, o segundo primer contém uma sequência que é complementar a fita de cDNA do primeiro primer. As enzimas e primers operam de maneira a amplificar a sequência nucleotídica alvo exponencialmente (Kievits *et al.*, 1991; Sooknanan & Malek, 1995). Devido às suas características, esse método constitui uma ferramenta potencial para a análise e detecção em larga escala de patógenos virais.

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2. OBJETIVOS

Esta tese apresenta como etapa inicial a caracterização molecular do ScYLV, na tentativa de viabilizar um melhor entendimento da organização do seu genoma, além de contribuir para a sua classificação filogenética (Capítulo 1). O conhecimento gerado com as informações da seqüência nucleotídica de parte do genoma viral foi empregado no desenvolvimento e otimização de um método preciso de diagnose baseado em técnicas de biologia molecular (Capítulo 2). Esse tipo de diagnóstico permite a detecção do vírus nos estágios iniciais da infecção, sob condições desfavoráveis, em infecções latentes, em material tolerante e também em afídeos virulíferos, possibilitando o estudo da epidemiologia e distribuição da doença no campo.

A segunda etapa consistiu no uso de um clone de cDNA infeccioso do *Potato leafroll virus* (PLRV), espécie tipo do gênero *Polerovirus* (família *Luteoviridae*), no estudo dos de alguns dos aspectos moleculares envolvidos na transmissão dos luteovirus. Foi proposta a avaliação e uso desse clone e de um mutante deletério da proteína RTD para a investigação do envolvimento dessa proteína na transmissão do PLRV por seu afídeo vetor *M. persicae* (Capítulo 3).

Capítulo 1

Molecular evidence that sugarcane yellow leaf virus (ScYLV) is a member of the *Luteoviridae* family [t]

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The nucleotide sequence data reported in this paper have been assigned GeneBank accession number AF141385 and AF160474

[t] Part of a dissertation presented by M.C.G. to the Universidade Estadual de Campinas in partial fulfillment of the requirements for the Ph.D. degree

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Summary

A previously uncharacterized virus was reported in southeast Brazil causing a yellowing leaf disease in sugarcane. The virus, termed sugarcane yellow leaf virus (ScYLV), shares features typical of the luteoviruses. To start the molecular characterization of ScYLV, the nucleotide sequence of the coat protein (CP), 17 kDa protein and C-terminus of the RNA-dependent RNA polymerase coding regions was determined from an RT-PCR amplification product. Comparisons showed that the deduced amino acid sequences share a considerable degree of identity and similarity with corresponding sequences of known luteoviruses, thus clearly establishing ScYLV as a member of the family *Luteoviridae*. The authenticity of the CP open reading frame was confirmed by its expression in *Escherichia coli*. The recombinant CP positively reacted in immunoblot assays with polyclonal antibodies raised against native ScYLV. Furthermore, phylogenetic analyses also suggest that the 5'and 3'coding blocks of the ScYLV genome possess different taxonomic affinities within the *Luteoviridae* family, as does also the genome of soybean dwarf virus.

Introduction

A new yellowing leaf disease of sugarcane in southeast Brazil has recently been reported to be associated with a small phloem-limited isometric virus (24 to 29 nm in diameter) [11, 15]. Symptoms are most commonly detectable on mature leaves where an intense yellowing of the abaxial surface of the midrib is observed. The pathogenicity of the virus and its ability to elicit typical yellowing symptoms in sugarcane was demonstrated in aphid transmission experiments [4, 11]. The virus, designated sugarcane yellow leaf virus (ScYLV), was tentatively classified as a member of the *Luteoviridae* family on the basis of particle morphology, biological properties and serology [11, 15].

The luteovirus genome consists of a single, positive-sense RNA of 5.6 to 6 kb which is devoid of a poly(A) tail [6, 8]. Luteoviruses have been classified into three major genera on the basis of their genomic organization, replication strategies, and expression mechanisms, the *Luteovirus* and *Polerovirus* genera, and the *Enamovirus* represented by pea enation mosaic virus [1]. The *Luteovirus* and *Polerovirus* genera differ mainly in both sequence and genetic organization of their 5' open reading frames (ORF). In contrast, an important sequence similarity is detected between ORFs located in the 3' coding block of the genome, i.e. the coat protein (CP), the overlapping 17 kDa and the readthrough (RTD) ORFs. Unlike other luteoviruses, for which complete nucleotide (nt) sequence and genome organization have been reported [2, 3, 7, 9, 14, 16, 17], to date no molecular data is available for ScYLV.

This paper reports the complete nt sequence of the CP and the 17 kDa coding regions of the ScYLV genome. The sequence coding for the C-terminus of the RNA-

dependent RNA polymerase (RdRp) was also determined. Analysis of the deduced amino acid (aa) sequence of these ScYLV-encoded proteins and comparison with those of other luteoviruses allowed us to clearly establish ScYLV as a member of the family *Luteoviridae*.

Materials and methods

cDNA cloning and sequencing

A Brazilian isolate of ScYLV was propagated in sugarcane in a greenhouse. Total RNA was extracted from 100 mg of leaf tissue from infected or healthy plants using Trizol reagent (Gibco BRL) according to the supplier's protocol. First strand cDNA was synthesized as described [10] using the primer Lu-CP, 5'-CGGAATTCGTCTACCTATTTNG-3', complementary to the conserved sequence flanking the UAG codon located between the CP and the RTD ORFs. The cDNA was then amplified by PCR using primer Lu-CP and the degenerated primer GDD, 5'-GC(C/G/T)ATGGG(C/G/T)GA(C/T)GATGCCC-3', whose sequence was based on the sequence similarity surrounding the conserved GDD amino acid motif of the RdRp of several luteoviruses. The PCR reactions consisted of one cycle at 94 °C (7 min), 54 °C (2 min) and 72 °C (20 min), followed by 30 cycles at 94 °C (1 min), 54 °C (2 min) and 72 °C (3 min), with a final cycle of 72 °C for 15 min. The PCR products were inserted into the pGEM-T Easy Vector (Promega) and their nucleotide sequence determined on both strands in an ABI 310 automatic sequencer (Perkin-Elmer). Reverse transcriptase (RT; Superscript II; Gibco BRL) and *Taq* polymerase (Gibco BRL) were used according to the manufacturers' instructions. Amino acid sequence alignments were performed on CLUSTAL W software [13].

Northern blot analysis

Northern hybridization analysis of total RNA isolated from infected and healthy tissues was carried out as described [5] using a restriction fragment (*Bam*HI and *Xho*I-digested) of the ScYLV-amplified cDNA as a probe. Briefly, RNA samples (10 μg) were electrophoresed in a 1% formaldehyde-agarose gel, transferred onto nylon membrane (Hybond N; Amersham) by capillary blot and fixed by UV cross-linking. The blot was hybridized for 16 h at 42 °C with the ScYLV ^{32}P-labeled probe, washed and the bands detected by autoradiography.

Cloning and expression of the ScYLV CP ORF

The CP ORF was obtained by PCR amplification from the cloned full-length cDNA using *Pfu* DNA polymerase (Stratagene) and the following specific primers: 5' CCGGAATTCATGAATACGGGCGC 3' and 5' GCTCTAGACTATTTTGGATTC 3'. The primers were supplemented with restriction sites for *Eco*RI and *Xba*I (underlined), respectively, for direct cloning of the resulting PCR fragment into the corresponding sites of pBluescript KS+ (Stratagene) to produce pKS-CP. For expression in *Escherichia coli*, an *Eco*RI/*Not*I CP fragment from pKS-CP was cloned in-frame into the similarly digested expression vector pET-28a (Novagen) to yield pET-CP. The correctness of this construct was verified by DNA sequencing. The recombinant plasmid was then transformed into *E. coli* BL21(DE3)pLysS and expression of the CP was achieved by means of the T7 expression system following isopropyl-β-D-thiogalactopyranoside (IPTG) induction essentially as described [12]. Cells were harvested after 2 h and total protein profiles analyzed by 0.1 % sodium dodecylsulfate-12 % polyacrylamide gels (SDS-PAGE) and immunoblotting using polyclonal antibodies raised against an isolate of ScYLV from Florida (a gift of S.

Scagliusi and B. Lockhart, University of Minnesota, USA) and CSPD (Tropix). The bands were detected by autoradiography.

Results

For unknown reasons, attempts to isolate viral RNA from purified ScYLV particles were ineffective. To start the molecular characterization of ScYLV, an RT-PCR on total RNA from infected and non-infected plants using degenerate primers was performed and resulted in the amplification of a 1200 nt long cDNA. The viral origin of the amplified cDNA was confirmed by Northern blot analysis (Fig. 1). Two different RNA species, probably corresponding to the viral genomic and subgenomic RNAs, were detected in total extracts of ScYLV-infected plants. In contrast, no hybridization could be observed in samples from total RNA extracts of non-infected plants. According to Scagliusi and Lockhart [11], the ScYLV RNA isolated from purified virions consists of a single component of about 5800 nt under denaturing conditions.

The inserts of three different clones were sequenced in both DNA strands and the same nt sequence was observed. Three distinct ORFs could be identified within the nt sequence of the amplified and cloned cDNA. As expected, a comparative sequence analysis with respect to other luteoviruses revealed that the cDNA comprised the coding regions for the ScYLV putative CP, 17 kDa protein and C-terminus of the RdRp, respectively.

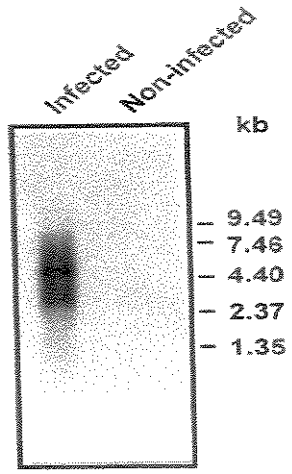

Fig. 1. Northern blot analysis of total RNA isolated from non-infected and ScYLV-infected tissues using a *Bam*HI- and *Xho*I-digested fragment of the cloned cDNA as a probe. Values (kb) to the right are from molecular weight markers (Gibco BRL) analyzed on a parallel lane of the same gel.

The 591 nt of the presumed ScYLV CP ORF encodes a predicted polypeptide of 196 aa with an estimated molecular mass of 21.7 kDa. The resulting aa sequence could be aligned with the CP of other luteoviruses, including different PAV-like isolates of barley yellow dwarf virus (BYDV) (Fig. 2). The sequence identity ranged from 43.6 % (BYDV-PAV-129) to 34% (soybean dwarf virus; SDV) while the similarity varied from 55.5 % (BYDV-MAV) to 43.3 % (bean leaf roll virus; BLRV). Recently, the nt sequence coding for the putative CP of an isolate of ScYLV from Florida has been deposited in the database (accession number). Comparison of the predicted CP aa sequence of the Brazilian isolate to that from the Florida isolate showed conservation of 195 invariant aa out of 196.

The aa sequence relatedness between the CP of ScYLV and other luteovirus CPs is shown as a predicted phylogenetic tree in Fig. 3. The phylogenetic analysis places ScYLV in an intermediate position between the two observed branches, but as being closer to viruses in the genus *Luteovirus*.

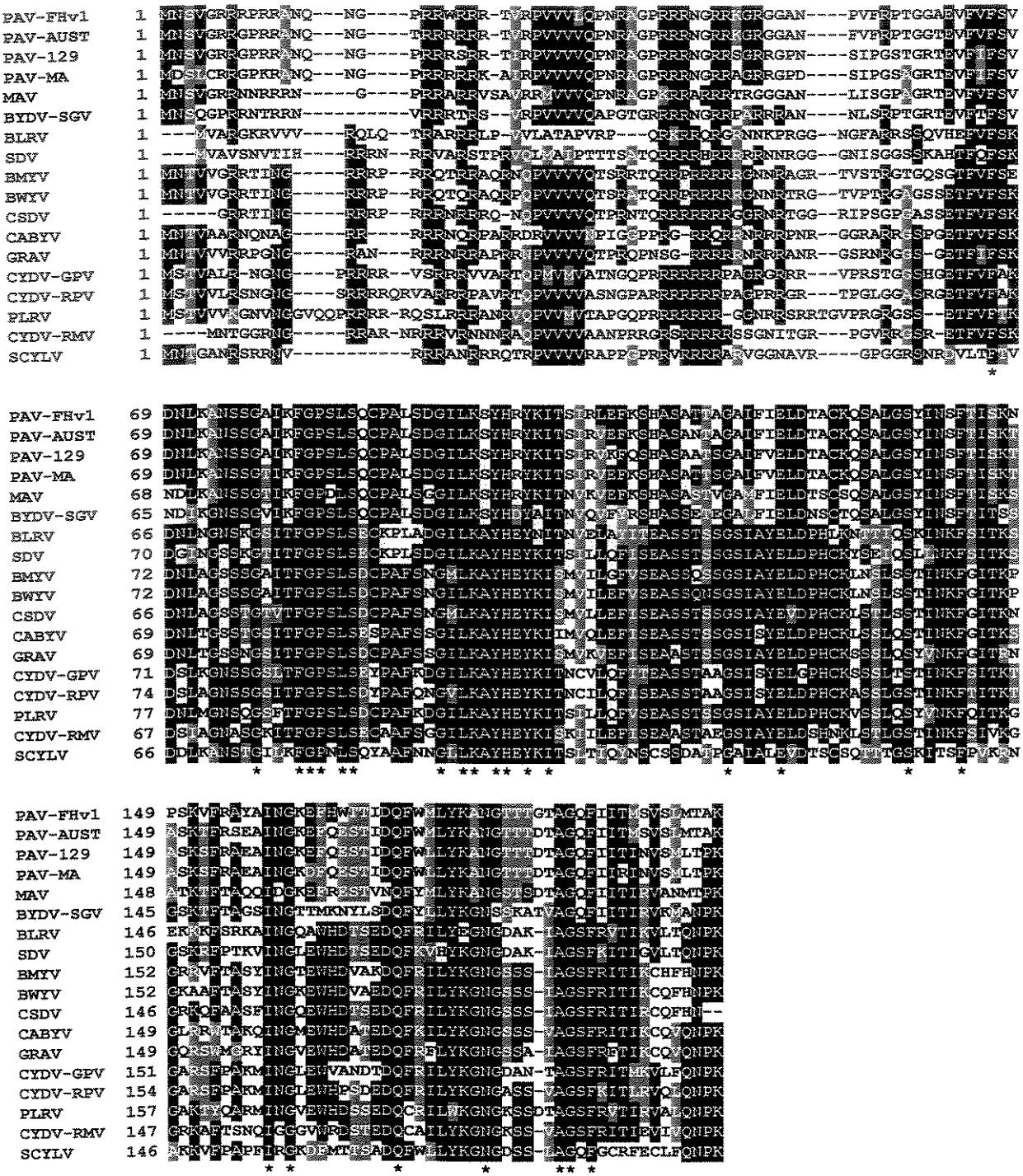

Fig. 2. Alignment of the deduced amino acid sequence of the coat protein (CP) of ScYLV with several luteoviruses CPs. Black and gray boxes indicate residues that are identical or similar in at least 50 % of the sequences. Asterisks indicate perfect matches within all sequences. The gaps introduced to optimize the sequence alignment are represented by dashes. Sequences were aligned using CLUSTAL W applying the Dayhoff PAM 250 matrix [13]. Shading was performed by the BOXSHADE program (ISRC Bioinformatics Group). Abbreviations and accession numbers are: Barley yellow dwarf virus (BYDV) strains PAV-

FHv1 (3660473), PAV-AUST (CAA30493), PAV-129 (903998), PAV-MA (3326950), MAV (P17966) and SGV (533393), Bean leaf roll virus (BLRV; P19126), Soybean dwarf virus (SDV; 1545979), Beet mild yellowing virus (BMYV; 1141681), Beet western yellows virus (BWYV; 1141701), Chickpea stunt disease associated virus (CSDV; 1877185), Cucurbit aphid-borne yellows virus (CABYV; 541316), Groundnut rosette assistor virus (GRAV; 1167834), Cereal yellow dwarf virus (CYDV) strains GPV (2329900), RPV (2429357) and RMV (58797), Potato leafroll virus (PLRV; 2289950), ScYLV (AF141385).

A second ORF (453 nt) within the CP coding region was also identified. This ORF has the potential to encode a 150 aa polypeptide with a calculated molecular mass of 16.6 kDa, which upon alignment with known luteovirus 17 kDa proteins (Fig. 4), showed 34 % (42 %), 29 % (37 %) and 25 % (33 %) aa identity (similarity) with BYDV-PAV, BYDV-MAV and potato leafroll virus (PLRV), respectively. The putative 17 kDa of ScYLV has less similarity to other luteoviruses than the CP. Additionally, the relatedness of ScYLV to other luteoviruses on the basis of the 17 kDa sequences resembles that exhibited by the CP (not shown).

The 375 nt from the RdRp ORF encoded 124 aa residues representing the C-terminal half of the polymerase. After alignment of the ScYLV RdRp deduced aa sequence with that of previously reported luteovirus polymerases, a phylogenetic analysis was performed. As observed in Fig. 5, the RdRp sequences clearly partitioned into two different groups with ScYLV clustered with viruses in the genus *Polerovirus*.

The cloned cDNA included an intergenic region of 130 nt located between the CP and the RdRp ORFs, which is also characteristic of other luteovirus genomes. Essentially, the ScYLV intergenic region is shorter than that of SDV (210 nt) [9] and

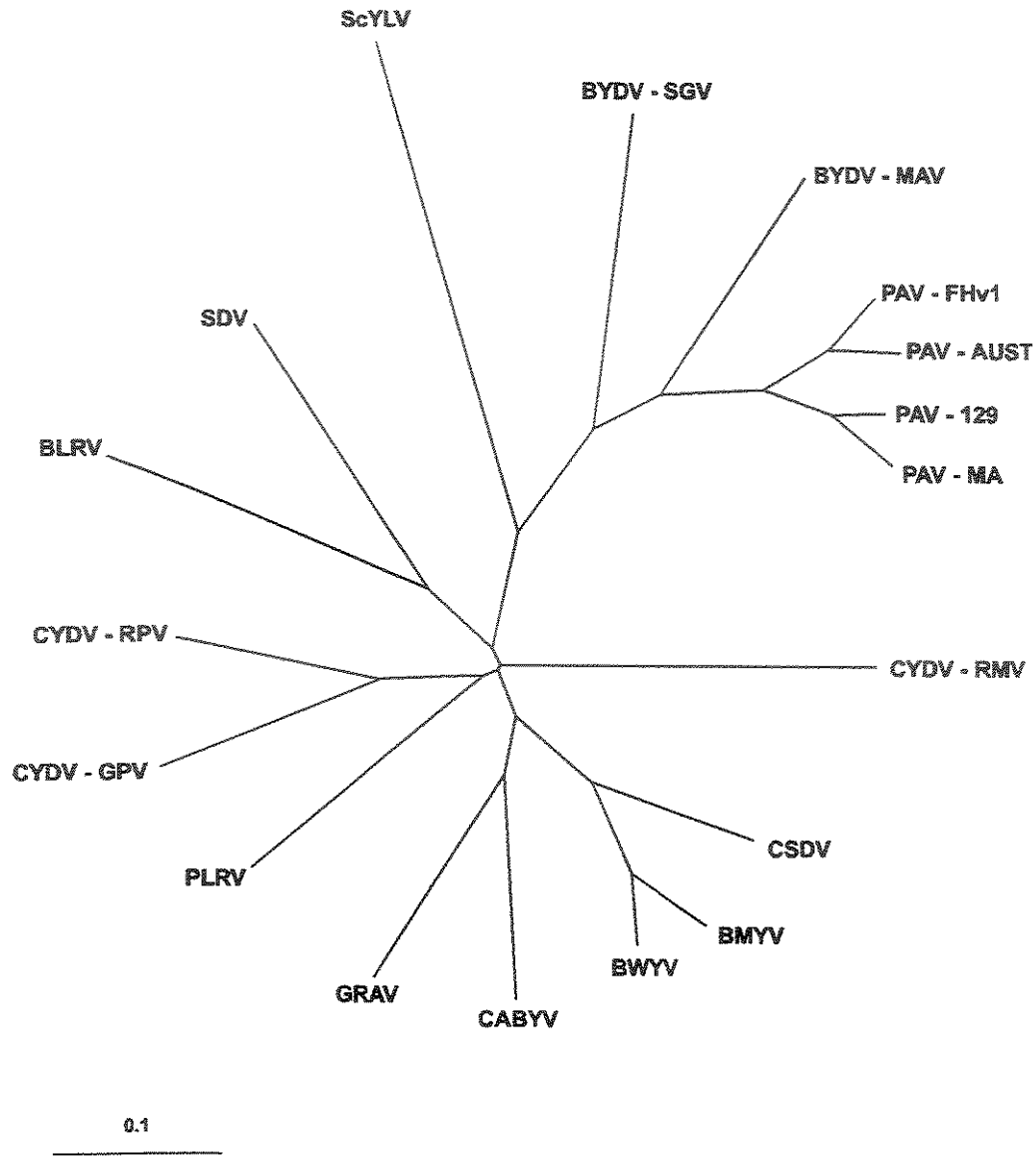

Fig. 3. Unrooted phylogenetic tree analysis of different luteovirus CP. The dendogram was constructed using the simultaneous alignment and phylogeny program CLUSTAL W [13] and graphically illustrated using TreeView 1.5. The scale bar indicates 0.1 substitution/site. Accession numbers are as described in Fig. 2.

cucurbit aphid-borne yellows virus (CABYV; 199 nt) [2] but is of about the same size as that of BYDV-PAV (113 nt) [7].

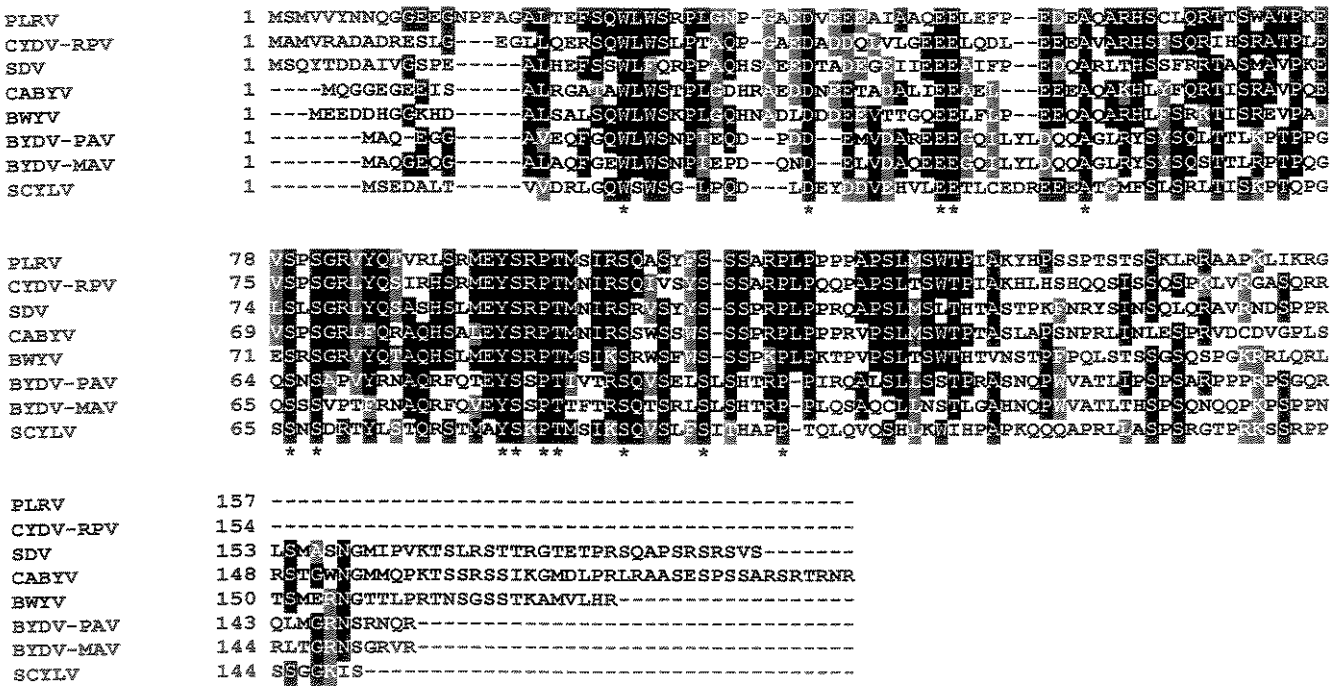

Fig. 4. Alignment of the deduced amino acid sequence of the putative 17 kDa protein of ScYLV with that of several luteoviruses. Black and gray boxes indicate residues that are identical or similar in at least 50 % of the sequences. Asterisks indicate perfect matches within all sequences. The gaps introduced to optimize the sequence alignment are represented by dashes. Sequence alignment and shading were performed as described in Fig. 2. Accession numbers are: PLRV (P10471), CYDV-RPV (P27579), SDV (1545980), CABYV (441273), BWYV (P09512), BYDV-PAV (P29047), BYDV-MAV (P29046), ScYLV (AF141385).

To confirm that the isolated cDNA really codes for the CP of ScYLV, the complete CP ORF was cloned under the control of the T7 promoter in pET-28a vector and overexpressed in *E. coli*. The presence of the CP in total cell lysates of non-induced and induced bacteria was verified by SDS-PAGE and immunoblotting. After induction by IPTG, an additional polypeptide with an apparent molecular mass of 27 kDa was present in cell lysates of induced bacteria (Fig. 6A, compare lanes 2 and 3). In immunoblot assays, the induced 27 kDa polypeptide was recognized by polyclonal antibodies raised against purified ScYLV (Fig. 6B, lane 3). In contrast, no band could

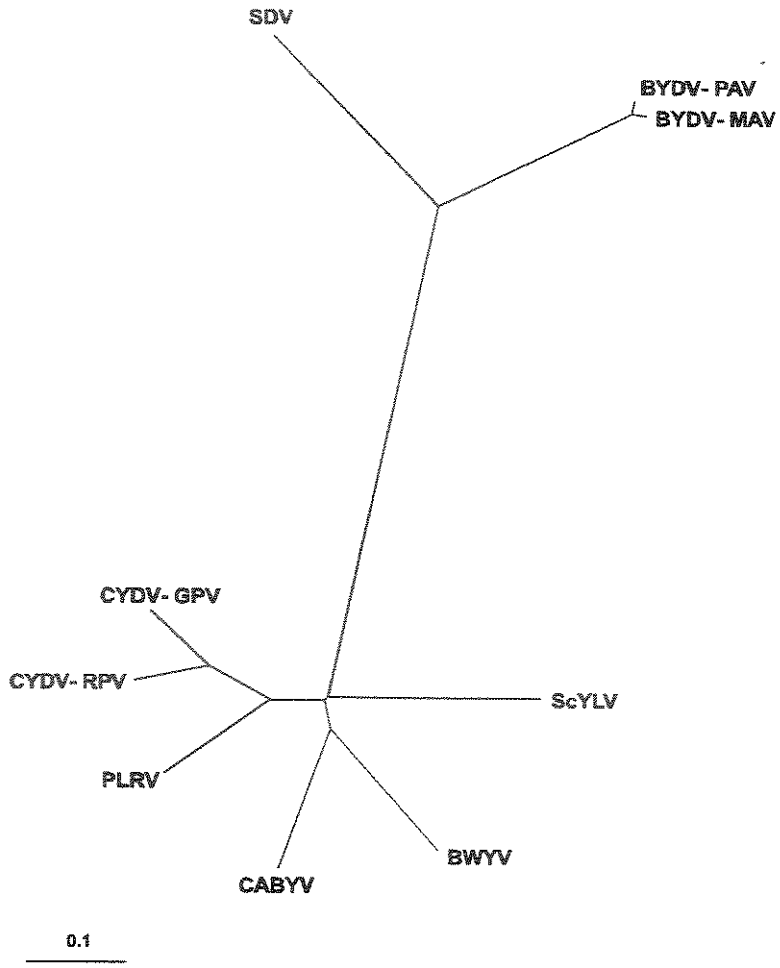

Figure 5. Unrooted phylogenetic tree analysis of different luteovirus RdRp. Only the nucleotide sequences coding for the C-terminus of the RdRp (from the third aa after the GDD motif to the stop codon) were aligned. The dendogram was constructed using the simultaneous alignment and phylogeny program CLUSTAL W [13] and graphically illustrated using TreeView 1.5. The scale bar indicates 0.1 substitution/site. Accession numbers are: CYDV-RPV (221095), BWYV (P09507), BYDV-PAV (P09505), CABYV (1334822), BYDV-MAV (P29044), PLRV (P11623), SDV (1364138), CYDV-GPV (2340970), ScYLV (AF160474).

be detected at an equivalent position to the expressed CP in total protein extracts from either induced bacteria transformed with empty vector, or non-induced bacteria (Fig. 6B, lanes 1 and 2 respectively). Two major immunoreactive polypeptides representing CP and RTD were observed in the control sample from purified virus

(Fig. 6B, lane 4). In addition, a minor product that probably arises from degradation of the RTD protein was also detected. The 4,000-*Mr* difference between authentic viral CP and its *E. coli*-expressed version can be accounted for by the 36 aa residues (including an His-tag) from the vector. These results confirm that the cloned ORF indeed codes for the viral CP.

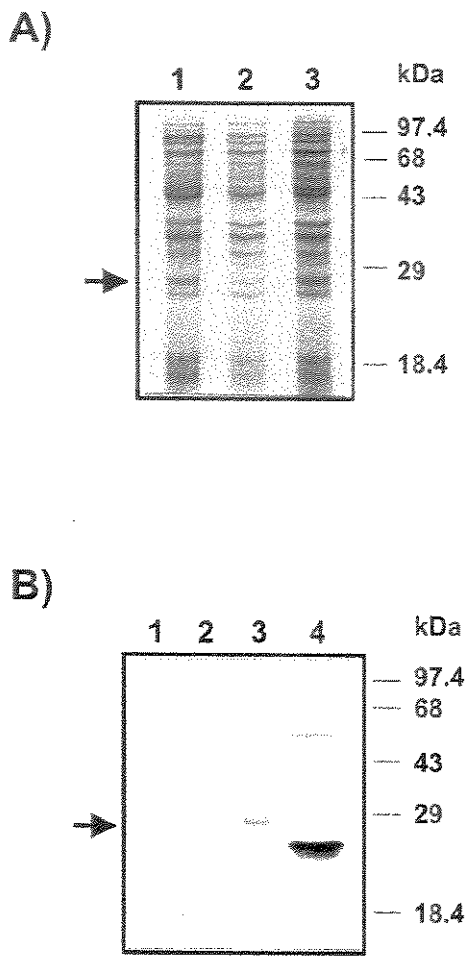

Fig. 6. Expression of ScYLV CP in *E. coli.* (A) Total cell lysates from non-induced (lane 2) or IPTG-induced (lane 3) *E. coli* harboring pET-CP were analyzed by 12% SDS-PAGE followed by Coomassie Blue staining. The control extract from IPTG-induced cells carrying empty pET-28a vector is shown in lane 1. (B) Immunoblot analysis of the gel shown in (A) using anti-ScYLV polyclonal antibodies and goat anti-rabbit IgG (alkaline phosphatase-conjugated). Purified ScYLV particles were used as control (lane 4). Molecular mass markers (kDa) are indicated on the right margin of each panel. The position of the CP is indicated by an arrow.

Discussion

The sequence data presented here are consistent with previously reported findings that include ScYLV, the causative agent of the yellow leaf disease in Brazil, as a possible member of the family *Luteoviridae* [11, 15]. The reaction of the antiserum raised against the isolate of ScYLV from Florida with the *E. coli*-expressed protein described here not only proved the authenticity of the CP ORF, but also established a link between viruses causing the yellow leaf syndrome of sugarcane in two different areas. Moreover, the detailed sequence comparisons reported in this study contribute to a better understanding of the taxonomic status of ScYLV.

The phylogeny of the RdRp sequence strongly suggests that ScYLV contains a sobemovirus-like rather than a carmovirus-like polymerase gene. Based on the data presented here, ScYLV should be included in the *Polerovirus* genus. On the other hand, the dendograns obtained from the comparison of the CP and 17 kDa sequences, revealed that ScYLV is more closely related to viruses in the genus *Luteovirus*. The serological cross-reactivity between ScYLV and BYDV-PAV [15] and the similarity in length of their intergenic region also support this classification. Taking these into consideration, one can reasonably suppose that the 5' coding block of ScYLV is more similar to that of the genus *Polerovirus* while its 3' coding block is closest to that of the genus *Luteovirus*.

The apparently different taxonomic affinities of the 3' and 5' coding blocks of the ScYLV genome, suggest that a possible recombination event has led to a genomic rearrangement as described for SDV. As opposed to ScYLV, SDV displays considerable sequence homology in its 5'-proximal ORFs with members of the genus *Luteovirus*, while sharing extensive homology to members of the genus *Polerovirus* in

its 3' half [9]. However, a better understanding of this possibility will be possible only when more sequence data become available.

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Capítulo 2

The use of molecular beacons combined with NASBA for the sensitive detection of *Sugarcane yellow leaf virus**

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Summary

Sugarcane yellow leaf virus (ScYLV) is widely distributed in Brazil and other sugarcane producing countries causing significant yield losses. Due to the high incidence of the aphid vector the virus is widespread in the field and in parental clones used in sugarcane breeding programs. Aiming to present a sensitive and reliable detection of ScYLV, we have developed an AmpliDet RNA system, compared it with the currently available detection methods and discussed its applicability for routine diagnosis. AmpliDet RNA consists of nucleic acid sequence based amplification (NASBA) of the target RNA with specific primers and simultaneous real-time detection of the amplification products with molecular beacons. The results showed that the system enabled the sensitive detection of the virus from different sources, such as highly diluted purified virus, plant tissue with low levels of infection (without the need of previous RNA extraction) and hemolymph of aphids. The method proved to be virus specific, testing negative for other species of the *Luteoviridae*. In conclusion, the system has a good potential to become a preferred detection method in routine diagnostic for sugarcane viruses.

Keywords: AmpliDet RNA, fluorescent probes, real-time detection, RT-PCR, sugarcane viruses.

Introduction

Sugarcane yellow leaf virus (ScYLV) is a newly characterised virus that infects sugarcane and causes one type of the Yellow Leaf Syndrome (YLS) in this culture (Vega *et al.* 1997; Lopes *et al.*, 1997; Scagliusi & Lockhart 2000). The symptoms consist of an intense yellowing of the abaxial surface of the midrib, reddening of its adaxial surface followed by tissue necrosis and reduction in plant growth. ScYLV was reported to occur in several sugarcane producing countries worldwide (Borth *et al.*, 1994; Schenck *et al.*, 1997; Vega *et al.*, 1997; Comstock *et al.*, 1998) causing significant yield losses. In Brazil, where the disease is widely distributed, losses as high as 50% were responsible for the decline of some of the most productive cultivars (Vega *et al.*, 1997).

On the basis of nucleotide sequence analysis it was recently proposed that ScYLV is a new member of the family *Luteoviridae*. The virus shares a number of characteristics with members of the genera *Polerovirus*, *Luteovirus* and *Enamovirus* (Moonan *et al.*, 2000; Maia *et al.*, 2000; Smith *et al.* 2000). However, it clearly represents a distinct species due to its unique biological properties and differences at the genomic level which suggest interspecies recombination (Moonan *et al.*, 2000; Maia *et al.*, 2000; Smith *et al.*, 2000).

The combination of the presence of the ScYLV in parental clones, clonal multiplication, and the high incidence of its aphid vector in the field necessitate the availability of a reliable method for the early detection of the virus in stock material. Sensitive and robust detection methods are required for supplying certified disease-free sugarcane varieties and are also essential tools in virus resistance breeding programmes. Currently, reverse transcriptase polymerase chain reaction (RT-PCR)

and serological assays based on Enzyme Linked Immunosorbent Assay (ELISA) or tissue printing have been used for screening the virus in plant material (Comstock *et al.* 1998; Scagliusi *et al.*, 1997; Scagliusi & Lockhart, 2000). However, the serological detection tests do not always offer the level of sensitivity and specificity required. On the other hand the gel-based RT-PCR systems are rather laborious and time demanding for routine diagnosis of a large number of samples.

The use of molecular beacons (Tyagi and Kramer, 1996; Eun & Wong, 2000) allows a gel-free approach in viral diagnostics, enabling sensitive, specific and fast detection. Molecular beacons are single-stranded oligonucleotides with a stem-loop structure and an internally quenched fluorophore. The loop portion contains a sequence complementary to the target nucleic acid, whereas the stem is unrelated to the target and has a double-stranded structure. One arm of the stem is labelled with a fluorophore, and the other is linked to a quencher. The double-stranded nature of the stem keeps the two moieties in close proximity to each other resulting in quenching of fluorescence. Upon hybridisation to its target the molecular beacon undergoes a conformational change separating the fluorophore and the quencher leading to fluorescence emission. The combination of nucleic acid sequence based amplification (NASBA; Kievits *et al.*, 1991) with molecular beacons is called AmpliDet RNA (Leone *et al.*, 1998). NASBA is a method that amplifies the target viral RNA isothermally at 41°C using two oligonucleotide specific primers and the enzymes AMV reverse transcriptase (AMV-RT), RNase H and T7-RNA polymerase. This combination permits the simultaneous amplification and detection of the viral RNA in one single reaction. Moreover, the assay is performed in a closed tube, thus minimising the risks of contamination.

In this paper we describe the development of an AmpliDet RNA system for the detection of ScYLV, and compare its sensitivity with that of Double Antibody Sandwich - ELISA (DAS-ELISA), RT-PCR and NASBA combined with Northern blotting analysis.

Materials and methods

Plant material, virus source and aphids

Healthy and naturally ScYLV-infected plants of two Brazilian sugarcane cultivars, SP 71-6163 and SP 82-3250, were grown in a greenhouse at 28°C under a photoperiod of 16 h. Both cultivars readily respond with symptoms upon an infection with ScYLV, and yield high virus titres. The virus was purified from frozen leaf material kept at –80°C using an enzyme-assisted procedure essentially as described by Van den Heuvel *et al.* (1991). Purified ScYLV (60-70 µg/ml) was stored in 0.1 M sodium citrate, pH 6.0, containing 20% sucrose at -80°C.

Colonies of the vector aphid *Mellanaphis sacchari* (Lopes *et al.*, 1997; Scagliusi *et al.*, 2000) were maintained on healthy sugarcane plants in aphid-proof cages at 23°C under a photoperiod of 16 h.

Samples of leaf material and aphids

Samples were prepared by grinding sugarcane leaf tissue in liquid nitrogen to which sample buffer (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_2O$, 0.05% Tween 20, 2% PVP 44,000, 0.2% ovalbumin, 0.1% Na-azide and 0.5% BSA, pH 7.4) was added in a ratio of 1:10 (w:v). Samples were aliquoted and either directly used or subjected to total RNA extraction using the RNeasy isolation kit (Qiagen).

The hemolymph of groups of 5 aphids which had been feeding on sugarcane plants was collected in 30 µl of deionised water by removing the siphons with a fine pair of forceps and applying some pressure on the abdomen of the insect. The samples were either directly used or stored at –80°C. RNA was extracted as described above.

NASBA and Northern blot detection

NASBA was carried out in reaction tubes as described by Kievits *et al.* (1991) using a reaction mix of 6 µl 3.3x NNX-buffer (200mM Tris-HCl, pH 8.5, 60 mM $MgCl_2$, 350 mM KCl, 2.5 mM DTT, 5 mM of each dNTP, 10 mM each of ATP, UTP and CTP, 7.5 mM GTP and 2.5 mM ITP), 4 µl 5x primer mix (75% DMSO and 1 µM of each primer in RNase free water) and 5 µl of sample extract per reaction. Primers used are listed in Table 1 and their position in the coat protein (CP) gene is shown in Figure 1. The tubes were incubated for 5 min at 65°C followed by 5 min at 41°C. NASBA was initiated by adding 5 µl of an enzyme mix (375 mM sorbitol, 2.1 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV reverse transcriptase) to each tube, incubating for 5 min at 41°C, followed by a 10 sec low speed centrifugation step, and continued incubation at 41°C for 90 min. Amplicons were separated on 1% pronarose gels containing in 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with 0.5 µg/ml EtBr, transferred onto Z-probe nylon membranes (BioRad) in 2X SSC (0.3 M NaCl, 30 mM Na-citrate), and exposed to UV light for cross-linking. For enhanced chemiluminescence detection (ECL detection reagents, Amersham Pharmacia Biotech) the blots were incubated with the biotinylated probe BIO_{ScYLV} (3 µM) for 60 minutes at 50°C in a hybridisation mix (5x SSC, 7% SDS, 20 mM Na-phosphate, pH 6.7, 10x Denhardt's reagent). The sequence and position of the probe BIO_{ScYLV} in the

CP gene of ScYLV are shown in Table 1 and Figure 1 respectively. Afterwards, the membranes were washed twice in 3X SSC with 1% SDS at 50°C for 5 minutes and once in 2X SSPE (20 mM Na_2HPO_4, 0.36 M NaCl, 2 mM EDTA) with 0.1% SDS, at room temperature for 10 minutes. The blots were then incubated with 2 µl streptavidin/peroxidase conjugate (Boehringer) in 10 ml of 5X SSPE with 0.5% SDS for 20 min at room temperature, extensively washed, and incubated in a substrate solution (ECL detection reagents). Signals were revealed by exposure to X-ray films.

Molecular beacon design and AmpliDet RNA

The molecular beacon MB_{ScYLV} was designed to carry 5' end and 3' end sequences of 6 nucleotides complementary to each other and an internal sequence of 21 nucleotides complementary to the ScYLV CP gene (Table 1). The molecular beacon was coupled with 6-carboxy-fluorescein (FAM; excitation wavelength: 494 nm; emission wavelength: 530 nm) and the quencher 4-[4'-dimethylaminophenylazo]-benzoic acid (DABCYL) at the 5' and 3' end respectively. The arms of 6 nucleotides form a double-strand structure at 41°C that avoid fluorescence emission in the absence of the target sequence. The position of the molecular beacon in the ScYLV genome is shown in Figure 1.

AmpliDet RNA was performed by adding 3 µl of sample, 1 µl of 8 mM ROX (5-(and –6)-carboxy-X-rhodamine) and 1 µl of 9 ng/µl of molecular beacon MB_{ScYLV} to the NASBA reaction mix described above. After incubation for 5 minutes at 65°C and 5 minutes at 41°C, 5 µl of the enzyme mix (previously described) was added to each tube. The reaction was immediately transferred to an ABI Prism 7700 Sequence Detector (Perkin Elmer). Amplification was performed at 41°C during 90 min while the fluorescence emission spectrum (530 nm) was measured in real-time every 2 minutes.

RT-PCR and DAS-ELISA

The antisense primer P2r (Table 1) was used to prepare cDNA from purified virus or total RNA extracted from healthy and infected leaf tissue. P2r and other PCR primers used (Table 1) were designed based on the coat protein coding region of ScYLV (Maia *et al.*, 2000). The reverse transcriptase was performed with 1 µl of antisense primer and 3 µl of samples and an usual pre-mix (4 µl of 5 x buffer, 1 µl of RNA guard, 2 µl 0,1 M DTT, 2 µl 10 mM dNTP mix and 200 U of Superscript - Gibco). The reaction mix for PCR consisted of 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 5 µl of 10 X Taq buffer, 8 µl of 25 mM $MgCl_2$, 5 µl 1mM dNTPs, 2,5 U of AmpliTaq Gold (Perkin Elmer), 5 µl of cDNA samples and 24,5 µl of H_2O. Several PCR protocols were tested for optimisation of the system.

DAS-ELISA was performed essentially as described by Scagliusi *et al.* (2000) with small modifications using ScYLV-specific polyclonal antisera supplied by the author.

Results

NASBA in a gel-based system

The NASBA method was first evaluated with Northern blot analysis before testing it in combination with molecular beacons. For determination of the sensitivity of NASBA, dilution series of purified virus were tested. The samples consisted of a 10-fold dilution series ranging between 100 pg and 10 fg of purified virus or total RNA from infected and healthy plants. Amplification of ScYLV RNA by NASBA was performed with primers N1r and N2f (Table 1) enclosing a sequence of 203

nucleotides within the coat protein open reading frame of the virus (Figure 1). The primer N1r has a 3' end target-complementary sequence and a 5' end T7-RNA polymerase recognition-site sequence. The NASBA products were analysed by Northern blot and ECL detection with the biotinylated probe BIO_{ScYLV}. Results showed the method was highly sensitive, enabling detection of 10 fg of purified virus (Figure 3A). Tests with dilution series of total RNA from infected plants in total RNA from healthy plants showed that this method permitted detection even after 1000-fold dilution of tissue samples (Figure 3B). No increase in fluorescence was observed with non-diluted total RNA from healthy plants.

Table 1: Oligonucleotide sequences and location of specific primers and probes in *Sugarcane yellow leaf virus* genome.

Oligonucleotide primers and probes	Sequences (5'→ 3')[1]	Positions[2]
RT-PCR sense primer - P1f	GCT.AAC.CGC.TCA.CGA.AGG.AAT.GT	3660-3882
RT-PCR antisense primer - P2r	GAA.GGG.GGC.CGG.GAA.GAC.T	4091-4109
RT-PCR sense primer - P3f	CAG.GTG.CAA.TCG.CAC.TTG.AAG.TGG.A	3997-4021
RT-PCR antisense primer - P4r	GAA.TTG.TCC.TGC.TAG.GCT.CGA	4179-4199
NASBA sense primer - N2f	CAG.GTG.CAA.TCG.CAC.TTG.AAG.TGG.A	3997-4022
NASBA antisense primer - N1r	AAT.TCT.AAT.ACG.ACT.CAC.TAT.AGG.GAG.GAA.TTG.TCC.TGC.TAG.GCT.CGA	4179-4199
Biotinylated probe - "BIO_{ScYLV}"	ATG.ACT.ACG.TCA.GCT.GAC.CA	4128-4147
Molecular beacon - "MB_{ScYLV}"	FAM **GCA.CCT**.ATG.ACT.ACG.TCA.GCT.GAC.CAG.**AGG.TGC** DABCYL	4128-4148

[1]Underlined letters in the NASBA primer sequence correspond to the 5'end T7-RNA polymerase recognition-site; bold letters in the molecular beacon sequence correspond to 5' and 3' end sequences complementary to each other; FAM: 6-carboxy-fluorescein, and DABCYL: quencher 4-[4'-dimethylaminophenylazo]-benzoic acid.

[2]Numbering refers to the corresponding positions of the oligonucleotides in the ScYLV genome.

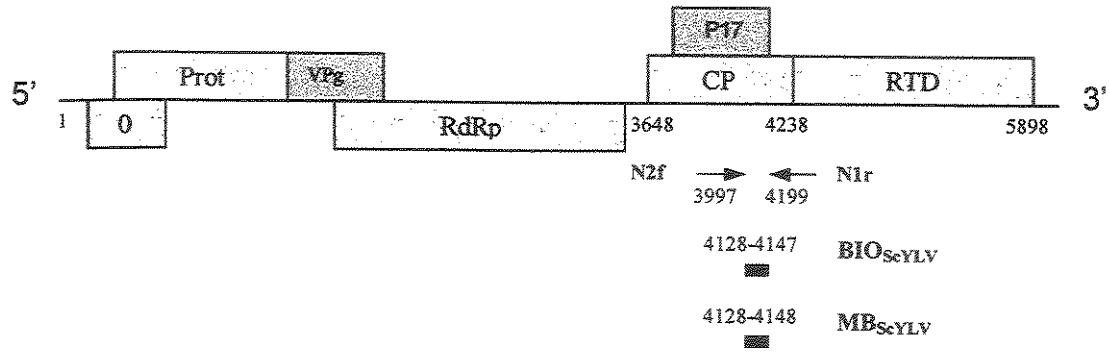


Fig. 1. Genomic organisation of *Sugarcane yellow leaf virus* showing the position of the NASBA primers N2f and N1f, the biotinylated probe BIO_{ScYLV} and the molecular beacon MB_{ScYLV}. 0: ORF 0, unknown function; Prot.: protease; VPg: putative viral protein genome-linked; RdRp: RNA dependent RNA polymerase; P17: putative movement protein; CP: coat protein; RTD: readthrough domain.

Sensitivity and specificity of the AmpliDet RNA system

To determine the sensitivity of the NASBA combined with the molecular beacon MB_{ScYLV} in a gel-free system (AmpliDet RNA), we used the same dilution series of purified virus and total RNA from infected plants as described above. The combination of NASBA with the molecular beacon was able to detect 100 fg of purified virus after less than one hour of amplification (Figure 4A). The system allowed detection of at least 1000-fold dilution of the total RNA from infected plants, and no fluorescence emission was detected for non-diluted total RNA from healthy plants (Fig. 4B). The method also allowed detection of the virus in fresh extracts from infected plants in sample buffer without previous RNA extraction (data not shown).

To determine the specificity of the molecular beacons to ScYLV, 5 different members of the family *Luteoviridae* were tested. Purified preparations of *Potato leafroll virus* (PLRV),

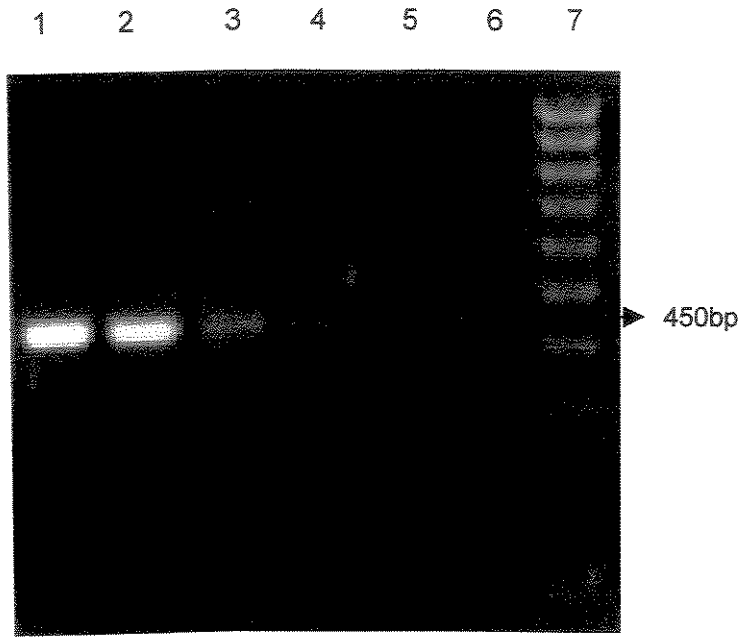


Fig. 2. Reverse transcription-PCR of *Sugarcane yellow leaf virus* from total RNA of infected plant. Lane 1: 100 pg of purified virus; lane 2: non-diluted RNA; lane 3: 10-fold dilution of RNA; lane 4: 100-fold dilution of RNA; lane 5: 200-fold dilution of RNA; lane 6: total RNA of healthy plant; lane 7: molecular weight marker. Dilutions were performed in total RNA of non-infected plants.

Beet western yellow virus (BWYV), *Barley yellow dwarf virus*-PAV (BYDV-PAV), *Beet mild yellow virus* (BMYV) and *Bean leaf roll virus* (BLRV) were assayed with the primers and molecular beacon developed for ScYLV. Three microliters (3 μl) of a 10 pg/ml dilution of each virus were used as samples in the reaction mix. Three microliters (3 μl) of 10 pg/ml of purified ScYLV and 3 μl of RNase-free water were used as positive and negative controls, respectively. All 5 luteoviruses (PLRV, BWYV, BYDV-PAV, BMYV and BLRV) tested negative by AmpliDet RNA, i.e. they presented a level of fluorescence similar to the negative control (RNase free water) whereas a high signal was observed for the positive control, ScYLV RNA (Figure 5).

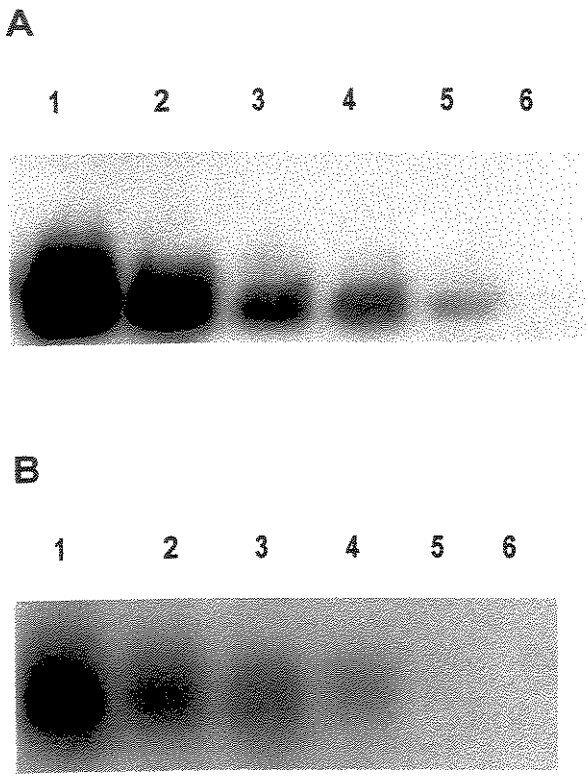
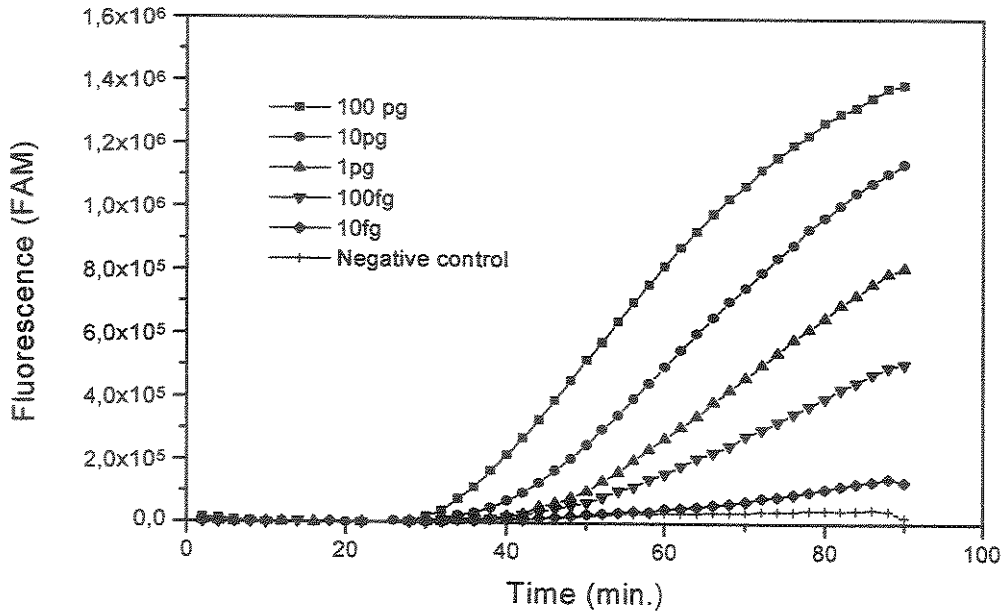


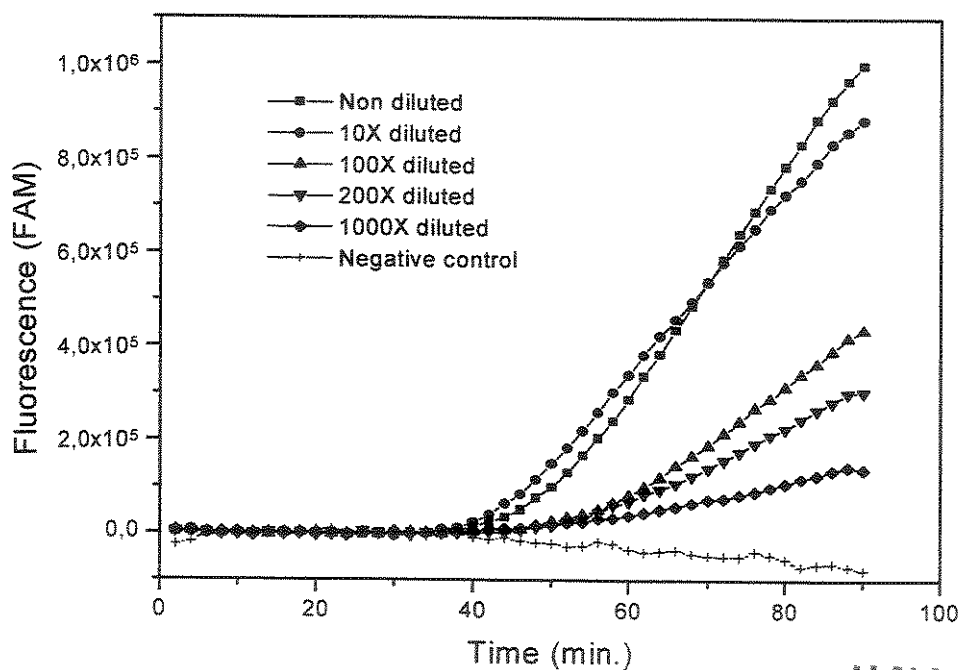
Fig. 3. Enhanced chemiluminescent detection after Northern blot analysis of the NASBA products using primers N2f and N2r, and BIO_{ScYLV} probe. **A)** 10-fold dilution series of purified ScYLV; lane 1: 100 pg; lane 2: 10 pg; lane 3: 1 pg; lane 4: 100 fg; lane 5: 10 fg; lane 6: RNase-free water. **B)** dilution series of total RNA of infected plant; lane 1: non-diluted RNA; lane 2: 10-fold dilution; lane 3: 100-fold dilution; lane 4: 1000-fold dilution; lane 5: 10000-fold dilution; lane 6: total RNA of healthy plant. Dilutions were performed in total RNA of non-infected plants.

Fig. 4. Real-time amplification and detection in a gel-free system using the molecular beacon MB_{ScYLV} (AmpliDet RNA). **(A)** 10-fold dilution series from 100 pg to 10 fg of purified ScYLV; **(B)** dilution series of total RNA from infected plant. Dilutions in (B) were performed in RNA from non-infected plants. Negative control consisted of total RNA from non-infected plant.

A



B



Detection in viruliferous aphids

The system was also assayed for detection of the virus in the aphid vector *M. sacchari*. Adult aphids were transferred to infected plants and confined to leaf cages allowing acquisition access periods (AAP) of 12, 24, 48 and 96 hours. Since the level of fluorescence when using the hemolymph from 5 aphids collected in deionised water or when extracting the RNA of those samples was similar, our tests were performed with freshly extracted hemolymph. After 24 hours feeding, aphids started to test positive for ScYLV presenting the fluorescent signal directly proportional to the AAP (Figure 6). Virus detection was not observed in the hemolymph of aphids with AAPs shorter than 24 hours or of aphids fed on non-infected plants (negative control). Hemolymph samples of a single aphid collected in 15 µl of deionised water after more than 48 hours AAP tested positive in the AmpliDet RNA system (data not shown).

Evaluation of the current diagnostic tools for ScYLV

A comparison of available DAS-ELISA and PCR diagnosis for ScYLV with our new system was carefully performed. The DAS-ELISA performed with the polyclonal antiserum raised against ScYLV presented best results when diluting infected leaf tissue 1:10 (wt/vol.) in 100 mM sample buffer. Immunoglobulin-alkaline phosphatase conjugate was diluted 1:500 in sample buffer and the readings were performed after 20 minutes of incubation with the substrate. Results showed a sensitivity of 1 ng in a 10-fold dilution series of purified virus from 100 ng to 1 pg.

The sensitivity of the RT-PCR for ScYLV was determined after optimisation of the system testing different primers and PCR protocols. The best set of primers was P2r and P1f (Table 1) using the following PCR protocol: 10 minutes 94°C, 40 cycles of 30 seconds 94°C, 1 minute 60°C, 2 minutes 72°C and 1 additional extension step of 7

minutes 72°C. A band of 450 bp, correspondent to the coat protein of ScYLV, was amplified from purified virus and from total RNA samples from infected leaf tissue (Figure 2). In a dilution series of total RNA from infected plants in total RNA of health plants, the method allowed detection up to 100-fold dilution (Figure 2). The sensitivity of the RT-PCR when using a 10-fold dilution series from 1 ng to 100 fg of purified virus was 1 pg (data not shown).

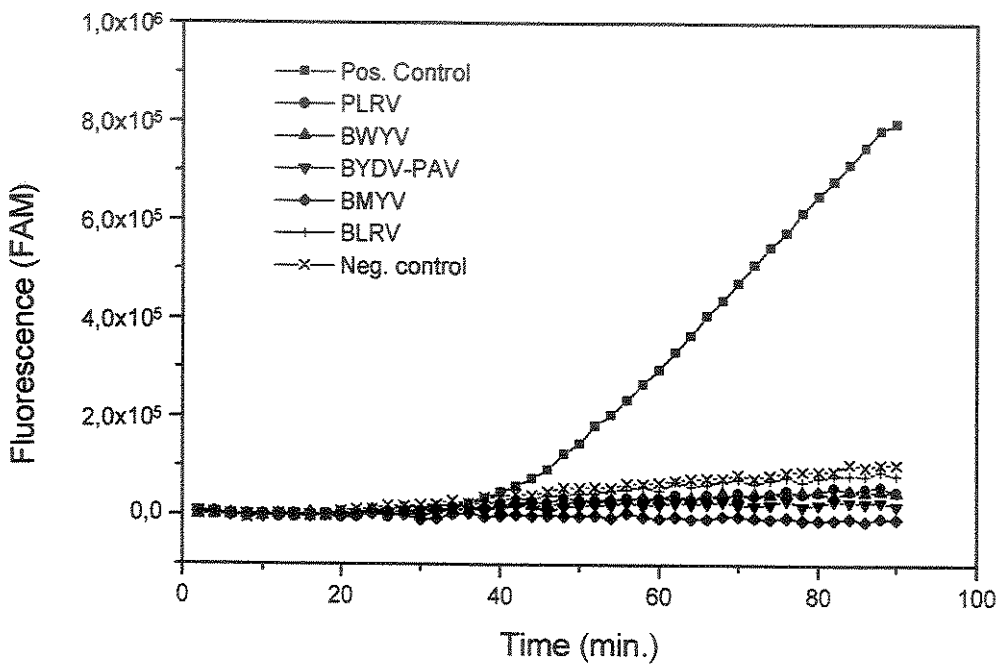


Fig. 5. Specificity of the AmpliDet RNA against 5 different luteoviruses. PLRV: *Potato leafroll virus*; BWYV: *Beet western yellow virus*; BYDV-PAV: *Barley yellow dwarf virus PAV*; BLRV: *Bean leaf roll virus*. Samples consisted of 3 µl of 10 pg/ml solution of each virus. Positive and negative controls consisted of 3 µl of 10 ng/ml of purified ScYLV and 3 µl of RNase-free water, respectively.

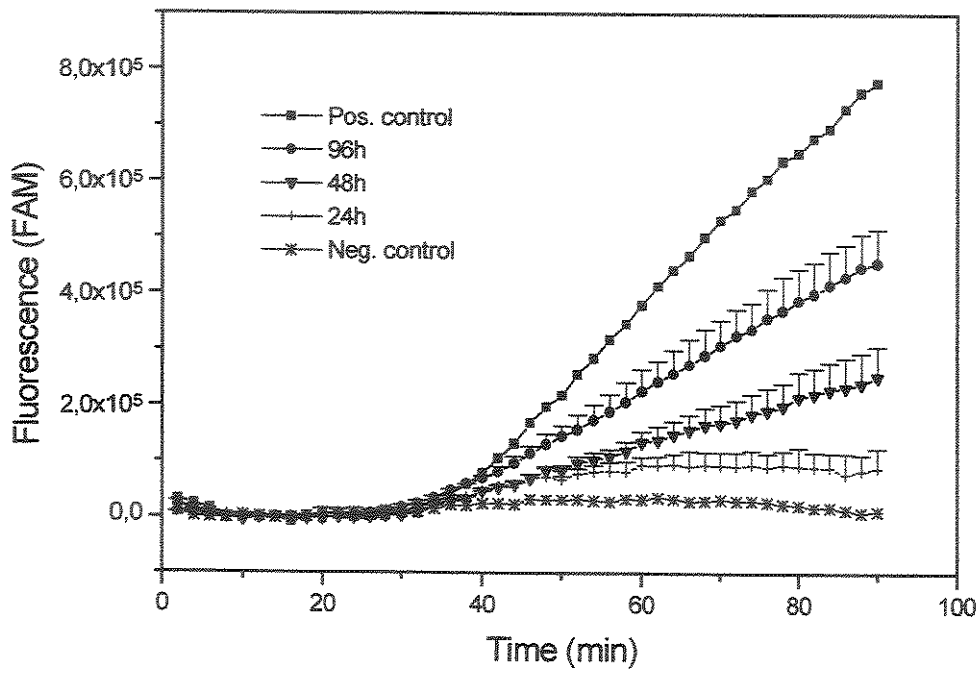


Figure 6. AmpliDet RNA of the hemolymph from 5 aphids *Mellanaphis sachari* collected in 30 µl of deionised water after 24, 48 and 96 hours acquisition access periods in infected plants. Each point represents the mean (± standard deviation) of 3 samples per acquisition access period. Three (3) µl of each sample was used in the assays. Positive and negative controls consisted of 3 µl of 10 ng/ml of purified virus and 3 µl of the hemolymph collected in water of 5 aphids fed in non-infected plants, respectively.

Discussion

The use of molecular beacons in virus diagnosis has increasingly received attention in the last few years on account of the broad potential of its application. In this study we have used the isothermal nucleic acid sequence-based amplification of RNA combined with molecular beacons in order to improve the detection of *Sugarcane yellow leaf*

virus. The results showed that the AmpliDet RNA developed for ScYLV allows for the highly sensitive and specific detection of the virus in plant material and in vector aphids.

The method shows several advantages when compared to the currently available diagnostic tools (ELISA and RT-PCR) for ScYLV. In addition to be more sensitive and specific than these methods, resulting in a more reliable diagnosis, the assay is also easier and faster to perform than RT-PCR analysis. The application of the molecular beacons allows detection in a single closed reaction tube, without need of running gels, the use of membranes or image recording. This results in reduction of labour and costs, speeds up the analysis, and minimises the risks of contamination and carry over errors during the process.

The NASBA developed allows in a gel-based system the detection of as little as 10 fg of purified ScYLV, as also in 1000-fold diluted total RNA extracts from ScYLV-infected plants. The combination of NASBA with the molecular beacon MB_{ScYLV} causes a 10-fold loss of sensitivity for purified virus. This indicates that the molecular beacon slightly inhibit the NASBA reaction. However, no sensitivity is lost in combination with the MB_{ScYLV} when analysing total RNA from plants. AmpliDet RNA is still 10-fold more sensitive than RT-PCR, which is currently used for ScYLV diagnosis in breeding programs in Brazil. On the other hand, the NASBA system generates single stranded RNA amplicons (Kievits *et al.*, 1991) enabling fluorescence measurement from hybridised molecular beacons (positive reactions) without the need of denaturation. This offers the possibility of using a relatively cheap fluorimeter for real-time or end-point readings instead of an ABI 7700 sequence detector. In case of end-point readings, the isothermal amplification can also be performed in a block heater, eliminating the necessity of a thermal-cycler. These features enable the application of

the method in laboratories with limited resources. The possibility of analysis of a large number of samples (a microtiter plate with 96 wells) and automation of the system makes it suitable for routine diagnostic purposes, like quarantine, certification and breeding programs for sugarcane.

We evaluated the AmpliDet RNA system against different conditions and different virus sources, such as highly diluted virus purification, plant tissue and viruliferous aphids. The dilution of total RNA from infected sugarcane in total RNA of non-infected plants indicates that virus detection in field-grown plants, under low pressure of inoculum and low levels of natural virus infection, is possible. The detection of the virus in the hemolymph of aphids just after an AAP of 24 hours offers the possibility of assessing the viruliferous nature of aphids from the field during the growing season of sugarcane, allowing monitoring the spread of the disease. Virus detection in crude extracts of infected plants without RNA extraction and in the hemolymph of a single aphid after 48 hours feeding on an infected plant reinforces the robustness and reliability of the method. Therefore, this system offers an excellent tool for screening and monitoring plant material from the field and for studying the epidemiological patterns of this relatively new disease.

Furthermore, the system can be developed into a multiplex real-time assay (Klerks *et al.*, 2001) for simultaneously screening of ScYLV and *Sugarcane mosaic virus* (SCMV), a worldwide-distributed potyvirus infecting sugarcane. These two viruses are of major concern regarding sugarcane breeding and quarantine programs before the introduction of new germplasm.

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M.C.Gonçalves is grateful to CAPES Foundation for a fellowship from PDEE programme for a research stay at Plant Research International. Thanks are also due to F. van der Wilk for his help with the RT-PCR protocols and Sandra M. M. Scagliusi for kindly supplying the ScYLV-specific antisera.

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Capítulo 3

Studies on Aphid Transmission and *Buchnera* GroEL Affinity of a *Potato leafroll virus* RTD Deficient Mutant*

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Summary

Potato leafroll virus (PLRV), genus *Polerovirus*, family *Luteoviridae*, is transmitted by aphids in a persistent and circulative manner. Members of the *Luteoviridae* (luteovirids) associate to a GroEL homolog produced by the endosymbiotic bacterium (*Buchnera* sp.) of aphids to avoid degradation in the hemolymph. Luteovirids purified particles contain two types of protein; a major 22 kDa coat protein (CP) and the minor capsid component of 54 kDa, the readthrough protein (RTD). The RTD contains determinants responsible for virus transmission and accumulation in agroinfected plants. An infectious cDNA full lengh clone of PLRV and a mutant devoid of the RTD were used to study and better understanding the molecular interactions between this luteovirid and its aphid vector *Myzus persicae*. The PLRV mutant lacking the entire RTD protein, was not transmissible by *M. persicae* and did not bind to *Buchnera* GroEL. Furthermore, the mutant was less persistent in the aphid's hemolymph than the wild type virus. These data greatly contrast with results on the role of RTD in the transmission process of this virus reported in previous work with PLRV VLPs produced in a baculovirus system. On the other hand our data corroborate previous observations with *Beet western yellow virus* (BWYV) and *Barley yellow dwarf virus* (BYDV) that the RTD domain is indispensable for luteovirids transmission and persistence in the aphid's hemocoel.

Introduction

Potato leafroll virus (PLRV) is the type member of the genus *Polerovirus* in the family *Luteoviridae* (Mayo and D'arcy, 1999; Pringle, 1998). PLRV virions are particles of 25 nm diameter carrying a positive-strand RNA of ~6kb with a viral linked genome protein, VPg, at the 5' end (Mayo et al, 1982). The genomic RNA encodes six open main reading frames (ORFs) (van der Wilk *et al.*, 1989; Mayo *et al.*; 1989) arranged into 5' end (ORF0, ORF1, AND ORF2) and 3' end (ORF3, ORF4, and ORF 5) gene clusters expressed by different mechanisms (Miller *et al.*, 1995). The 3' end proximal ORFs are expressed via subgenomic RNA synthesised in host cells during the infection process. ORF3 encodes the major capsid protein (CP) of about 23 kDa, ORF4 encodes a 17 kDa putative movement protein, and ORF5 encodes the minor capsid component (RTD) expressed by translational readthrough of the ORF 3 stop codon. The full length resulting protein is about 74 kDa but in preparations of purified virus the RTD is detected in a C-terminally truncated form of about 54 kDa (Bahner *et al.*, 1990).

Members of the *Luteovidae* ("luteovirids", Smith *et al.*, 2000) replicate mainly in the phloem tissue of their host plants and are transmitted by aphids in a persistent and circulative, non-propagative manner (Gildow, 1999). The process of acquisition and transmission of these viruses by their specific vectors has been thre object of extensive studies in the last decade. After ingestion with the phloem sap during aphid feeding on an infected plant, the virus is actively transported through the epithelial cells to the hemocoel by receptor-mediated endocytosis and exocytosis (Gildow, 1993; Garret *et al.*, 1996). The virus particles are retained in an infective form in the hemolymph of the aphid and

upon contacting with the basal lamina of the accessory salivary gland (ASG) they may be transported through the underlying plasmalemma into the salivary canal, from where they are excreted with the saliva while the aphid feeds. The high degree of specificity among luteovirids and the aphid species at this level determines the successful transport across the ASG basal lamina and plasmalemma, and consequent virus transmission (Gildow and Gray, 1993; Pfeiffer *et al.*, 1997).

Symbionin , a GroEL homolog synthetized by the primary endosymbiotic bacteria (*Buchnera* sp) of aphids is abundantly present in the hemolymph and is essential for luteovirids persistence in the aphid's body (van den Heuvel *et al.*, 1994, 1997). In ligand binding assays, PLRV and *Barley yellow dwarf virus* (BYDV) show a high specific affinity for GroEL homologs of vector and non vector species (van den Heuvel *et al.*, 1994; Filichkin *et al.*,1997). Decreasing *Buchnera* GroEL levels in the hemolymph by antibiotic treatment inhibited transmissibility of PLRV and caused loss of capsid integrity, suggesting that the association of luteovirids with this protein retards proteolytic breakdown (van den Heuvel *et al.*, 1994, 1997).

The role of the viral capsid proteins (CP and RTD) in determining the efficiency of aphid transmission has been an important object of study of luteovirids. Previous studies with BWYV RTD mutants have shown that this protein is indispensable for transmission by *Myzus persicae* (Sulz.), harbouring determinants which mediate circulation of the virus in the aphid (Brault *et al.*, 1995). The recognition and acquisition process of BWYV into the aphid vector hemocoel is greatly reduced in RTD deficient mutants, suggesting an important role for this protein in the virus recognition and transcellular transport process

(Reinbold *et al.*, 2001). Several subdomains can be identified in the readthrough domain (Mayo and Ziegler-Graff, 1996). Located in the N-terminal half of the RTD, downstream of the coat protein suppressible termination codon, is a cytidine-rich sequence encoding a tract of alternating proline residues. This proline tract is followed by a region of 200 amino acids which displays considerable homology throughout the *Luteoviridae* (Bruyère *et al.*, 1997; Brault *et al.*, 2000). The C-terminal half of the RTD is poorly conserved, although it contains a region of homology unique to luteovirids efficiently transmitted by *M. persicae* (Guilley *et al.*, 1994). The presence of the N-terminal region of the RTD in BWYV virions determines the virus binding with the endosymbiont bacteria *Buchnera GroEL* and the persistence of the virus in the aphid's hemolymph (van den Heuvel *et al.*, 1997). Brault *et al.* (2000) have identified specific amino acids in the conserved part of the RTD involved in virus accumulation in plants and in transmission of BWYV by *M. persicae*. Successful aphid transmission of the BWYV RTD mutants accompanied by compensatory mutations in this protein suggests that the RTD possesses structural redundancy involved in transmission by *M. persicae*.

In this study we have used an infectious cDNA clone of a Dutch isolate of PLRV (van der Wilk *et al.*, in publication) to show the role of the RTD of PLRV in the transmission by *M. persicae*. For this purpose, a mutation that abolishes RTD expression was constructed and its consequences were followed in microinjected aphids and throughout the transmission process.

Materials And Methods

Virus strain and aphid colony

PLRV-Wageningen (van der Wilk *et al.*, 1989) was maintained on *Physalis floridana* for use in our experiments. The virus was purified from frozen *P. floridana* leafs by an enzyme assisted procedure according van den Heuvel *et al.* (1997). Purified virus was stored at -80°C in 0.1M sodium citrate (pH 6.0) containing 20% sucrose. Nonviruliferous *M. persicae* aphids reared on cabbage (*Brassica napus* subsp. *oleifera*) at 20°C ± 3°C under a photoperiod of 16 hours a day were used in all microinjection and transmission experiments.

Mutant of PLRV

The recombinant vectors containing wild-type PLRV full-length cDNA, PLRV-H6 (van der Wilk et. al, in preparation), and RTD deletion mutant PLRV (PLRV-Seam F) are represented in Figure .1 The entire RTD protein was eliminated by deletion and frameshifting in the mutant PLRV-Seam F. This mutant was constructed by PCR mutagenesis under control of the T7 promoter using PLRV-Wageningen as template.

Infection of protoplasts and virus purification

Cowpea (*Vigna unguiculata* L.) protoplasts were inoculated with viral RNA transcripts according van Bokhoven *et al.* (1993) with modifications. One million protoplasts plus 2 μl of 2 μg/μl transcripts were precipitated with 40% PEG 6000, 0.5 M Mannitol and 0.1 M $Ca(NO_3)_2$ in MES/Mannitol buffer (0.5 M Mannitol, 15 mM $MgCl_2$ and 0.1% MES, pH 5.6). Protoplasts were incubated for 20 minutes at room temperature and centrifuged for 3 minutes at 600 rpm. After washing with 0.5 M

Mannitol and 0,1% CaCl2, protoplasts were centrifuged for 3 minutes at 600 rpm and the pellet resuspended in Rottier medium (Rottier *et al.*, 1979). Protoplasts were incubated for 48 h at 24°C under weak light and centrifuged for 2 minutes at 1000 rpm. Pellets were stored at –20°C.

The wild-type PLRV-H6 and the mutant PLRV Seam F were purified from infected protoplasts by resuspending 1.000.000 protoplasts in 1 ml of 0.1 M sodium citrate, pH 6.0 containing 0.5% cellulase, 0.5% macerozyme, 1 µl of thioglycolic acid and 5 µl of ethanol 100%. After incubation for two hours with gentle agitation at room temperature, 0.5 ml of a mixture of chloroform:butanol (1:1) was added, mixed and the suspension was centrifuged for five minutes at 1000 rpm. To the aqueous phase was added 10 µl of Triton X-100 followed by incubation and agitation for 30 minutes at room temperature. The solution was loaded onto a 20 to 50% linear sucrose gradient and centrifuged in a Beckman SW41 rotor at 35.000 rpm for 5 h. Fractions of 0.5 ml were collected in an automatic fraction collector and peaks at 254 nm tested for the presence of viral antigen by antigen-coated plate enzyme linked immunossorbent assay (ACP-ELISA) with a polyclonal antiserum raised against PLRV. Virus fractions were combined, diluted in sodium citrate 0.1 M, pH 6.0 and sedimented by centrifugation for 3 h at 40000 rpm in a Beckman SW55 rotor. The pellet was resuspended in sodium citrate 0.1 M, pH 6.0 containing 20% of sucrose. Virus concentration was determined by ACP-ELISA, and purified virus was stored at –80°C.

Analysis of viral RNA and capsid proteins

Infection of protoplasts by cDNA full-length clones was monitored by transmission electron microscopy (TEM). Protoplasts were sonicated for 1 minute and

submitted to negative contrast with 2% (wg/vol) uranyl acetate before observation by TEM.

Viral structural proteins of purified virus from protoplasts were separate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in 10% gels, as previously described (Brault *et al.*, 1995) and detected by Western blotting using a specific antiserum raised against PLRV.

Purification of Buchnera GroEL and GroEL ligand assay

The endossimbiotic bacteria of *M. persicae*, *Buchnera* GroEL, was purified from 6-day old aphids as previously described (van den Heuvel *et al.*, 1997). Samples collected from bands of a 10 to 50% linear sucrose gradient were diluted (1:10) in SYMbuffer (50 mM Tris-HCl, pH 7.6 containing 35 mM KCl, 25 mM NH_4Cl, 10 mM MgAcetate and 1mM DTT), boiled for 5 minutes and the proteins were separated by SDS-PAGE in a 8.5% polyacrilamide gel. The GroEL-containing bands were identified by Western Blot analysis with an antiserum raised to native *Buchnera GroEL* from *M. persicae* (van den Heuvel *et al.*, 1997). Blotted proteins were stained with 0.5% Ponceau stain and their position marked with fine forceps. Concentration of GroEL was estimated using an immunoplate by the Comassie Plus Protein Assay (Pierce, Rockford, IL, USA) with a protein dilution series ranging from 2000 to 10 μg/ml. Optical density was measured at 630 nm in a Bio-Kinetics reader EL 312 (Biotech Instruments Inc. Winooski, Vt.) and purified GroEL was stored at -80°C.

The affinity binding of PLRV-H6 and PLRV-Seam F for Buchnera GroEL ("GroEL ligand assay") was determined using immunoplates as described by van den Heuvel *et al.* (1997) with small modifications. Plates were coated with 100 μl of 6 μg/ml purified GroEL 0.05 M in sodium carbonate, pH 9.6 (coating buffer), and incubated

overnight at 4°C. Samples, consisting of 100 µl of 40 to 80 ng/ml of purified virus from protoplasts in sample buffer (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_2O$, 0.05% Tween 20, 2% PVP 44,000, 0.2% ovalbumin, 0.1% Na-azide and 0.5% BSA, pH 7.4), were incubated overnight at 4°C. Subsequent steps were followed as described previously (van den Heuvel *et al.* 1997).

Aphid transmission assays and microinjection

Six-days old unviroliferous *M. persicae* starved for 5 to 10 hours were microinjected with 530 and 800 nl of purified virus from protoplasts at 15 and 10 µg/ml respectively, using calibrated glass capillaries (Gabay Instruments, Geneva, Switzerland). Positive controls consisted of aphids microinjected with 800 nl of purified PLRV (20 µg/ml) from frozen leaf material. Fifteen to 20 microinjected aphids were transferred to healthy *P. floridana* or to cabbage plants maintained at 20°C under a photoperiod of 16 hours. After a 96 hours inoculation access period (IAP), *P. floridana* plants were sprayed with insecticide and symptoms were monitored periodically. Plants were analysed for virus infection by DAS-ELISA after 3 weeks of inoculation. Batches of 2 aphids were collected from cabbage plants after 0, 24, 48, 72, and 96 h and stored at –80°C for further analysis by triple antibody sandwich (TAS)-ELISA.

Detection of virus in microinjected aphids

For TAS-ELISA analysis, immunoplates were first coated with 100 µl of 1 µg/ml anti-PLRV IgG in coating buffer for 3 h at 37°C. Samples, consisting of 2 aphids frozen at different times after microinjection, were ground in 110 µl of SEB and incubated overnight at 4°C. Intact virus particles were detected with 100 µl of 2 µg/ml monoclonal antibody WAU-B9 (van den Heuvel *et al.*, 1990) in SEB by incubation for 3 h at 37°C. Immunoplates were then incubated for 3 h at 37°C with 100 µl goat anti-mouse IgG

linked to alkaline phosphatase (Sigma, St. Louis, Mo.) in SEB buffer following manufacturer instructions. The enzyme was assayed with 1 mM p-nitrophenyl phosphate in 10% diethanolamide (pH 9.8). Readings at 405 nm determined the amount of immobilized enzyme.

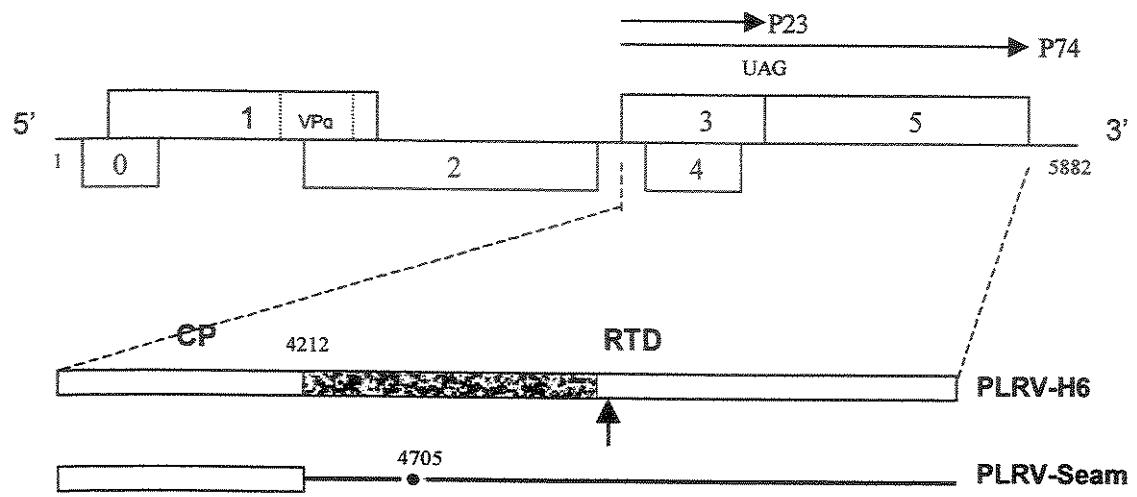

Figure 1. Genomic organisation of *Potato leafroll virus* and structure of the readthrough protein (RTD) of wild-type PLRV (PLRV-H6) and RTD deletion mutant PLRV-Seam F. Open reading frames (ORFs) are represented by numbered boxes and the viral structural proteins P23 and P74 by arrows. Viral genome linked protein (VPg) coding sequence is represented between dashed lines in ORF 1.The conserved N-terminal half of the RTD is shaded; the predicted site of cleavage of P74 to generate the C-terminally truncated form of the RTD is indicated by an arrow. The deletion in mutant PLRV-Seam F is indicated by a dot and numbering refers to the nucleotide positions in the PLRV genome. The deletion of a single nucleotide in the mutant caused a frameshift and non-translation of the RTD, represented by a thick line.

Results

Biological activity in protoplasts

The infectivity of wild type and mutant transcripts in cowpea protoplasts was first assayed by TEM. After sonication of protoplasts and staining with uranyl acetate, intact particles of PLRV-H6 and PRLV-Seam F transcripts were observed in similar amounts and were indistinguishable by TEM (Figure 2).

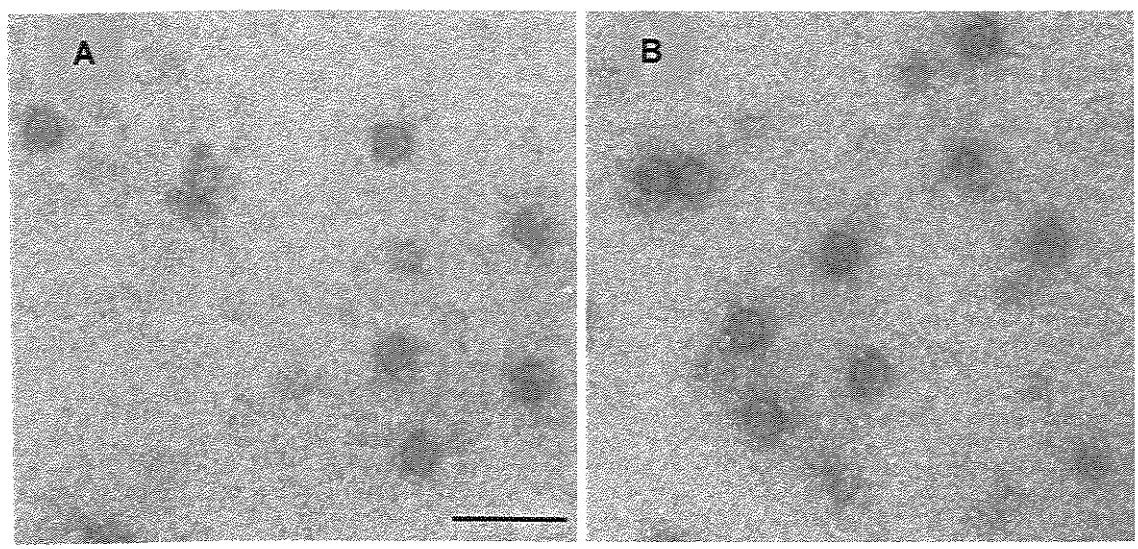

Figure 2. Transmission electron micrographs of PLRV particles purified from infected protoplasts. Particles were stained with 2% uranyl acetate. A: wild type full length clone H6; B: RTD deletion mutant Seam F. Scale bar = 100 nm.

The PLRV-Seam F transcripts were sequenced with specific primers (van der Wilk *et al.*, in preparation) in the RTD region, from position 3334 to the end of the PLRV genome sequence. Sequencing was performed in an ABI 373 automated sequencer using an AmpliTaq polymerase kit (Applied Biossystems). Sequencing revealed a point mutation of one nucleotide (guanine) in the position 4705 of the PLRV genome (Figure 1). Western blot analysis following SDS-PAGE of virus purified from

infected protoplasts revealed that the point mutation of one nucleotide in the N-terminal region of the RT domain of PLRV genome, suppressed the formation of the entire RTD protein. In Figure 3 the two structural proteins of the capsid of PLRV can be observed in lanes corresponding to wild-type PLRV purified from infected *P. floridana* and to PLRV-H6, purified from transcript-infected cowpea protoplasts. The major coat protein of about 23 kDa (CP), encoded by the open read frame (ORF) 3 is present in all lanes. In contrast, the RTD of about 74 kDa, which is expressed as a result of translational readthrough of the ORF 3 stop codon into the ORF 5, is not present in the lane corresponding to the mutant Seam F. The RTD proteins shown here are present in a truncated form of about 54 kDa, lacking the C-terminal region, which is characteristic of purified virus particles (Bahner *et al.*, 1990; Brault *et al.*, 1995; Wang *et al.*, 1995).

The deletion of the entire RTD of PLRV did not inhibit viral RNA replication in transcript-infected cowpea protoplasts. However, the absence of the RTD had significant effects in subsequent steps of the virus life cycle as will be discussed bellow.

SAMPLES	SYMPTOMS SEVERITY[1]	ELISA DO 405nm[2]
Wild-type PLRV[3]	3 plants ++++	0.957 ± 0.195
PLRV-H6	3 plants +++	0.430 ± 0.023
PLRV-Seam F	3 plants -	0.008 ± 0.003
Positive control[4]	++++	0.840
Negative control[5]	-	0.006

[1] Scale varies from – (negative) to ++++ (maximum intensity)
[2] Mean of 3 plants ± standard deviation
[3] Purified from leaf material
[4] Physalis inoculated with wild-type PLRV
[5] Healthy Physalis plant

Table 1. Symptoms expression in *P. floridana* and amount of virus given by DAS-ELISA (DO 405nm) after 3 weeks of inoculation with microinjected *M. persicae*. Aphids were allowed a 96 hours IAP.

SAMPLES	SYMPTOMS SEVERITY[1]	ELISA DO 405nm[2]
Wild-type PLRV[3]	10 plants ++++	1.094 ± 0.186
PLRV-H6	10 plants ++++	0.508 ± 0.172
PLRV-Seam F	10 plants -	0.007 ± 0.003
Positive control[4]	++++	0.960
Negative control[5]	-	0.002

[1] Scale varies from – (negative) to ++++ (maximum intensity)
[2] Mean of 10 plants ± standard deviation
[3] Purified from leaf material
[4] Physalis inoculated with wild-type PLRV
[5] Healthy Physalis plant

Table2. Symptoms expression in *P. floridana* and amount of virus given by DAS-ELISA (DO 405nm) after 3 weeks of inoculation.One-day old *M. persicae* nymphs were allowed an AAP of 48 hours in *P. floridana* inoculated with microinjected aphids and 96 hours IAP.

Transmission by microinjected aphids

It is already known that BWYV and BYDV virions lacking the readthrough are stable in the intestine and able to cross the gut epithelium (Brault *et al.*, 1995, Chay *et al.*, 1996), but these particles are not able to cross the ASG basal lamina, and therefore are not transmissible by the aphid (van den Heuvel *et al.*, 1997). On the other hand, RTD protein-deficient PLRV VLPs produced in a baculovirus system were found in the vector in the same locations as observed for wild-type PLRV (Gildow *et al.*, 2000). In this case, the absence of the RTD protein did not greatly affect the ability of virions to penetrate the accessory salivary gland basal lamina. In order to better understand this question we microinjected purified PLRV-H6 and the RTD-deficient mutant PLRV-Seam F in the aphid's hemocoel and tested their transmission by *M. persicae.* Unviroliferous and wild-type PLRV microinjected aphids were used as negative and positive controls respectively. PLRV-H6 was efficiently transmitted by aphids in all tested plants (3/3 test plants with 15 to 20 aphids/plant), while PLRV-Seam F was not transmissible (3 test plants with 15 to 20 aphids/plant). Symptoms

and DAS-ELISA results with an antiserum raised against PLRV are shown in Table 1. Two of the three tested plants used for inoculation with microinjected aphids (PLRV-H6, PLRV-Seam F, and positive control) were randomly chosen as source of inoculum for a new transmission by *M. persicae*. One-day old nymphs were allowed a 48 hours acquisition access period (AAP) in tested plants and aphids were transferred to *P. floridana* seedlings (10 seedlings with 3 aphids/seedling) for a 96 hours IAP. Symptom evaluation and DAS-ELISA showed that transmission was maintained for PLRV-H6 after passage through *P. floridana* (Table 2). All the 10 PLRV-Seam F inoculated plants showed no symptoms and tested negative in DAS-ELISA.

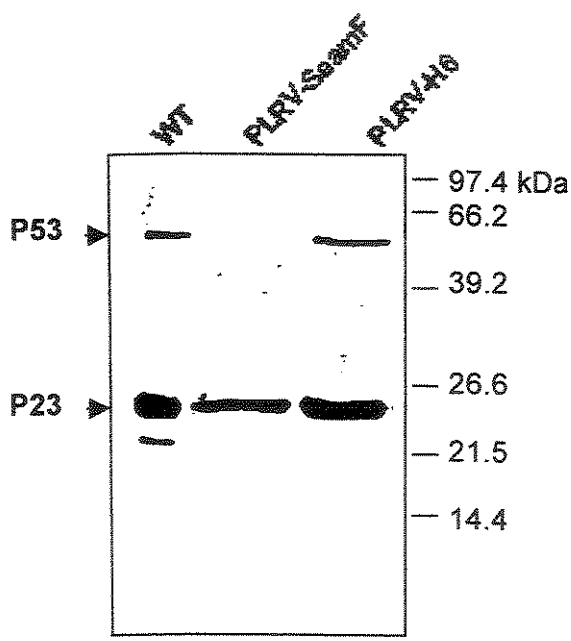

Figure 3. Western blot analysis of PLRV structural proteins CP (23 kDa) and RTD (74 kDa) in virus purified from transcript-infected *V. unguiculata* protoplasts (PLRV-H6 and PLRV-Seam F) and from virus purified from frozen *P. floridana* leaves (WT). Proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a specific antiserum raised against PLRV. The positions of the major capsid protein (P23) and the C-terminally truncated form of the RTD protein (P53) are indicated to the left. Molecular markers are shown to the right.

Stability in microinjected aphids

The acquired PLRV particles are retained in an infective form in the hemolymph of *M. persicae* for the lifespan of the aphid (Eskandari *et al.*, 1979). One of the characteristics that determine the persistent nature of PLRV in its vector body is its association with Buchnera GroEL. This protein is responsible for prevention of proteolytic degradation of virions in the hemolymph. We have used microinjected aphids to check the stability of wild type PLRV-H6 and RTD-deficient mutant in the aphid hemolymph. Results showed that 24, 72 and 96 hours after injection mutant Seam F particles were less stable than the H6 particles (Figure 6). However the fastest degradation occurred in the first 24 hours post-injection, after wich virus titer remained at similar levels until 96 hours post-injection.

Discussion

In the present study we have demonstrated some of the roles of the RTD protein in the transmission process of PLRV by its main vector. Earlier reports have already postulated that the RTD is important and indispensable for aphid transmission, as reviewed in the introduction. However, the molecular aspects of luteovirids-vector interactions and the involvement of RTD in the transmission process are mainly based on work with BYDV and BWYV. Most of the studies with PLRV are related to the interaction of the particles with *Buchnera* GroEL (van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998; 2000), with a few exceptions. Perhaps the main reason for this observation was the difficulty for constructing infectious transcripts from cloned cDNA of PLRV until recently.

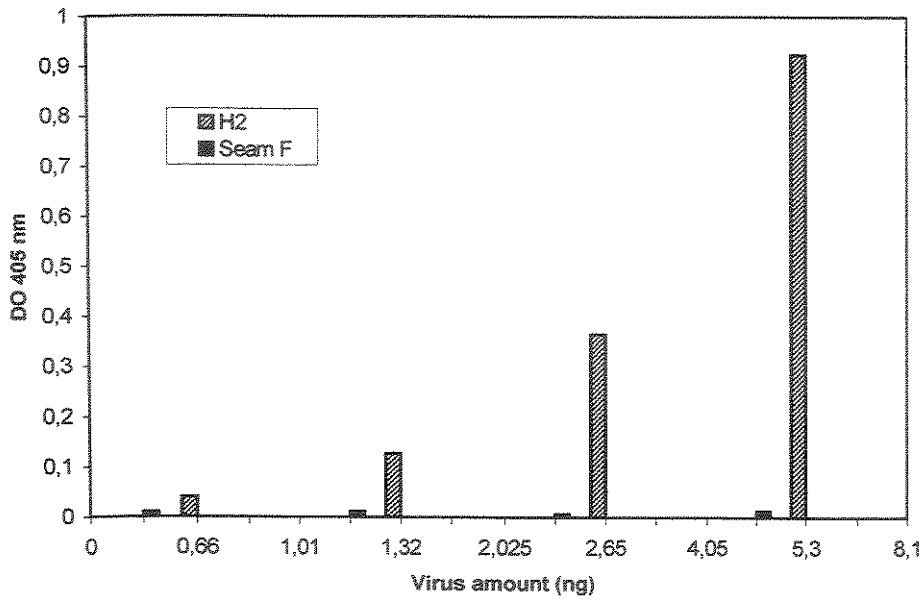

Figure 5. Affinity binding of PLRV-H6 and RTD deficient mutant PLRV Seam F to *Buchnera* GroEL purified from *M. persicae*. Microtiter plates were coated with 100 µl of 6 µ g/ml of purified GroEL in coating buffer and incubated overnight at 4°C. Each virus sample was tested with 3 different dilutions in sample buffer. Other steps were performed following GroEL ligand assay (see text). Absorbance measurements at 405 nm were performed after 15 minutes of addition of the substrate. Negative controls (sample buffer) showed absorbance values of less than 0,015.

In an attempt to overcome this limitation Gildow *et al.* (2000) have worked with PLRV virus-like particles (VLPs) produced in a baculovirus system to elucidate some roles of this protein in the transmission of PLRV by *M. persicae*. However, the VLPs were unable to infect plants when inoculated by aphids and the studies focused on acquisition and circulation of RTD deficient VLPs through aphid tissues. These authors reported that the absence of the RTD did not greatly affect the stability of VLPs in the hemolymph and the ability of crossing the accessory salivary gland basal lamina, considered a specific barrier for transmission by aphids (Pfeiffer *et al.*, 1997, Gildow, 1999). Furthermore, no structural evidence was encountered for GroEL binding to

either PLRV or to VLP. Our results with cDNA infectious clones of PLRV were in total contrast to these observations. The introduction of one point mutation in the N-terminal half of the RTD conferred the non-translation of this protein, consequently the RTD deficient mutant was less stable in the hemocoel and was not transmitted by *M. persicae*. The smaller stability of the RTD deficient mutant in the hemolymph, compared to the wild type clone H6, is explained by its inability to bind to GroEL (as shown in Figure 4), leading to its degradation in this hostile environment. The differences observed between our results and those with PLRV VLPs can be explained by the possibility that the histidine tag fused at the N-terminus of the CP could be exposed on the surface of the VLPs and mimic the function of RTD, interacting with *Buchnera* GroEL. As raised by Gildow *et al.* (2000) "the addition of this N-terminal sequence on the CP may have influenced particle recognition and survival in the aphid hemocoel".

The role of the RTD in luteovirids-vector interactions seems to involve different steps in the transmission process. Amino acid changes in the conserved region of the RTD of a PLRV isolate account for its poor transmissibility (Rouzé-Jouan *et al.*, 2001). In this case the barrier for no or only poor transmission was the gut membrane that regulates the passage of virus particles from the gut into the hemocoel on *M. persicae*. Moreover, when microinjected in the hemolymph of aphids, these particles crossed the ASG basal lamina and were transmitted by the vector. Similar observations were reported for a BWYV cDNA infectious clone, in which mutations at amino acids 267-268 in the conserved region of the RTD, resulted in lost of ability to cross the gut membrane of *M. persicae* (Brault *et al.*, 2000). However, no transmission was observed when these particles were microinjected in the hemocoel, indicating that these mutations in the RTD also impeded subsequent events in the transmission

process, probably the passage through the ASG basal lamina. We have shown in recent work that the RTD of BWYV is not strictly required for recognition and acquisition of virions into the aphid hemocoel, but its presence greatly enhances the efficiency of these processes (Reinbold *et al.*, 2001). On the other hand, this protein was absolutely necessary for maintenance of BWYV particles in the hemolymph and their passage through the acessory salivary gland.

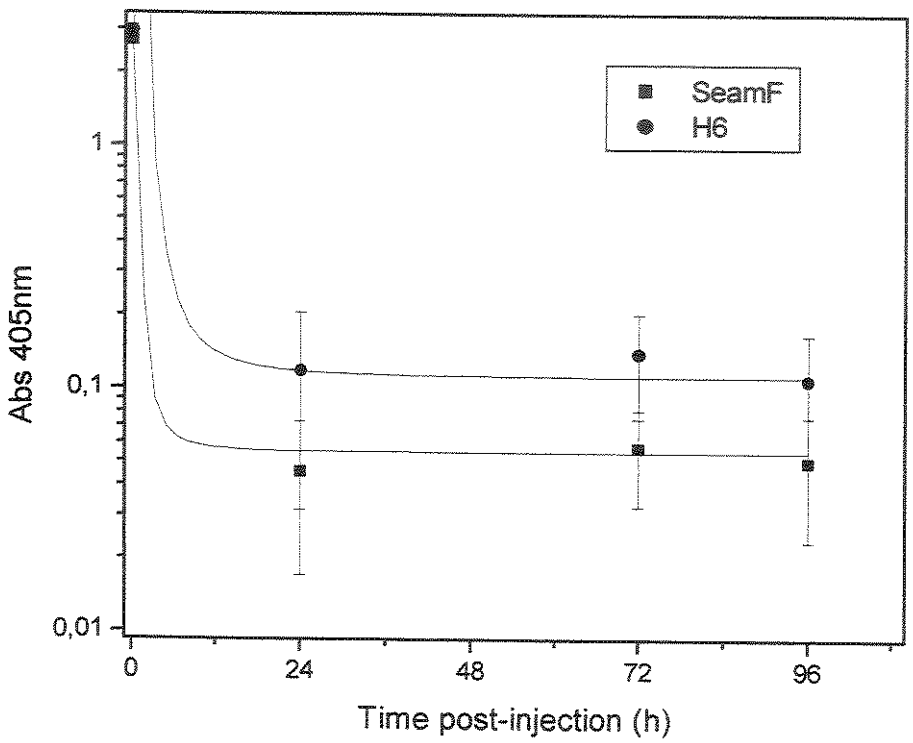

Figure 6. Stability of PLRV-H6 and PLRV-Seam F RTD deletion mutant in microinjected *M. persicae*. Aphids were analysed by ELISA (A_{405}) after the indicated times post-injection. Each point represents the mean (± standard error) of 3 samples from 2 microinjected aphids. Negative controls (unvirouliferous aphids) gave $A_{405} \approx 0,003$.

In spite of the great volume of information generated, contrasting results have been obtained in recent years on the specific roles of the luteovirid RTD. As a

consequence several questions remain unclarified. In summary, our results with a RTD deletion mutant of a PLRV cDNA infectious clone establish this protein as indispensable for virus transmission, association with *Buchnera* GroEL and stability of particles in the hemolymph, corroborating earlier findings on the role of this protein for BWYV and BYDV transmission by *M. persicae*.

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3.DISCUSSÃO GERAL

Baseado nos dados de seqüência apresentados nessa tese, nós confirmamos observações recentes (Vega *et al.*, 1997, Scagliusi *et al.*, 2000) e concluímos que o ScYLV é um novo membro da família *Luteoviridae*. As análises das seqüências deduzidas de aminoácidos das proteínas CP e P17 mostraram que o ScYLV possui alta porcentagem de identidade com o outros membros dessa família, em especial o *Barley yellow dwarf virus* (BYDV), pertencente ao gênero *Luteovirus*. A filogenia baseda na seqüência de aminoácidos da proteína CP também posiciona o ScYLV próximo aos membros do gênero *Luteovirus*. Por outro lado, a filogenia baseada na seqüência de aminoácidos da região C-terminal da RdRp sugere que o ScYLV contém o gene da polimerase mais semelhante aos dos membros do gênero *Polerovirus*.

Esses dados permitem identificar um possível evento de recombinação com o ScYLV, de maneira similar ao que ocorre com o SDV (Rathjen *et al.*, 1994). Porém, o ScYLV representa um caso distinto, uma vez que a porção 3' do seu genoma possui características do gênero *Luteovirus* e a porção 5' do gênero *Polerovirus*, de maneira inversa ao que ocorre com o SDV. De acordo com os critérios mais lógicos para a classificação dentro da família *Luteoviridae*, a similaridade de seqüência na região da polimerase deve ser considerada como prioritária, uma vez que a região 3' do genoma dos membros dessa família é altamente conservada. Com base nesse critério o ScYLV deve ser classificado como uma nova espécie do gênero *Polerovirus*.

Após o depósito das nossas seqüências no GenBank e quase que simultaneamente a publicação do artigo referente ao Capítulo 1, a seqüência

completa e organização genômica de um isolado norte-americano do ScYLV foi publicada por Moonan *et al.* (2000) e Smith *et al.* (2000). A sequência desse isolado difere apenas em dois nucleotídeos das sequências parciais do isolado brasileiro, aqui relatadas. As substituições ocorrem nas posições 4201(G por T) e 4232 (A por C) do genoma do ScYLV, correspondentes respectivamente à região codificadora da proteína P17 e a capa protéica do vírus. A primeira substituição resulta na troca de uma Glicina na seqüência deduzida de aminoácidos do isolado brasileiro, por uma Valina, na seqüência deduzida de aminoácidos do isolado norte-americano. Esses autores identificaram dois possíveis sítios de recombinação de RNA no genoma do ScYLV, os quais coincidem com os sítios de transcrição de RNAs subgenômicos de outros membros da família *Luteoviridae*. Apesar das semelhanças de sequência da RdRp com os polerovirus, há uma tendência para situar o ScYLV com um "unassigned member" (D'arcy and Mayo, 1997) da família *Luteoviridae*, como ocorre com o SDV. Independentemente da classificação filogenética, essas informações vêm confirmar nossa afirmativa de que o ScYLV é um novo membro da família *Luteoviridae*, dividindo características comuns entre os gêneros *Luteovirus* e *Polerovirus*.

No Capítulo 2 é abordado o emprego dos dados de seqüência do isolado brasileiro no desenvolvimento do AmpliDet RNA para o ScYLV. A combinação da técnica NASBA com o uso de molecular beacons permitiu a obtenção de uma ferramenta de diagnóstico altamente específica e sensível para o vírus. O uso dessa técnica oferece inúmeras vantagens, conforme abordado anteriormente, e possibilita a transposição de barreiras freqüentemente encontradas no diagnóstico de fitoviroses. Atualmente, no Brasil, a detecção do ScYLV tem sido especialmente requerida em material vegetal utilizado para o melhoramento genético de cana-de-

açúcar, na busca por fontes de resistência duráveis. Nesse caso, variedades que não apresentam sintomas da doença ou com baixo título de vírus, podem constituir um reservatório viral, escapando à detecção por técnicas menos sensíveis, como ELISA e, conseqüentemente, sem a percepção imediata do melhorista. No caso da introdução de material propagativo, procedimentos de quarentena, ou avaliações epidemiologicas da doença, muitas vezes é necessário um diagnóstico altamente sensível, que possa ser empregado em larga escala. Nesses casos a técnica de RT-PCR vem sendo usada, porém a sua principal limitação consiste no tempo de execução e a necessidade do uso de géis de eletroforese, dificultando a análise de um grande número de amostras. O AmpliDet RNA apresentado aqui, além de ser mais sensível e específico que a técnica de PCR, permite a automação do processo e conseqüentemente a análise de um grande número de amostras simultaneamente.

Por se tratar de uma metodologia nova envolvendo ferramentas complexas de biologia molecular, essa técnica vem ganhando espaço somente nos últimos anos no diagnóstico de fitoviroses. Todavia, o seu potencial de aplicação é bastante vasto. O investimento inicial é relativamente alto, principalmente no que diz respeito ao desenvolvimento do diagnóstico para um determinado patógeno. No caso da cana-de-açúcar, as duas principais viroses ocorrendo no Brasil e em diversos outros países produtores são o ScYLV e o vírus do mosaico da cana, *Sugarcane mosaic vírus* (SMV). O fato do SMV também ser um vírus cujo genoma é constituído por uma molécula de RNA, oferece a possibilidade do desenvolvimento de um sistema múltiplo de detecção para esses dois vírus, denominado "multiplex AmpliDet RNA" (Klerks *et al.*, 2001). Essa técnica consiste na introdução de diferentes sondas com diferentes marcações fluorescentes na reação, possibilitando a detecção simultânea de mais de uma seqüência alvo. Essa constitui uma etapa futura da exploração do

conhecimento adquirido com o AmpliDet RNA desenvolvido nessa tese, estendendo sua aplicação para as duas principais viroses da cana-de-açúcar.

A abordagem dos aspectos moleculares envolvidos na relação entre os membros da família *Luteoviridae* e os seus afídeos vetores tem despertado grande atenção devido ao contundente número de informações obtidas sobre o assunto nos últimos anos. A proteína RTD está fortemente envolvida na associação de partículas do BWYV com a proteína *Buchnera* GroEL encontrada no vetor *M. persicae* e é indispensável para a transmissão do vírus (Brault *et al.*, 1995). A maioria das informações recentes sobre o assunto tem sido obtida ainda com o BWYV e em menor escala com o BYDV, devido a disponibilidade de clones infectivo de cDNA desses vírus, possibilitando a construção de mutantes da proteína de RTD. Recentemente foram usadas partículas semelhantes ao PLRV ou "virus like particles" (VLP), produzidas em um sistema de baculovirus recombinante e desprovidas da proteína RTD para estudar o assunto com esse polerovirus (Gildow *et al.*, 2000). Os resultados encontrados foram controversos, pois as VLPs do PLRV sem a proteína de RTD apresentaram-se estáveis na hemolinfa e foram encontradas nas mesmas regiões dos afídeos que partículas normais do PLRV, mais precisamente em canais e ductos salivares, órgãos considerados uma barreira no processo de transmissão. Uma hipótese explicativa para esse acontecimento provém da possibilidade da cauda artificial de histidinas ("histidine-tag"), ligada à região N-terminal da proteína da capa das VLPs, pudesse estabilizar o capsídeo dessas partículas pela interação com a proteína GroEL, substituindo funcionalmente a proteína RTD (Reinbold *et al.*, 2001). Isso invalidaria a observação de que partículas do PLRV sem a RTD são tão estáveis quanto partículas normais do vírus no corpo do inseto. Convém salientar que devido a incapacidade das VLPs em infectar plantas, não foi testada a sua

transmissão por afídeos. No Capítulo 3 desta tese é relatado o uso de um clone infectivo de cDNA do PLRV para o melhor entendimento da função da proteína RTD na transmissão desse vírus por *M. persicae*. Os testes de transmissão com o mutante deletério da RTD mostraram que a ausência dessa proteína não só impede a transmissão por afídeos dos mutantes microinjetados, como também inviabiliza a associação dessas partículas com a proteína GroEL. Aliada a essas observações, a menor estabilidade dos mutantes deletérios no corpo de *M. persicae* confirma os dados previamente relatados com o BWYV que evidenciam que a proteína de RTD está intimamente ligada ao processo de transmissão. Todavia, esses experimentos não permitiram diferenciar entre a capacidade do afídeo em transmitir o vírus para a planta e a habilidade dessas partículas mutantes em infectar a planta inoculada. Mutações na proteína RTD do BWYV possuem um efeito inibitório no acúmulo de partículas virais em plantas agroinoculadas (Bruyere *et al.*, 1997; Brault *et al.*, 2000), o que levanta a possibilidade que essa proteína também esteja envolvida nas etapas iniciais do processo de infecção da planta hospedeira.

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