PRISCILLA DE SOUSA GERALDINO

ESTUDO GENÔMICO DE DOIS ISOLADOS DE *Potyvirus* E *Carlavirus* E PURIFICAÇÃO DE PROTEÍNAS ENVOLVIDAS NA FORMAÇÃO DE TÚBULOS DE MOVIMENTO POR UM *Comovirus*

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia para a obtenção do título de Doutor.

Orientadora

Profa. Dra Antonia dos Reis Figueira

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RESUMO

Viroses de plantas podem causar perdas consideráveis em diversas culturas e são consideradas um dos maiores desafios na produção em larga escala em diversos países, incluindo o Brasil. Neste trabalho foram estudados dois vírus, o *Potato virus S* (PVS) e o *Soybean yellow shoot virus* (SYMV). Adicionalmente, também foi estudado o mecanismo de movimentação do *Cowpea mosaic virus* (CPMV), utilizado-se protoplastos de caupi (*Vigna unguiculata*). No estudo do PVS, a estirpe andina denominada BB-AND foi completamente sequenciada e analisada. A comparação entre BB-AND e isolados das estirpes comum e andina de PVS mostrou que o BB-AND é diferente, tendo a menor similaridade de aminoácidos sido com a ORF1 (82%) e ORF6 (87%), quando o único isolado de PVS andino do banco de dados foi utilizado na comparação das sequências. A análise de recombinação mostrou que o isolado alemão Vltava (AJ863510) é um recombinante entre a estirpe andina e a comum de PVS, sendo o evento de recombinação entre os nucleotídeos 6125 e 8324. No estudo do SYMV, a região 3'do genoma, incluindo a cauda poli A, 3'UTR, capa proteica e parte das regiões NIb e CI, foi sequenciada e analisada. As sequências de nucleotídeos e aminoácidos revelam que o SYSV é um membro distinto do gênero *Potyvirus* e apresentara uma similaridade de aminoácidos de 44% a 47%, entre a região CI e 29% a 32%, entre região 3' e os demais Potyvirus. A análise filogenética mostrou que o SYMV não permanece no mesmo clado que os demais *Potyvirus* empregados na análise. Quando apenas sequência parcial da NIb foi analisada, o SYSV permaneceu no mesmo clado que o *Glycine virus Y*, um *Potyvirus* descrito na Austrália. A análise do genoma do SYMV deixa clara sua singularidade, indicando ser esse uma espécie de *Potyvirus* diferente de qualquer outra já descrita no Brasil e no mundo. No estudo do envolvimento de proteínas da membrana plasmática do hospedeiro no movimento célula-a-célula do CPMV, as estruturas de movimento (túbulos) foram purificadas e analisadas em espectrômetro de massa. Além das proteínas virais, 19 proteínas do hospedeiro foram identificadas e caracterizadas como pertencentes a 11 grupos diferentes de proteínas conservadas. A identificação destas novas proteínas pode ajudar no entendimento do processo de transporte viral, ainda pouco conhecido para a maioria das famílias virais.

ABSTRACT

Plant viruses cause considerable losses in several crops and are considered one of the biggest challenges in large-scale production in all countries. In this work we described two viruses isolate, *Potato virus S* (PVS) and *Soybean yellow shoot virus* (SYMV). In addition, in this work was studied the movement mechanism of *Cowpea mosaic virus* (CPMV) using cowpea protoplasts (*Vigna unguiculata*). For the PVS analysis, the Andean strain called BB-DNA was completely sequenced and analyzed. The comparison among BB-AND and other Andean and common PVS isolates showed that the genome of this isolate is quite distinct. The lowest amino acid identity with the only other fully sequenced Andean isolate was in ORF 1 (82%) and ORF 6 (87%), which code for RdRp and 11K, respectively. Recombination analysis, including ordinary and Andean isolates, was also performed and showed that the isolate Vltava (AJ863510), from Germany, is a recombinant between PVS^{O} and PVS^{A} isolates, with the recombination event located between the nucleotides 6125 and 8324. In the study of SYMV, the 3'end of the genome was sequenced and analyzed and corresponds to the poly A tail, 3'UTR, coat protein and part of the NIb and CI regions. The nucleotide and amino acid sequences reveal that the SYMV is new member of the genus *Potyvirus* and showed an identity of amino acids ranging from 44 to 47% CI and from 29 to 32% between the 3'end of other *Potyvirus* from Gen BanK. Phylogenetic analysis showed that the SYMV did not remain in the same clade as other *Potyvirus* employed in the analysis. When only the partial NIb sequence was analyzed, the SYSV remained in the same clade *Glycine virus Y*, a potyvirus described in Australia. To study the involvement of host plasma membrane proteins the virus cell to cell movement, the movement structures (tubules) of CPMV were purified and analyzed in a mass spectrometer. In addition to viral proteins, 19 protein from the host were identified and characterized as belonging to 11 different groups of conserved proteins. The identification of these new proteins may help the understanding of the viral movement process.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Viroses de plantas constituem um dos maiores desafios na produção em larga escala de alimentos, biocombustíveis e derivados, em todo o mundo. O fato de os vírus utilizarem as mesmas organelas, rotas metabólicas e mecanismos genéticos que as plantas empregam na sua divisão e multiplicação celular faz com que o controle curativo desses patógenos seja impossível, pois qualquer interferência na sua replicação afeta também a sua hospedeira.

As perdas causadas por vírus em plantas variam com o vírus, a planta e as condições ambientais, podendo chegar a 100% (ALMEIDA et al., 2005; KAWUKI; ADIPALA; TUKAMUHABWA, 2003). Os métodos de controle são, principalmente, de caráter preventivo, direcionados, principalmente, a evitar sua introdução e disseminação no campo e a utilizar plantas portadoras de algum tipo de resistência (STEINLAGE; HILL; NUTTER JUNIOR, 2002). Esses métodos têm uma eficiência limitada, uma vez que nem sempre é possível evitar a sua introdução no campo, principalmente via vetores e, uma vez presente, nem sempre as medidas para evitar a sua disseminação são efetivas. O uso de plantas resistentes tem como limitação a grande variabilidade genética que os vírus podem apresentar. Assim sendo, conhecer as estirpes e os variantes genéticos que ocorrem no campo é fundamental para embasar os programas de melhoramento, visando à obtenção de plantas resistentes a doenças.

Atualmente, com a disponibilidade de técnicas moleculares que permitem o sequenciamento dos genomas virais, diversas estirpes e variantes genéticos têm sido descritos (CHEN et al., 2008; GONG et al., 2011; MONGER et al., 2007; MOREIRA; KITAJIMA; REZENDE, 2010; ORILIO et al., 2009). Relatos de grandes prejuízos econômicos, causados por novos vírus que ainda não foram descritos ou ainda por novas estirpes, são cada vez mais frequentes.

Um exemplo relevante é o do *Potato virus Y* (PVY), do qual novas estirpes têm sido descobertas com frequência, após a intensificação nas investigações envolvendo o sequenciamento e a caracterização dos isolados encontrados na cultura da batata (GALVINO-COSTA et al., 2010).

Os vírus são capazes de cruzar as barreiras entre as espécies, permitindo a infecção de novos hospedeiros. A replicação contínua possibilita a produção rápida de diversidade genética, incluindo as mutações que facilitem a adaptação ao hospedeiro. Portanto, estudos de caracterização de isolados e análise genômica permitem acompanhar esta evolução viral, bem como compreender alguns aspectos da interação vírus-planta. O surgimento de novas espécies virais é resultante dessa interação, sem a qual o sucesso da infecção viral e translocação de partículas virais para todas as partes da planta não é possível. Por exemplo, uma infecção bem sucedida exige um transporte de partículas virais célula-a-célula eficiente (SCHOELZ; HARRIES; NELSON, 2011).

Neste trabalho, foram caracterizados dois novos vírus encontrados no Brasil, que apresentam características singulares e com potencial de indução de grandes perdas de produtividade, quando comparados com os isolados de vírus do mesmo gênero ou espécie já descritos. O primeiro foi descrito como um isolado pertencente à estirpe andina do *Potato virus S* (PVS), pela primeira vez identificado no Brasil, e o primeiro da América do Sul (centro de origem da estirpe) a ser sequenciado. O sequenciamento deste isolado possibilitou o estudo de recombinação entre as duas estirpes de PVS. O segundo isolado de vírus estudado neste trabalho foi identificado como sendo uma nova espécie do gênero *Potyvirus*, apresentando características moleculares únicas na região 3' do genoma, ainda não descritas no Brasil e no mundo.

Além do estudo de novos isolados de vírus de plantas, foi desenvolvido um protocolo para o isolamento dos túbulos de movimento formados pelo *Cowpea mosaic virus*, em protoplastos de *Vigna unguiculata*. Foram também

identificadas proteínas da membrana plasmática do hospedeiro nessas estruturas tubulares que, possivelmente, estariam envolvidas no transporte do vírus célula-a-célula.

2 REFERENCIAL TEÓRICO

2.1 Caracterização molecular de isolados virais e de sua translocação célula-a-célula

O uso de plantas resistentes aos fitovírus tem sido o método mais desejável, principalmente nesse momento em que a produção sustentável tem sido uma exigência universal (STEINLAGE; HILL; NUTTER JUNIOR, 2002). Além de diminuir o custo de produção, dispensando o uso adicional de medidas de controle, como gastos com defensivos agrícolas, evita a poluição do meio ambiente. Entretanto, a variabilidade genética e a capacidade que esses patógenos têm de superar a resistência da planta hospedeira têm constituído um grande desafio para os melhoristas de plantas. Assim, a caracterização molecular e a correta classificação dos vírus em gêneros e estirpes genéticas são indispensáveis para garantir uma solução que, ainda que não seja definitiva, seja o mais duradoura possível (GONG et al., 2011; MOREIRA; KITAJIMA; REZENDE, 2010).

Outro aspecto que deve ser considerado nas abordagens que visam o controle de doenças viróticas é a interação do vírus com a célula hospedeira e a sua capacidade de translocação de uma célula para outra, na planta, possibilitando a invasão sistêmica da mesma. Estudos detalhados desse mecanismo demonstraram que a maioria dos vírus codifica uma ou mais proteínas de movimento (MP) que facilitam o transporte do vírus por meio dos plasmodesmata (CARRINGTON et al., 1996; LAZAROWITZ; BEACHY, 1999). Como o limite de exclusão dos plasmodesmas é insuficiente para a passagem de vírions ou mesmo do genoma viral desencapsulado, eles precisam

desenvolver um mecanismo para modificar esses canais e permitir o seu transporte. A amplitude dessa modificação vai depender do tamanho do vírus ou do mecanismo de translocação, envolvendo apenas o genoma ou o vírion completo.

No caso do *Tobacco mosaic virus* (TMV), em que apenas o seu ácido nucleico é translocado, ocorre um aumento do limite de exclusão do plasmodesma com mudanças morfológicas consideráveis. Entretanto, outros vírus, como as espécies de *comovirus*, cuja translocação é na forma de vírion intacto, induzem uma completa transformação estrutural do plasmodesma, formando túbulos de transporte que se projetam de uma célula para outra. Compreender melhor esse mecanismo faz parte da estratégia necessária para idealizar a construção de plantas resistentes, notadamente as transgênicas.

2.1.1 *Potato virus S* –PVS

O *Potato virus S* (PVS) pertence ao gênero *Carlavirus*, família *Betaflexiviridae* e possui partícula flexuosa com, aproximadamente, 650 nm de comprimento por 12 nm de diâmetro, composta de uma fita simples de RNA positivo (ssRNA), com cerca de 8,5 Kb (MATOUSEK et al., 2005; MONIS; ZOETEN, 1990; WETTER, 1971). Possui uma região não codificadora nos primeiros 62 nt, seguida por 6 ORFs (do inglês *open reading frames*), sendo a primeira do nucleotídeo 63 ao 5983, codificando a proteína de replicação característica para os *Carlavirus*, com domínios para metiltransferase, helicase e RNA polimerase dependente de RNA (RdRp) (MATOUSEK et al., 2005). Em seguida, localiza-se um bloco com 3 ORFs, denominado de bloco triplo, na região do nucleotídeo 5970 ao7169, que codifica as proteínas 25K, 12K e 7K, envolvidas no movimento do vírus célula-à-célula (FOSTER, 1992). A quinta ORF está localizada entre os nucleotídeos 7211 a 8095 com, aproximadamente, 34K, e codifica a capa proteica. Finalmente, na região 3’ se encontra a última

ORF, que codifica uma proteína com aproximadamente 11K, seguida por uma cauda poliA (MACKENZIE; TREMAINE; STACE-SMITH, 1989). Acredita-se que a proteína 11K dos *Carlavirus* poderia atuar de maneira similar à Hc-Pro dos *Potyvirus*, que atua na transmissão por afídeos (FOSTER; MILLS, 1990).

O PVS é considerado um dos vírus mais comuns nos campos de cultivo de batata, em outras partes do mundo (CHIKH; MAOKA; NATSUAKI, 2008; COX; JONES, 2010; HINOSTROZA-ORIHHUELA, 1973; SALARI et al., 2011). Os sintomas induzidos por este vírus variam desde latentes à forte rugosidade, mosaico, bronzeamento dependendo da cultivar e da estirpe do vírus, porém, para a maioria das cultivares, os sintomas são latentes, dificultando a sua detecção no campo.

Duas estirpes são descritas para o PVS: a estirpe comum (PVS^{O}) e a estirpe Andina (PVS^{A}). A PVS^{A} difere da PVS^{O} principalmente por ser mais facilmente transmitida por afídeos (SLACK, 1983) e por induzir sintomas sistêmicos em *Chenopodium* spp. (CHIKH; MAOKA; NATSUAKI, 2008; DOLBY; JONES, 1987; FLETCHER, 1996; FOSTER, 1992; HINOSTROZA-ORIHUELA, 1973; ROSE, 1983; SLACK, 1983), o que não ocorre com a estirpe comum. Além disso, a estirpe Andina pode alcançar concentrações mais altas que PVS^{O} e sintomas secundários mais fortes, como necrose em plantas de batata (DOLBY; JONES, 1987; ROSE, 1983).

Recentemente, tem sido proposto um novo grupo de PVS, denominado $PVS^{O\text{-}CS}$. Estes variantes, ao serem estudados, apresentaram sintomas que os levaram a ser classificados como pertencentes à estirpe andina e características moleculares que os agrupavam com isolados de PVS^{O} (MATOUSEK et al., 2005). Tendo em vista esta variação entre isolados de PVS, em trabalhos recentes tem sido demonstrada a existência de diferentes genótipos de PVS (COX; JONES, 2010; SALARI et al., 2011). A diferença das propriedades biológicas entre as estirpes de PVS tem sido atribuída a blocos de aminoácidos

no N- terminal da capa proteica e nas proteínas 11K e 7K (FOSTER, 1991; FOSTER; MILLS, 1992; MATOUSEK et al., 2000).

Até 2008, apenas o PVS^{O} era detectado em campos de cultivo de batata do Brasil, porém, Ribeiro e Figueira (2008), ao realizar estudos de diversos isolados provenientes de diferentes regiões produtoras do país, detectaram, pela primeira vez, a presença do PVS^{A} em campos de cultivo brasileiro. Estudos da capa proteica deste isolado mostraram que ele tinha características moleculares diferentes das demais descritas no Brasil e no mundo. A capa proteica deste isolado apresentou características semelhantes às de outro isolado detectado em *Solanum phureja* proveniente da região andina (FIGUEIRA et al., 2008).

Geraldino (2009) iniciou a caracterização molecular deste isolado andino sequenciando a região 3' e parte da ORF1 e, mesmo com o genoma incompleto, observou-se que o isolado brasileiro pertencia à estirpe Andina, porém, mostrando clara distinção dos demais isolados disponíveis no GenBank.

2.1.2 *Soybean yellow shoot virus* – SYSV

O vírus do amarelo do broto da soja (SYSV) foi detectado em Lavras, MG, na estação experimental da EPAMIG, em 1984. Os sintomas induzidos pelo SYSV iniciam-se com mosaico, clareamento de nervuras, evoluindo até os sintomas mais característicos, como amarelecimento e encrespamento dos brotos. Além disso, ocorre paralização do crescimento dos ponteiros, causando enfezamento da planta, que apresenta encurtamento de entrenós e superbrotamento (DESLANDES; COSTA; FIGUEIRA, 1984; FIGUEIRA; COSTA; REIS, 1987). Santos (2000) detectou que o SYSV também afeta as sementes, causando perdas consideráveis, devido a manchas e deformação.

O SYSV possui uma ampla gama de hospedeira e lesões locais induzidas em mamoeiro e *Chenopodium quinoa* são características deste vírus. Em estudo realizado por Deslandes, Costa e Figueira (1984) foi demonstrado

que se tratava de um *Potyvirus* ainda não descrito no Brasil e no mundo. Na análise de tecido infectado foram identificadas partículas de 750 a 780 nm de comprimento (DESLANDES; COSTA; FIGUEIRA, 1984; FIGUEIRA; ALVES; KITAJIMA, 1991) e, juntamente com as inclusões lamelares, apenas reforçam o fato de o SYSV ser descrito como novo *Potyvirus*.

Pouco ainda se sabe sobre o SYSV, porém, em estudos sorológicos foi demonstrado que o SYSV é relacionado com estirpes de *Potyvirus* do maracujá, canavalia, mas não com BCMV e SMV (FIGUEIRA; ALVES; KITAJIMA, 1991). O gênero *Potyvirus* pertence à maior família dos vírus de planta, a família *Potyviridade*.

2.1.3 Cowpea mosaic virus (CPMV) e movimento célula-a-célula

O CPMV é membro da família *Comoviridae*, gênero *Comovirus*. Possui um ssRNA+ bipartido encapsidado separadamente, o RNA1 e o RNA2, e tem sido considerado vírus modelo no movimento célula-a-célula túbulo guiado. O RNA1 codifica as proteínas envolvidas na replicase, enquanto o RNA2 codifica as duas subunidades da capa proteica (maior-L e menor-S), a proteína de movimento 48K e o cofator 58K envolvido na replicação do RNA2 (MUPHY et al., 1995).

Dois principais mecanismos para transporte viral entre células têm sido descritos. No primeiro, o genoma viral é transportado sem a capa proteica, sendo o principal exemplo o TMV (WAIGMANN et al., 1994) e, no segundo tipo, partículas virais maduras são transportadas célula-a-célula. Nas células infectadas com o CPMV, há a formação de longas estruturas tubulares que atravessam o plasmodesmata, levando as partículas virais de uma célula infectada para uma célula sadia (LENT; WELLINK; GOLDBACH, 1990). Estes túbulos são formados também em protoplastos. Neste caso, os túbulos são formados na ausência do plasmodesmata e crescem junto à membrana

plasmática celular (LENT; WELLINK; GOLDBACH, 1990). A membrana plasmática da célula do hospedeiro envolve os túbulos e pesquisadores têm sugerido o envolvimento de proteínas da membrana no processo de formação dos túbulos (POUWELS et al., 2004).

A proteína de movimento é o único componente dos túbulos (LENT; WELLINK; GOLDBACH, 1990). O C- terminal desta proteína é essencial na inclusão das partículas virais, durante a formação dos túbulos de movimento; mutações nesta região demonstram que os túbulos são formados, porém, vazios, sem partículas virais. As regiões central e N- terminal da proteína de movimento estão envolvidas na formação dos túbulos (GOPINATH et al., 2000; LEKKERKERKER et al., 1996), que mostram que a proteína de movimento sozinha pode induzir a formação dos túbulos e nem mesmo a capa proteica é necessária durante o processo.

No movimento célula-a-célula túbulo guiado, as células do hospedeiro são drasticamente modificadas. No plasmodesmata de uma célula infectada ocorre a remoção dos desmotúbulos (extensões tubulares do retículo endoplasmático) e há a montagem dos túbulos dentro do plasmodesma pelo qual vírions são transportados, aumentando significativamente o limite de exclusão dos plasmodesmas. A maioria dos vírus tem forma icosaédrica, mas a forma não afeta a formação dos túbulos ou o transporte de vírions (CHENG et al., 1998; STORMS et al., 1995).

O envolvimento de proteínas do hospedeiro no processo de formação de túbulos ainda é pouco conhecido, porém, fica claro, durante todo o processo, que a participação do hospedeiro é essencial. Em alguns trabalhos, como o de Carvalho (2003) foram identificadas algumas proteínas de membrana, assim como as subunidades H, D e E da v-ATPase e aquaporina.

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SEGUNDA PARTE – ARTIGO CIENTIFICOS

ARTIGO 1: Complete genome sequence of the first Andean strain of *Potato virus S* from Brazil and evidence of recombination between PVS strains

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Complete genome sequence of the first Andean strain of *Potato virus S* from Brazil and evidence of recombination between PVS strains

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ABSTRACT

An isolate of the Andean strain of *Potato virus S* (PVS), named BB-AND, was detected for the first time in a Brazilian potato crop, fully sequenced and analyzed. The comparison among BB-AND and other Andean and common PVS isolates showed that the genome of this isolate is quite distinct, being usually grouped in the same clade as the other Andean isolates but always in different branches. The lowest amino acid identity with the only other fully sequenced Andean isolate was in ORF 1 (82%) and ORF 6 (87%), which code for RdRp and 11K, respectively. BB-AND is the first Andean isolate from South America to be completely sequenced. Recombination analysis, including ordinary and Andean isolates, was also performed and showed, for the first time, that an isolate named Vltava (AJ863510), from Germany, is a recombinant between PVS^{O} and PVS^{A} isolates, with the recombination event located between the nucleotides 6125 and 8324.

Introduction

Potato virus S (PVS) belongs to the genus *Carlavirus* within the family *Betaflexiviridae,* and it consists of flexuous particles, approximately 610-700 nm in length, containing a single-stranded, positive-sense RNA genome of approximately 8400 nucleotides, a cap structure at the 5' terminus, and a poly-A tail at the 3' terminus [2,17]. The genome contains six ORFs: ORF1 encodes a

putative replication protein (RPT) as well as characteristic motifs for *Carlavirus*, such as methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRp) [31]; ORFs 2, 3 and 4 form the triple gene block encoding 25K, 12K and 7K, respectively, which have been shown to be involved in cell-to-cell movement [35]; ORFs 5 and 6 encode coat protein and 11K protein, respectively. There is some evidence that ORF 6 may be involved in the transmission of *Carlaviruses* by aphids [16,17].

Two PVS strains are known: the ordinary strain (PVS^{O}) and the Andean strain (PVS^{A}). The main difference between the two strains is based on the non-systemic or systemic infection in *Chenopodium* ssp, respectively. The Andean strain was first described by Hinostroza-Orihuela [22], who observed its ability to induce systemic infection in plants of the species *Chenopodium quinoa*, instead of the local lesions induced in those plants by PVS^{O}. Later, the transmission of PVS by aphids was described, and it was reported that PVS^{A} was more readily transmissible by contact and aphids [38,41,46]. However, Mautosek et al. [31] suggested the existence of potentially divergent variants within the Central European PVS isolates (PVS^{CS}) that systemically infected *Chenopodium quinoa*. The PVS^{CS} isolates analyzed were more closely related to the PVS^{O} European isolates but were distant from the original PVS^{A} partially sequenced by Mackenzie et al. [30]. Recently, Cox and Jones [11] suggested that the biological characterization did not combine with the genetic features of

PVS^{A}. Therefore, the acronym $PVS^{O\text{-}CS}$ was proposed to be used for isolates that systemically infect *Chenopodium* ssp. but are not within the clade PVS^{A}. For many plant viruses, this variability was associated with RNA recombination, with typical strains of the virus generating uncharacterized virus genotypes [20,37,40,45].

In Brazil, PVS is widespread in potato crops and has been found in low incidence. Its indexation in seed potatoes is intended to prevent the introduction of imported potato seeds with higher incidences, preventing unwanted losses. Until recently, PVS^{O} was the only strain of PVS detected in Brazilian potato fields. In 2008, PVS^{A} was detected for the first time in Brazil, and preliminary studies of the coat protein showed that this isolate, called BB-AND, had different molecular characteristics when compared with the other Andean isolates described in other parts of the world. The presence of PVS^{A} in Brazilian crops is worrying, considering its transmissibility by aphids and the high population of vectors in Brazilian fields all year long, which could facilitate its spread in the potato fields. The losses could become worse with the co-infection of PVS and other viruses, such as *Potato virus Y* (PVY) and *Potato virus X* (PVX), that further reduce the yield by 10-20% [28,47,48].

The studies have shown a great variability among the CP genes of PVS isolates. The variability among the strains of PVS has been attributed to differences between the blocks of amino acids located at the N-terminal of the

coat protein and in the 11K and 7K protein sequences [16, 17, 32]. Additional information about the 5' region of the PVS genome is scarce, and only four complete nucleotide sequences of PVS isolates have been published. Three of them are PVS^O: Leona [31] from Germany (access nº AJ863509), and Id4106 (access nº FJ813513) and WaDef (access nº FJ813512) from the United States [27]. The isolate Vltava (access nº AJ863510), from the Czech Republic, is the only complete sequence of PVS^A [31].

To better understand the differences among PVS isolates, it would be useful if a higher number of complete PVS^A sequences were known. In this work, the efficiency of aphid transmission of BB-AND was determined to evaluate its potential risk for Brazilian potato fields. Furthermore, the genome of this isolate was fully sequenced, analyzed and compared with other PVS isolates from GenBank. Finally, the genome recombination potential was analyzed, using the PVS^O (Id4106, WaDef and Leona) and PVS^A (Vltava and D00461) isolates.

Materials and methods

Virus isolates

The BB-AND isolate was detected in 2008 in Bueno Brandão, Minas Gerais state, Brazil, in plants that originated from potato seeds imported from Chile. The isolate was maintained and multiplied in plants of *Chenopodium*

quinoa, *C. amaranticolor* and potato plants/tubers, either in greenhouses or dried in silica gel and stored at -20°C. The infected tubers were stored at -8ºC for planting when necessary.

For comparison with the BB-AND isolate, the following complete sequences of PVS isolates were used: isolate Id4106-US (FJ813513) and isolate WaDef-US (FJ813512), both from the United States, isolate Leona (AJ863509) from Germany, and isolate Vltava (AJ863510) from the Czech Republic. Additionally, the following incomplete sequences were used: the ordinary isolates AJ889246 from China, DQ000234 and DQ786653 from India and Y15625 from the Czech Republic; and the Andean isolates AF493951 (unknown origin), DQ000231 from the Czech Republic, and D00461 from Peru. A Brazilian isolate of *Potato virus P* (EU338239) was used as outgroup for the phylogenetic analysis.

Aphid transmission

To investigate aphid transmission, plants of *Chenophodium quinoa* and potato cv. Monalisa were chosen as the virus hosts. Two species of aphids commonly found in the potato field were tested, *Myzus persicae* Sulz. and *Aphis gossypii* Glover. After a starving period of 3 hours, the aphids were settled for 30 min on infected leaves to allow virus acquisition. Next, 10 aphids were transferred to healthy *C. quinoa* plants at the 4 leaf stage and left for 10 to 12

hours. Three weeks after inoculation, the virus infection was detected by DAS-ELISA. The experiments were performed in triplicate, using 10 plants per test.

***Primer* design**

The primers for the genome amplification were designed, based on the alignment of the complete sequences of the PVS^{A} isolates available in GenBank, to amplify fragments of approximately 1-1.5 kbp, with an overlap of approximately 100 bp between each fragment [9], each fragment obtained were sequenced three times to obtain the consensus sequence. The 3' terminal was obtained using an oligo-dT primer and the 5'terminal was obtained using primers designed based on this region for other isolates. The nucleotide positions and the annealing positions of primers are indicated in Table1.

Table 1 Various internal primers designed for the determination of the genomic RNA of the BB-AND isolate

Primer	Sequence (5'-3')	Genome Position (nt)	Gene
PVSF1	GATAAACACTCCCGAAAATAA	1-21	UTR
PVSR493	CCATGGTGCCGCTTGAGTTCG	472-493	ORF1
PVSF430	GGTATGTGAGCAGTGCCG	430-448	ORF1
PVSR1949	CGACCATCGTGCCCCC	1933-1949	ORF1
PVSR2910	CGATGATGGCCTCCT	2896-2910	ORF1
PVSF2811	CGAGGATTGCAACAG	2811-2825	ORF1
PVSR3780	TTTCTTCAGTAGCGCTCT	3766-3780	ORF1
PVSF3664	CCCCAAGGAAGCATT	3664-3678	ORF1
PVSF4532	GGGTGATCCGTGGTT	4532-4546	ORF1
PVSR5512	GCCTCACCRGAGAAG	5498-5512	ORF1
PVSF5413	GGGCYTGCCCAATGA	5413-5427	ORF1
PVSR6373	TGCCAAAGCGATGGC	6359-6373	ORF2
PVSF7955	CGCTCACAAGAGCATGGC	7955-7972	ORF5
PVSR8094	CGTTCCGCTTTCATTGG	8078-8094	ORF5
PVSR8464	ATGCTAAAATATTTTAAAAAC	8444-8464	UTR

Y= T or C; R = G or A

RNA extraction, RT-PCR and sequencing

Total RNA was extracted from symptomatic leaves of *Chenopodium quinoa* using the method described by Chang et al. [7]. Two different steps were performed for the RT-PCR reactions, with M-MLV and GoTaq® Flexi DNA Polymerase (Promega, Madison, WI), both used according to the manufacturer's instructions. The cDNAs were synthesized using the reverse primers, and the reaction was incubated at 42ºC for 60 min, then at 95ºC for 5 min and, finally,

on ice for at least 5 min. Then, for the PCR, the thermocycling conditions were 1 cycle at 95ºC for 2 min followed by 30 cycles at 95°C for 40 s, 42-54°C for 55-60 s and 72°C for 1 min per 1 kb, depending on the fragment size. The PCR products were analyzed in 0.7% agarose gel, and the bands with the correct size were purified with a GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Amersham Biosciences) according to the manufacturer's instructions. The fragments were then cloned into a pGEM-T Easy Vector (Promega Corp. Madison, WI, USA) for sequencing. The resulting recombinant plasmids were checked for insert presence by digestion with EcoRI or NotI (Invitrogen Carlsbad, CA). The clones were sequenced by Macrogen Inc, Seoul, using walking primers for fragments longer than 1,000 bp.

Sequence analysis

The assembly of the genomic sequence was performed using BioEdit (ver. 7.0.90), NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the identification of the coding regions was carried out with the Open Reading Frame (ORF) finder program provided by the NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide and protein sequence alignments were performed using the CLUSTAL W program (ver. 2.0). The phylogenetic analysis and phylogenetic trees were constructed using the neighbor-joining algorithm for amino acids and the UPGMA algorithm for

nucleotides, with bootstrap values determined by 2,000 replicates in the MEGA 4.0 software package [11, 43]. The detection of potential recombinant sequences, the identification of likely parental sequences, and the localization of possible recombination break points were determined using the recombination detection program RDP3 [29], using the default parameters for all of the programs implemented (RDP, BOOTSCAN, MAXCHI, GENECONV, 3SEQ, SISCAN and CHIMAERA)

Results

Aphid transmission

The results of the analysis of BB-AND transmission by aphids are shown in Table 2. Both *M. persicae* and *A. gossypii* transmitted this isolate from infected potato plants and/or *C. quinoa* to uninfected plants of the same species. When *C. quinoa* was used as the source of inoculum, the aphid *M. persicae* transmitted the virus to 46.6% of the *C. quinoa* plants and 20% of the potato plants inoculated (Table 2).

Table 2 The transmission rate of BB-AND by two different species of aphids, *Myzus persicae* and *Aphis gossypii*.

Plant tested source of inoculum	Number of inoculated plants (INO)/Number of infected plants (INF) and percentage of infection (%) in host plants inoculated by two different species of aphids.							
	Myzus persicae				***Aphis gossypii***			
	Potato		*Chenopodium quinoa*		Potato		*Chenopodium quinoa*	
	INO/INF	%	INO/INF	%	INO/INF	%	INO/INF	%
C. quinoa	30/06	20.0	30/14	46.6	30/01	3.3	30/04	13.3
Potato	30/03	10.0	30/07	11.6	30/0	0	30/01	3.3

Maximum temperature of 28ºC and minimum temperature of 13ºC

However, when the potato plants were used as the source of inoculum, the percentages of transmission were lower: 11.6% and 10% for *C. quinoa* and potato plants, respectively. The aphid *A. gossypii* showed a lower efficiency, transmitting the virus to 13.3% of *C. quinoa* and 3.3% of inoculated potato plants when *C. quinoa* was used as the source of inoculum. Using potato plants as the source of inoculum, this aphid was not able to transmit the virus to potato plants and transmitted the virus to only 3.3% of the *C. quinoa*.

The organization and genomic structure of BB-AND isolate

The genome of the BB-AND isolate was 8,485 nucleotides (nt) long, excluding the 3' poly-A tail, organized into six putative ORFs and 5'- and 3'-

untranslated regions (UTRs) of 61 and 102 nt, respectively. The genomic structure and organization were similar to those of the PVS genomes already sequenced: ORF1 overlapped ORF2 by 13 nt and was preceded by the 5' UTR; ORF2 overlapped ORF3 by 22 nt and ORF4 overlapped ORF5 by 42 nt. Only 3 nt were shared by ORF5 and ORF6. The number of nucleotides in BB-AND was the same as in the ordinary isolates WaDef-US (FJ813512) and Id4106-US (FJ813513), from the United States; however, compared with two isolates from Central Europe that have been described by Matoušek *et al.* [31], it was 21nt longer than the Andean isolate Vltava (AJ863510) and 7 nucleotides longer than the ordinary isolate Leona (AJ863509). This sequence variability of Andean and ordinary PVS genomes has been reported by Matoušek *et al*. [32], based on a detailed characterization of the 3' ends of the genomes of several Central European PVS isolates.

Analyses of the nucleotide and deduced amino acid sequences

The genome sequence of BB-AND showed a nucleotide identity ranging from 79 to 81% compared with four previously described isolates (Leona-GE, Vltava-CZ, WaDef-US and Id4106-US). The comparison among these four isolates from Genbank showed greater similarity, and the identity was between 90 and 97% (Table 3). The degree of nucleotide sequence conservation in the individual ORFs, between BB-AND and the other isolates used in the

comparison, follows the decreasing order ORF 2 > ORF 3 > ORF 4 > ORF 6 > ORF 5 > ORF 1, and the most variable among the ORFs was ORF1. Compared with the PVS isolates from GenBank, the nucleotide identity of the 5' UTR region was 98%, showing a high degree of conservation, and the nucleotide identity of the 3' UTR was slightly lower, ranging from 83% to 96%.

ORF1 of the BB-AND isolate consisted of 5925 nt and coded for a protein with 1975 aa. This ORF shared 78% nucleotide identity with the isolates Vltava-CZ, WaDef-US, and Id4106-US and 77% with Leona-GE (Table 3). The comparison among the isolates from GenBank showed an identity ranging from 93% to 98%.

Table 3 Comparison of nucleotide (nt) and amino acid (aa) sequence identities (%) in the individual open reading frames (ORFs) between the BB-AND isolate and other PVS isolates from GenBank.

Isolate	ORF1		ORF2		ORF3		ORF4		ORF5		ORF6	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
AF493951 [A]	-	-	-	-	-	-	-	-	84	93	-	-
AJ863509 [O]	77	83	85	95	83	94	82	90	79	92	81	84
AJ863510 [A]	78	82	90	94	89	98	90	90	88	95	89	87
AJ889246 [O]	-	-	-	-	85	97	84	89	80	93	81	84
D00461 [A]	-	-	90	95	89	98	90	90	88	95	89	87
DQ000231 [A]	-	-	-	-	-	-	-	-	-	-	81	84
DQ000234 [O]	-	-	-	-	84	96	82	90	-	-	-	-
DQ786653 [O]	-	-	-	-	-	-	-	-	81	93	-	-
FJ813512 [O]	78	84	86	95	82	95	83	89	81	92	80	79
FJ813513 [O]	78	85	86	94	82	95	84	90	80	93	80	80
Y15625 [O]	-	-	86	85	83	96	81	87	81	93	80	84

[O] – indicates Ordinary isolates and [A] – indicates Andean isolates

The ORF1 amino acid sequence analysis showed conserved regions corresponding to methyltranferase (MTR), NTP-binding helicase (HEL), RNA-dependent RNA polymerase (RdRp), the typical motifs $G_{1181}X_{1182}G_{1183}K_{1184}S_{1185}$, within the HEL domain, and the putative $G_{1851}D_{1852}D_{1853}$ (GDD) motif, conserved among most positive-stranded RNA viruses [27]. The amino acid sequence identity between BB-AND and both American and European isolates ranged from 82 to 85% (Table 3). It was lower than the amino acid sequence identity seen among the isolates from GenBank, which ranged from 92% to 98%. In ORF1, the lowest identity was found in the region between amino acids 435 and 889, and the highest similarity was observed in the 5' and 3' ends. BB-AND presented a specific block of amino acids in ORF1, from amino acid 1473 to 1491, with one deletion at position 1486 (Figure 1). The isolates Leona and Vltava contain this conserved block in another position, from amino acid 1208 to 1235, with one deletion in position 1206.

```
FJ813513        VTRLYQNRVLGRFLLKTARIDDLKMLLPGRPCFKEGFGGERIGADEGKREFKLEGDPWLK 1500
FJ813512        VTRLYQNRVLGRFLLKTARIDDLKLLLPGRPCFKEGFGGERIGADEGKREFKLEGDPWLK 1500
AJ863510        VTRLYQNRVLGRFLLKTAKIDDLKMLLPGRPRFKEGFGGERIGADEGKREFKLEGDPWLK 1496
AJ863509        VTRLYQNRVLGRFLLKTAKIDDLKMLLPGRPRFKEGFGGERIGADEGKREFKLEGDPWLK 1498
BB-AND          VTRLYQNRALGRFLLRTARVEDLKSLLPGRPNFKRVSKVKELGQMR-EEGIQLEGDPWLK 1499
                ********.******:**:::*** ****** **.    :.:*  . :. ::********
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Fig.1 Alignment of amino acid sequences corresponding to ORF1 from the four isolates of PVS that have been completely sequenced. The region highlighted represents the block of specific amino acids for BB-AND.

ORF2 encode a 243-aa protein and corresponds to the first ORF of the Triple Gene Block (TGB), which has been shown to be responsible for viruses'

cell-to-cell and long-distance movement [33, 34]. Its nucleotide sequence analysis showed identity ranging from 85% to 90% with the other genomes available in GenBank (Table 3). The lowest identity was observed between BB-AND and Leona-GE (85%), and the highest was shared with the isolates Vltava-CZ and D00461 (90%). The isolate Vltava-CZ shared 99% identity in the same genome region compared with the known Andean isolate D00461 and 86% with the ordinary isolate Kobra (Y15625). The amino acid sequence analysis of ORF2 of BB-AND showed that not all changes in the nucleotide sequences generate amino acid mutations, resulting in silent mutations [36], and that the range of similarity was from 94% to 95%. The positions of two amino acids were characteristic in the sequence of PVS^{A} isolates: the D at position 168 of the PVS^{O} isolates was replaced by an E, and the G at position 212 was replaced by an S. The conserved NTPase/helicase domain [27] was also present and conserved in ORF2.

ORF3 (328 nt) of BB-AND encodes a protein of 12 kDa that corresponds to the second protein of the TGB and contains two hydrophobic regions [27]. It shares 89% identity with the other two Andean isolates, Vltava-CZ and D00461, at the nucleotide level and 98% at the amino acid level. Analyzing the amino acid sequence, it was possible to find a $Y_{73}T_{76}O_{97}$ block, which is characteristic of the PVS^{A} isolates. At the same position, in the PVS^{O} isolates, the block is $H_{73}A_{76}P_{97}$. The alignment of this ORF with the ordinary

isolate sequences from GenBank showed a nucleotide identity ranging from 82% to 85% and an amino acid identity from 94% to 97% (Table 3). The hydrophobic regions in ORF3 and ORF4 are believed to be involved in cell-to-cell movement [23].

ORF4 is the third ORF of the TGB and encodes a polypeptide of 7 kDa that contains a hydrophobic N-terminal region. The nucleotide identity ranged from 82% to 84% with PVS^{O} isolates and was 90% with both Andean strains from GenBank (Table 3). The amino acid similarity varied from 87% to 90% when compared with PVS^{O} and was exactly the same as that observed for nucleotide identity (90%) when compared with PVS^{A}, demonstrating that all of the substitutions were synonymous. The amino acid sequence shows only two characteristic differences between the Andean sequences and the ordinary sequences: an I at position 13 and an R at position 61 in the PVS^{A} isolates were replaced by an M and a G, respectively, in the PVS^{O} isolates.

ORF5 encodes the coat protein (CP) [16] of 34 kDa, and it is 885 nucleotides long. The CP, together with the nucleotide-binding protein (11K) and 7K protein, has been considered to be responsible for the differences in biological properties between PVS^{A} and PVS^{O} [17,32]. This ORF had nucleotide and amino acid identities of 88% and 95%, respectively, with the Andean isolates Vltava-CZ and D00461 and 84% and 93% with AF493951, which is also an Andean isolate (Table 3). The identities between BB-AND and some

ordinary PVS sequences from GenBank ranged from 79% to 81% for nucleotides and from 92% to 93% for amino acids.

ORF6 has 285 nt and encodes an 11-kDa protein that is potentially involved in aphid transmission, viral protein replication and host gene transcription, due to a cysteine-rich nucleic-acid-binding site [31]. It shares 81–89% nucleotide identity with the other two Andean isolates and 84% to 87% at the amino acid level (Table 3). The identities of the nucleotide sequence with the ordinary isolates range from 80% to 81% and for the amino acid sequences from 70% to 84%. A block of characteristic amino acids, $E_4D_{23}I_{26}K_{27}S_{45}V_{65}P_{86,}$ was found for PVS^A. The block located at the same region in the known PVS^O isolates is composed of $D_4E_{23}V_{26}N_{27}$ $A_{45}I_{65}Q_{86}$.

The phylogenetic analysis of the complete genome sequence showed that BB-AND did not cluster with the other isolates from GenBank and was located in a different branch, separate from the other PVS^A isolate (Figure 2A). However, when we analyzed the ORFs separately, most of the time, BB-AND was clustered with the Andean isolates D00461 and Vltava (AJ863510), but in a different branch. Additionally, the phylogenetic tree constructed using the nucleotide and amino acid sequences of ORF1 of BB-AND indicated that this isolate is neither in the same cluster as the Andean isolate AJ863510 nor in the group of ordinary isolates (Figure 2B).

Both phylogenetic trees based on the nucleotide and amino acid sequences of ORF2 showed three subclades: one containing the PVS^{O} isolates, another containing most of the PVS^{A} isolates and the third containing only BB-AND (Figure 2C). In phylogenetic trees based on ORF3, the PVS isolates were located in three different groups. The first group contained only the ordinary isolates used for analysis; the Andean isolates D00461 and AJ863510 are in the second group; and BB-AND is in the last group, separated from all of the other isolates (Figure 2D). The same situation happens for the last ORF of the TGB, ORF4 (Figure 2E). Thus, the phylogenetic analyses of the TGB reveals that BB-AND was different from the other PVS isolates in GenBank.

The phylogenetic analysis of ORF5 showed, in general, two main clades; one consisted of the PVS^{O} isolates analyzed and other of the PVS^{A} isolates. Again, BB-AND was grouped in the PVS^{A} clade but was in a different branch from the isolates AJ863510, AF493951 and D00461 (Figure 2F). The same situation happened when we analyzed ORF6: the isolates AJ863510 and D00461 remained together but in a different cluster than BB-AND, which appears isolated, both in the tree based on nucleotides and in the tree based on amino acids (Figure 2G). The PVS^{O} isolates were in a larger group, and isolate DQ000231, described as PVS^{A}, was in the same group.

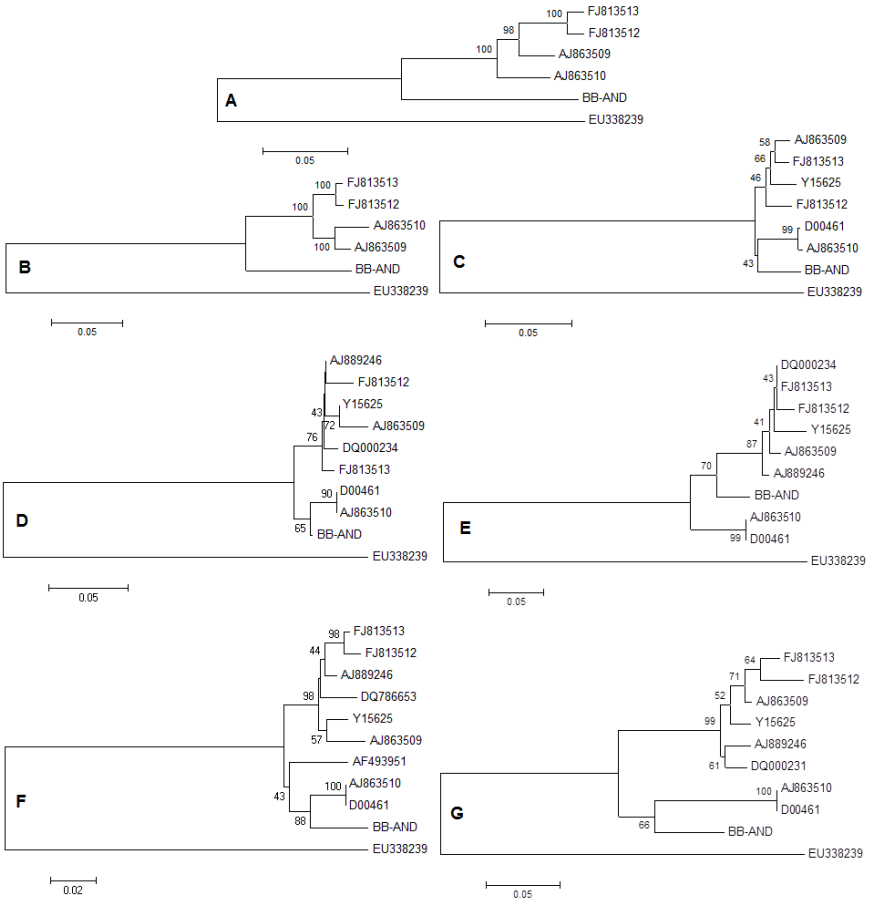

Fig. 2 Phylogenetic analysis of **A**. the complete nucleotide sequence, and several amino acid sequences: **B**. ORF1, **C**. ORF2, **D**. ORF3, **E**. ORF4, **F**. ORF5, and **G**. ORF6 of BB-AND with other available genome sequences of PVS isolates. The tree was constructed using MEGA 4.1, and the numbers at the nodes indicate the bootstrap values. The data set was subjected to 2,000 bootstrap replicates.

Recombination analysis

The recombination analysis of BB-AND and all four complete PVS genomes available in GenBank detected traces of past recombination events.

The recombination analysis performed to find out the specific genetic features of BB-AND compared with other ordinary and Andean isolates did not show any recombination events in its genome. However, one recombination event was detected in the isolate Vltava (AJ863510), with Leona (AJ863509) as the major parent (97.4%) and BB-AND as a minor parent (88.4%). In this recombination event, the region from nucleotide 6125 to 8324 in Leona was replaced by the BB-AND sequence that includes the C-terminal 25K protein, ORF 3 and ORF4, which are involved in viral transport, the coat protein and almost all of protein 11K [35]. In this recombination analysis, there was a discontinuity of unknown origin from nucleotide 6511 to 6611 (Figure 3A and B).

The recombination event showed a high degree of trust in all methods used for recombination detection, although BB-AND and Vltava were the only Andean isolates completely sequenced. To include another isolate, and based on the fact that the recombination between the isolates was seen in ORF2, another analysis was performed including another Andean isolate, D00461, whose available nucleotide sequence lacks ORF1. For this analysis, all of the sequences were cut off at nucleotide 4914, which corresponds to the first nucleotide of the D00461 sequence.

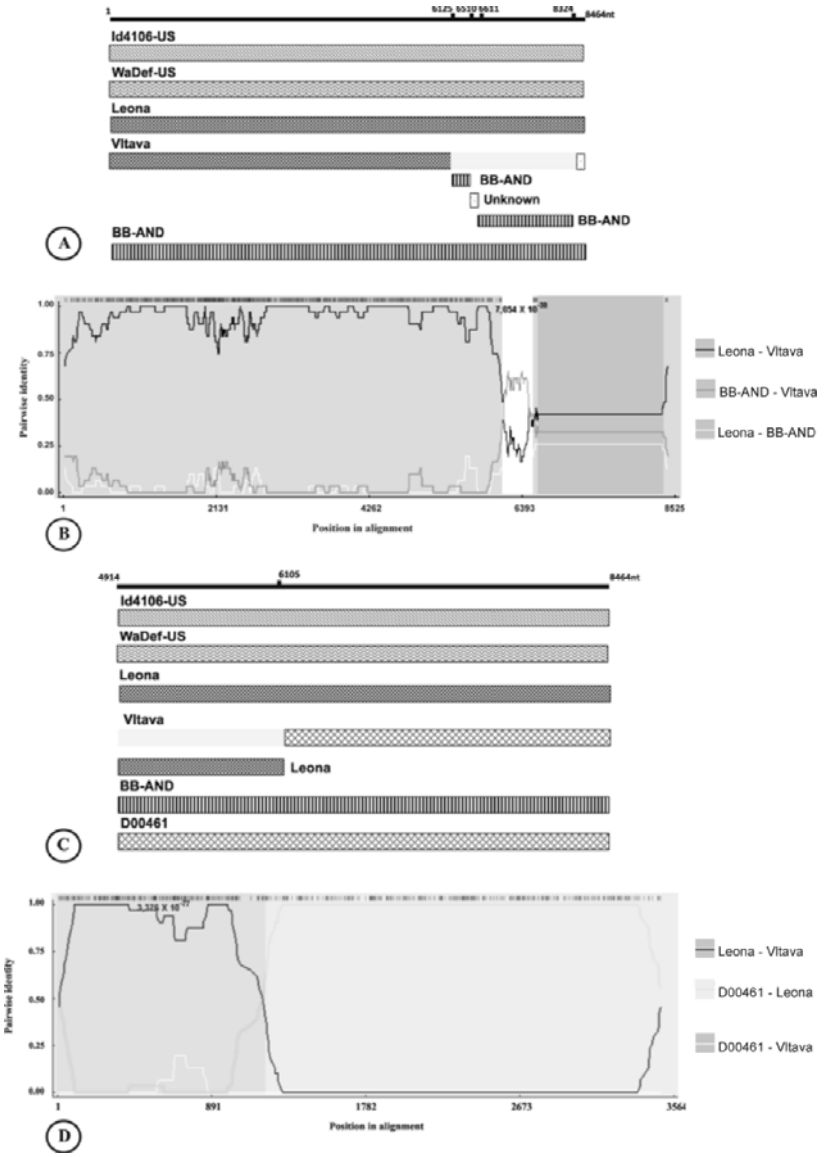

Fig. 3 Recombination events identified by RDP3. **A**. Analysis using BB-AND as the only PVSA isolate. The breakpoint lies between positions 6125–8324nt. **B**. RDP screenshot shows the estimation of the recombination breakpoints in the white region. **C**. Analysis indicating the recombination events when partial genomes were analyzed. The breakpoint lies between positions 4914–6102 nt. **D**. RDP screenshot shows the estimation of the recombination breakpoints, including the isolate D0046, in the light grey region.

When the recombination analysis included D00461, the recombination was confirmed, showing that the Vltava isolate acquired the genome fragment from 4914 to 6105 from Leona (minor parent, 99.1%) and the rest of its genome from D00461 (major parent, 100%; Figure 3C and D). In this analysis, the mismatching that was seen with BB-AND proposed as a parent, from 6511 to 6611 nt, was absent. This event was clearly identified by RDP (average P-value = $3.376.10^{-77}$), BOOTSCAN (average P-value = $1.056.10^{-74}$), MAXIMUM CHI SQUARE (average P-value = $7.668.10^{-34}$), CHIMERA (average P-value = $7.622.10^{-33}$), SISTER SCAN (average P-value = $2.315.10^{-36}$), 3SEQ (average P-value = $8.591.10^{-113}$), and GENECONV (average P-value = $7.622.10^{-33}$). The genomic region in which the recombination event was reported is that encoding the triple gene block, coat protein and 11K, proteins that are reported to be involved in cell-to-cell movement, transmission and differentiation between the two strains of PVS [17, 35]. Partial sequences of the isolates were used to construct the trees that confirmed the recombination between these two isolates. The phylogenetic tree clearly supports the evidence that Vltava is a recombinant and that the isolates Leona and D00461 are the parental sequences (Figure 4A and 4B). The first tree represents the grouping of the PVS isolates when only the first part of the sequence, before the breakpoint, was compared (Figure 4A). This part of the Vltava genome (AJ863510) was identified by the recombination analysis as coming from Leona (AJ863509), and the tree showed the

recombinant Vltava (considered to be PVS^{A}) in the same cluster as the PVS^{O} Leona.

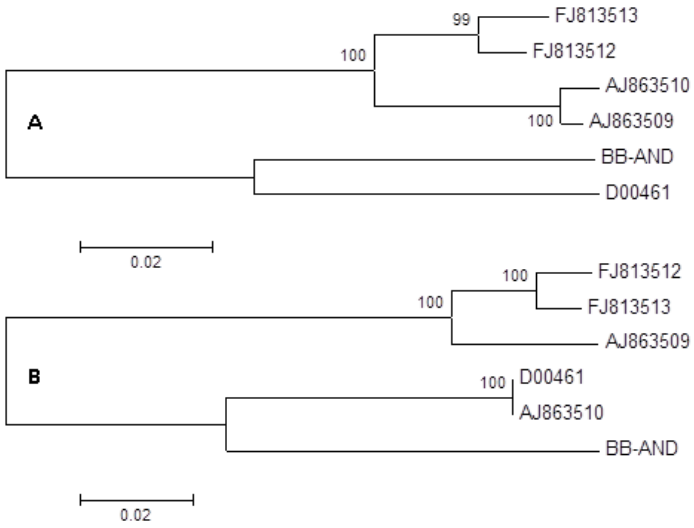

Fig. 4 Phylogenetic relationships among the isolates of PVS. **A**. Sequence used from nucleotide 4914 until the breakpoint at approximately nucleotide 6105. **B**. Sequence from the breakpoint until the end of the genome sequence.

The second tree represents the analysis of the sequence from the breakpoint until the end of the genome (Figure 4B). The recombinant Vltava is clustered with D00461, and the RPD3 program detected this region of the recombinant coming from the isolate D00461, as is clearly indicated in the tree. All of the clades were supported by 100% bootstrap values.

Discussion

In this paper, the presence of the Andean strain of PVS in Brazil (BB-AND) was reported for the first time. Analyzing its whole sequence, it was possible to detect that this isolate is different from the other PVS^{A} available in GenBank. This paper is the first report of a complete PVS^{A} sequence from South America, where this type of PVS isolate, inducing systemic infection in *C. quinoa*, was first described [22].

The aphid transmission rate of BB-AND was shown using two species, *Myzus persicae* and *Aphis gossypii*. Both aphids transmitted BB-AND, but *M. persicae* was more efficient, reaching a rate of 46.6% of infected *C. quinoa* plants when the inoculum source was *C. quinoa*. The rates were lower when the source of the virus was potato plants, and this lower rate may have been due to the concentration of virus in potato plants, where the aphids were left to feed. When the ELISA test was performed in the infected plants that were used as the source of the virus (data not shown), the absorbance average for the potato plants was almost 50% lower than the absorbance average for *C. quinoa*. *A. gossypii* was much less efficient as a vector and failed to transmit BB-AND from infected potato plants to uninfected potato plants. Similar rates of transmission were reported by Slack [41] who tested *M. persicae* as a PVS vector from infected potato to uninfected potato and *C. quinoa*. In New Zealand, 13% of the potato plants were infected by PVS^{A} after being inoculated with

aphids that fed in infected potato plants [15]. However, lower rates of transmission were reported by Wardrop et al.[46] and Kostiw [25], ranging from 5.9% to 2.9%, respectively. In laboratory conditions, it was found that *Aphis nasturtii* also transmitted PVS to healthy plants, with rates of 5.9% and 14.3%, respectively [46]. This variation in transmission rates is expected due to the possible differences among the aphids' biotypes, especially among those that are kept in the laboratory [46], and because of the different periods for virus acquisition used in the various experiments. Kostiw [24] drew a graph showing that the feeding time of *A. nasturtii* can affect the rate of transmission of PVS.

Most of the reports of PVS transmission by aphids tested *M. persicae* as a vector, and some reported transmission by *A. nasturtii* [24]. However, there is no information in the literature about transmission of PVS^{A} by *A. gossypii*, which is a very widespread important aphid in Brazilian fields. This is the first report that demonstrates PVS transmission by *A.gossypii.*

PVS has not been considered important in the Brazilian potato fields because most of the isolates are not easily transmitted by vectors, but only by infected seeds. Thus, if the seeds used are free of viruses, virus control in the fields is accomplished by exclusion principles and there is no risk of virus introduction from outside the crop. However, once the virus is introduced via seed potato, it becomes important due to its easy mechanical transmission and synergistic effect with other viruses, which may lead to significant yield losses

[3,47]. The Andean strain isolates present an additional risk of yield losses for Brazilian fields, if one considers that, in addition to being transmitted by tubers, they can easily be transmitted by insect vectors. Because the virus can spread to alternative hosts and survive between potato growing seasons, it can act as source of inoculum and reach the plants in the potato fields even when virus-free tubers are used for planting. In addition, dispersion of the virus inside the culture will also be increased by the vector. All of these factors taken together explain the importance of Andean PVS for Brazilian potato crops and the greater potential to cause potato yield losses.

The analysis of the complete genome revealed that BB-AND is an Andean isolate, but it is distinct from the other PVS isolates already described as PVS^{A}. It is likely that this result occurred because BB-AND is the first South American isolate that has been completely sequenced, and it had a different evolutionary pathway compared with the European PVS^{A} isolates. Cox and Jones [11], analyzing the genetic diversity of the PVS coat protein gene, reported that isolates from North America were found in the same clade, whereas the isolates of PVS^{O} from Europe and Asia were displayed in five different sub-clades. However, due to the lack of available sequences from South American PVS isolates, there is currently no way to evaluate its regional diversity, which would be very important, considering that South America is the center of potato domestication. Salari et al., [39], studying the Iranian PVS

isolates, noted some degree of genotype/geographical region specificity for isolates from Iran, Europe and Australia.

Most of the recent studies that have tried to differentiate PVS^O from PVS^A only compare the 3' ends of the sequence, including CP and 11K [10,11,39]. In this work, the nucleotide and amino acid sequences of ORF1 of BB-AND revealed that this ORF was the most distinctive region in the entire genome, compared with the other four sequences available in GenBank [27,31]. The analysis of the amino acid sequence for this ORF revealed that the most variable region was between the conserved motifs for MTR, HEL and RdRp. These results are consistent with the previous findings of Matousek et al.[31]. A broad variability in PVS isolates had been reported, and some blocks of 11 and 8 amino acids, at the N-terminal regions of the coat protein and the 11K protein, respectively, were described as major differences between PVS^A and PVS^O [16,32]. Some recent studies support the idea that differences in 11K alone might determine the systemic infection of *C. quinoa* by PVS^A [11, 39]. Other works report that the 3' end of the genome of PVS^A differed from PVS^O in 582 loci, 568 single-nucleotide substitutions and 14 deletions/insertions, [31, 32]. Amino acid variations, such as an A replacing a G or C at position 232 of the C-terminal part of 7K and the N-terminal of the coat protein and also as well as at position 17 combined with a polar amino acid at position 34 of the N-terminal part of CP were frequently associated with the PVS isolates that systemically

infect *C. quinoa* [31]. In this study, many characteristic blocks of amino acids highly agree with the data reported by Mautosek et al.[31], and some were reported as potential differences between the isolates of PVS^A and PVS^O studied. Because only four full sequences of PVS isolates are available in the GenBank and none of them could be described as the original Andean isolate [22,30], there are not enough data to confirm the locations of the genetic variations that define the biological differences between the strains of PVS.

Besides the nucleotide and amino acid sequence studies, phylogenetic analyses of the whole sequence and of the ORFs separately always clearly indicate two main clades, one containing the PVS^O isolates and another containing the PVS^A isolates, as observed in previous works [11,27,31,39]. BB-AND, in all of the phylogenetic analyses, was separate from the other PVS isolates that were reported to be able to systemically infect *Chenopodium* ssp, which demonstrates the uniqueness of BB-AND, the first isolate completely characterized from Brazil and from South America. It seems possible to conclude that BB-AND may be the first original Andean isolate described, due to the proximity of the Andean region to Brazil and the ease of importing potatoes from this region.

The most interesting result was obtained with the recombination analysis performed in this study. It was demonstrated, using the RDP3 package [29], that the ordinary strain was able to recombine with the Andean strain. Analyzing

only the four complete genomes of PVS available on Gen Bank, it was possible to detect that isolate Vltava is a recombinant, with parental sequences from BB-AND as the minor parent and Leona as the major parent. BB-AND was considered the parental when it was used as the only Andean isolate in the recombination analysis, showing that the Vltava isolate was a recombinant PVS isolate. In this first analysis, the percentage of confidence was lower for both the major parent (97.4%) and the minor parent (88.4%). However, when the analysis was repeated including a partial sequence from D0046, this isolate became a major parent, with 100% identity, and Leona became the minor parent, with 99.1% identity. This result can be explained if we consider that the sequence of BB-AND is quite different from those of the other Andean isolates, but it was still useful to identify the breakpoint at the same Vltava genome region, revealing that recombination events between isolates of PVS^{O} and PVS^{A} can occur. The phylogenetic analysis also supports this recombination evidence, showing that Vltava, as a recombinant, was grouped once with PVS^{O} Leona and another time with PVS^{A}, depending on the sequence region used to build the trees.

Recently, some works studying the genetic diversity of PVS isolates propose that these isolates have a broad diversity and could be grouped in different genotypes [11,39]. One evolutionary process that might facilitate emergence by generating novel variants is recombination. There are many

reports on natural recombination in a number of plant viruses, including *Potyvirus* [6,19,20,44], *Luteovirus* [21], *Nepovirus* [26], *Cucumoviruses* and *Bromovirus*es [1,5,18], and *Carlavirus* [40]. Chare and Holmes [8] suggested that recombination is a relatively common process in positive-sense plant RNA viruses, occurring in more than one among the three genome sequence alignments studied, and stated that the recombination in some plant viruses occurs at a sufficiently high frequency to enhance their potential for evolutionary change. Recent studies show that recombinant strains of *Potato virus Y* (PVY) are dominant and have replaced the non-recombinant strains [4,12,20].

These recombinations can potentially generate new virus isolates, more adapted and competitive in the field, leading to significant epidemiological changes. One example happened in Brazil with PVY. Until the mid-90s, this virus was not considered to be a problem for the potato crop, whereas *Potato leafroll virus* (PLRV) was primarily responsible for the yield losses for almost a century. However, with the introduction, via imported seed potatoes, of recombinant necrotic strains such as $PVYN^{Wi}$, which is more easily transmitted by the aphid vectors, and PVY^{NTN}, which presented good adaptation to the Brazilian environment, this scenario has changed, and PVY became the major cause of yield losses in the Brazilian potato crops [13,14,42]. Since then, PVY remains a major problem for the potato crop, not only in Brazil but in all countries where the potato is grown, illustrating that recombination events may

have a role in radical changes in the epidemiology of viruses within particular crop species. Therefore, this first report of recombination between strains of PVS is considered very important because, as has occurred with PVY in Brazil, the emergence of recombinant viruses could produce isolates that multiply and spread easily in the field. Considering also the fact that in Brazil the potato is cultivated up to 3 times a year and that the vector population is high throughout the year, the introduction of a more virulent, recombinant isolate could have an impact similar to that already experienced with PVY.

According to previous works, the biological definition of PVS^{A} does not match its genetic definition because some European isolates that infect *Chenopodium* ssp. systemically were more related to PVS^{O} than to Andean isolates [11,31]. However, as this statement was made based on only the 3' end regions of PVS isolates, if more details about these isolates were available, it might be possible to identify these isolates as recombinants emerging in Europe. Unfortunately, these sequences, named PVS^{CS}, are not available for analysis, and without having more PVS^{A} sequences, it is impossible to compare and determine the real origin of recombinants.

Despite the geographical distance between PVS isolates, due to the trade, import and export of tubers there is a great chance of improving the recombination environmental conditions between native and exotic isolates growing on different continents. The knowledge of recombination events in

complete genomes of PVS isolates can be useful for developing new procedures to avoid the spread of plant viruses, such as PVS^{A}, with the potential to cause damage in crops where the aphid population is always present, such as in Brazil.

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ARTIGO 2: Caracterização da região 3’ do genoma do vírus do amarelo do broto da soja: um novo *potyvirus* descrito no Brasil

Artigo preparado de acordo com as normas da Revista Tropical Plant Pathology

Caracterização da região 3' do genoma do vírus do amarelo do broto da soja: um novo *Potyvirus* descrito no Brasil

Resumo

O vírus do amarelo do broto da soja, VABS (*Soybean yellow shoot vírus*, SYMV), foi detectado, pela primeira vez, em 1984, em área experimental da EPAMIG, região de Lavras. Estudos preliminares mostraram se tratar de um *Potyvirus* infectando soja, porém, mais severo e com características biológicas e sorológicas diferentes dos *Potyvirus* já descritos. Desde então, as características moleculares desse vírus permaneceram desconhecidas. Neste trabalho, a região 3'do genoma do SYSV, englobando a cauda poli A, 3'UTR, capa proteica e parte das regiões NIb e CI, foi sequenciada e analisada. Essa região foi amplificada por RT-PCR, empregando-se o RNA extraído de partículas virais parcialmente purificadas e oligonucleotídeos universais, desenhados para amplificar a região CI, NIb e CP. As sequências de nucleotídeos e aminoácidos revelam que o SYSV é um membro distinto do gênero *Potyvirus* e apresentaram identidade e similaridade de 51% a 63% e de 44% a 47%, respectivamente, entre a região CI sequenciada e os demais *Potyvirus* já estudados. A comparação da sequência da região 3' indicou identidade variando de 55% a 59% para nucleotídeos e similaridade de 29% a 32% para aminoácidos. A análise filogenética da região amplificada mostrou que o SYMV não permanece no mesmo clado que os demais *Potyvirus* empregados na análise. Quando apenas sequência parcial da NIb foi analisada, o SYSV permaneceu no mesmo clado que o *Glycine virus Y*, um *Potyvirus* descrito na Austrália, mas ausente em outras partes do mundo. A análise do genoma do SYMV deixa clara sua singularidade, indicando ser essa uma espécie de *Potyvirus* diferente de qualquer outra já descrita no Brasil e no mundo.

Abstract

The *Soybean yellow shoot virus* (SYMV) was first detected in 1984 in the experimental area EPAMIG, Lavras. Preliminary studies showed it is a *Potyvirus* infecting soybean, however, more severe and with different biological and serological characteristics of *Potyvirus* already described. Since then, the molecular characteristics of this virus remained unknown. In this work the region 3'of SYSV genome, including the poly A tail, 3'UTR, coat protein and part of the NIb and CI regions, was sequenced and analyzed. This region was amplified by RT-PCR, using the RNA extracted from partially purified virions and universal primers designed to amplify the region CI and CP NIb. The nucleotide and amino acid sequences revealed that SYSV is a distinguished member of the genus *Potyvirus* and had, respectively, a nucleotide identity and amino acid similarity of 51-63% and 44-47%, between CI the region and the other sequenced potyvirus already studied. The comparison of the sequence of the 3' region indicated a nucleotide identity ranging from 55 to 59% and from 29 to 32% for amino acids similarity. Phylogenetic analysis of the amplified region showed that the SYMV does not remain in the same clade other *Potyvirus* employed in the analysis. When only part of the NIb sequence was analyzed SYSV remained the same clade of *Glycine virus Y*, a *Potyvirus* described in Australia, but absent in other parts of the world. Analysis of the genome SYMV showed its uniqueness, indicating that it is a new species of *Potyvirus* different of any previously described in Brazil and in world.

Introdução

A soja (*Glycine max* (L.) Merrill) é considerada uma das culturas mais importantes do mundo. Nativa da Ásia, foi introduzida no Brasil em meados 1882, quando se iniciaram estudos para utilização da planta como forrageira e na rotação de cultura. Aproximadamente depois uma década, a soja já era amplamente cultivada na região sul do país, adquirindo importância econômica a partir dos anos 1940.

Atualmente, o Brasil é o segundo maior produtor de soja do mundo. A produção estimada para 2011/12 é de 71,75 milhões de toneladas e área plantada total de mais de 24 milhões hectares (Conab, 2012). Mesmo com essa alta produção, os agricultores necessitam vencer diversos desafios causados por fatores que limitam a produtividade da soja, entre os quais os patógenos ocupam lugar de destaque, por diminuir drasticamente os rendimentos da cultura, gerando grande impacto econômico.

Aproximadamente 40 doenças causadas por fungos, bactérias, nematoides e vírus já foram identificadas em lavouras de soja no Brasil, com perdas anuais que variam de 15% a 20%, podendo chegar a 100%, de acordo com a região e as condições climáticas (Almeida et al., 2005; Kawuki et al., 2003).

Os vírus não são considerados causadores de grandes perdas na cultura, devido à existência de cultivares resistentes, obtidas por meio de melhoramento genético das cultivares. Porém, nos últimos anos, pelo menos cinco viroses foram identificadas em plantios de soja no Brasil. São elas: mosaico cálico (*Alfalfa Mosaic Virus* - AMV), mosqueado do feijão (*Bean Pod Mottle Virus* - BPMV), mosaico comum da soja (*Soybean Mosaic Virus* - SMV), necrose da haste (*Cowpea Mild Mottle Virus* - CPMMV) e queima do broto (*Tobacco Streak Virus* - TSV) (Fleysh et al. 2001, Giesler et al. 2002, Clark and Perry, 2002).

O SMV pertence à família *Potyviridae*, gênero *Potyvirus* e tem sido considerado o vírus mais prejudicial, sendo amplamente distribuído em regiões produtoras de soja do mundo todo. As perdas causadas pelo SMV variam com a cultivar e a época de plantio, podendo chegar a mais de 70% (Almeida et al., 1994, Farias et al., 2001,Hill et al., 1987, Silva et al., 2003). Além da redução da produção, o SMV contribui para o descarte de lotes de sementes, pois a maioria das sementes infectadas apresenta manchas que depreciam sua qualidade. Um controle efetivo para esse vírus em soja tem sido obtido pelo uso de cultivares resistentes (Silva et al., 2003), porém, com a expansão do plantio de soja para novas áreas, principalmente na forma de monocultura, novas viroses têm surgido, podendo ocasionar perdas significativas na produção.

Em 1984, um novo vírus com partículas flexuosas de 750 a 780nm de comprimento foi detectado, na EPAMIG de Lavras, MG (Deslandes et al. 1984), na época denominado de vírus do amarelo do broto da soja –SYSV e, posteriormente, denominado *Soybean yellow shoot virus* – SYSV. Devido às suas características sorológicas, biológicas e citológicas, o SYSV foi descrito como sendo um *Potyvirus*, diferente de qualquer outro já relatado para a cultura da soja no Brasil (Figueira et al., 1991; Santos, 2000).

O SYSV apresenta um grande potencial para causar perdas significativas na cultura devido à severidade dos sintomas que induz em plantas de soja, considerados muito mais drásticos que os causados pelo SMV. Os sintomas variam de cultivar para cultivar, mas, em geral, inicia-se com um mosaico e evolui para amarelecimento e encrespamento dos brotos. Ocorre a paralisação do crescimento dos ponteiros, levando ao enfezamento e à superbrotação da planta, e a produção se torna reduzida ou quase nula. Possui ampla gama de hospedeiros infectando plantas das famílias *Fabaceae*, *Chenopodiaceae*, *Solanaceae*, *Caricaceae* e *Amaranthaceae*. As plantas de *Chenopodium amaranticolor*, *C. quinoa*, *Carica papaya e Alternanthera tenella* são consideradas diferenciadoras

do SYSV, devido ao aparecimento de lesões locais nas folhas inoculadas (Vega et al., 1985; Santos, 2000).

Durante muitos anos, a gama de hospedeiras, o modo de transmissão e as propriedades antigênicas foram considerados as características mais importantes para diagnose de um vírus. Entretanto, sabe-se, atualmente, que estas características não podem ser analisadas separadamente, desconsiderando as propriedades genômicas da partícula, que têm se tornado um dos critérios mais importantes na classificação de vírus.

Diversos trabalhos envolvendo o estudo das propriedades biológicas e sorológicas do SYSV e o seu efeito em diversas cultivares de soja já foram realizados (Deslandes et al., 1984; Figueira et al., 1986; Figueira et al., 1987; Figueira et al., 1991; Vega, et al., 1985). Porém, nada ainda era conhecido sobre o seu genoma. Neste trabalho, realizaram-se o sequenciamento e a análise da região 3' do genoma do SYMV, contendo 1.600 pb, e parte da região CI com 682 pb, visando determinar a possível espécie desse vírus e sua semelhança com outras já descritas no GenBank.

Material e métodos

Vírus e manutenção do isolado

O SYMV foi detectado em 1984, na subestação da EPAMIG, na região de Lavras, MG e tem sido mantido dessecado a -20ºC, sendo multiplicado em plantas de soja, em casa de vegetação, sempre que necessário, para a realização de estudos.

Purificação parcial das partículas virais e extração do RNA viral

O RNA viral foi extraído pelo método de Lane (1992): 8 g de folhas de soja infectadas foram maceradas em nitrogênio líquido e ressuspendidas em 15

ml de tampão 0,1 M citrato de amônia (pH6,5), acrescido de 150 µL de 0,15 M Na-dieca e 50 µL de β mercaptoetanol. O extrato foi filtrado e centrifugado, por 10 minutos, a 8000 g. O sobrenadante foi coletado e clarificado com 0,5 mL de Triton-X100. Após agitação, o sobrenadante foi centrifugado sobre almofada de 20% sacarose, por 3 horas, a 29.000 rpm. O sobrenadante foi descartado e o pellet lavado em água ultrapura e ressuspendido em 500 µL de 0,02 M tampão fosfato (pH 7,2).

O RNA viral foi extraído a partir de 200 µL de suspensão de vírus parcialmente purificados, adicionando-se 50 µL de tampão de extração (0,2 M Tris-glicina, 0,2 M NaCl, 20 mM EDTA – pH 9,5), 20 µL de SDS 20% e 2,7 µL de proteinase K (20mg/mL). Após agitação, a mistura foi incubada, a 37 ºC, por 1 hora e, então, centrifugada, por 2 minutos, a 11.000 rpm. Ao sobrenadante foi adicionado fenol/clorofórmio (1:1 v/v) e, após agitação, a mistura foi submetida à centrifugação, por 15 minutos, a 11.000 rpm. O sobrenadante foi transferido para um novo tubo e novamente submetido ao tratamento com fenol/clorofórmio (1:1 v/v), centrifugado, por 15 minutos, a 11.000 rpm. O RNA foi precipitado pela adição de 1/20 volume de 3 M NaOAc (pH 5,5) e 2,5 volume de etanol absoluto ao sobrenadante. Após 1 hora a -20 ºC, a mistura foi centrifugada, por 15 minutos, a 11.000 rpm e o pellet contendo o RNA foi lavado com etanol 70% e centrifugado, por 2 minutos, a 11.000 rpm. O pellet foi ressuspendido em 20 µL de água ultrapura tratada com DEPC e armazenado a -20 ºC, para análises posteriores.

RT-PCR e sequenciamento

Os pares de *primers* degenerados *Potyvirus* específicos utilizados para amplificação dos fragmentos do genoma do vírus foram descritos por Ha et al. (2008) e estão listados na Tabela 1. As reações de RT-PCR foram realizadas em duas etapas utilizando M-MLV transcriptase reversa e GoTaq Flexi DNA

Polymerase (Promega, Madison, WI), de acordo com as instruções do fabricante. Os cDNAs foram sintetizados utilizando os *primers* reversos CIRev e N1T (Tabela 1) e a reação incubada, a 42 °C, por 60 minutos, 95°C, por 5 minutos e resfriada em gelo por, pelo menos, 5 minutos. Para o PCR, as reações foram incubadas, a 95 °C, por 2 minutos, seguida por 30 ciclos, a 95 °C, por 40 segundos, 42-50 °C, por 55 segundos e 72 °C, por 1 minuto, por 1 kb. Os produtos de PCR foram analisados em gel de agarose 0,7% e purificados utilizando-se o kit GFX PCR DNA *and Gel Band Purification* (GE Healthcare, Amersham Biosciences), de acordo com as instruções do fabricante. Os fragmentos foram clonados em pGEM-T Easy Vector (Promega Corp. Madison, WI, USA) e enviados para sequenciamento pela Genewiz, USA, utilizando-se os *primers* universais T7 e SP6.

Tabela 1. *Primers* utilizados para amplificação dos fragmentos do SYMV

Primer	Sequência (5'-3')	Domínios Conservados	Uso
CIFor	GGIVVIGTIGGIWSIGGIAARTCIAC	GxVGSGKST	CI gene potyvirus
CIRev	ACICCRTTYTCDATDATRTTIGTIGC	ATNIIENGV	CI gene potyvirus
NIbFor1	GGICARCCITCIACIGTIGT	GQPSTVV	NIb gene potyvirus
N1T	GACCACGCGTATCGATGTCGAC(T)17V		3' end primer

Nas sequências dos *primers*, I=inosina, Y=C/T, R=G/A, W=A/T, V=A/C/G, S=C/G e D=A/G/T.

Análise das sequências

A análise das sequências obtidas foi realizada utilizando-se o NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) e a identificação das regiões codificadoras foi realizada com o programa Open Reading Frame (ORF) finder, disponível no National Center for Biotechnology Information - NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Para o alinhamento das sequências de nucleotídeos e aminoácidos, foi utilizado o programa CLUSTAL W (ver. 2.0). As relações filogenéticas foram estudadas utilizando-se o

algoritmo neighbor-joining para aminoácidos e UPGMA para nucleotídeos, utilizando bootstrap com 2.000 repetições com o programa MEGA 5.0 (Cox and Jones, 2010; Tamura et al., 2007).

As sequências dos *Potyvirus* selecionadas e utilizadas nas análises de nucleotídeos e aminoácidos foram: *bean common mosaic virus* - BCMV (NC003397), *beet mosaic virus* – BTMV (AY206394), *east asian passiflora virus* - EAPV (AB604610), *glycine virus Y* – GYV(DQ098902), *lily mottle virus* - LiMoV(JN127341), *narcissus yellow stripe virus* - NYSV(AM158908), *onion yellow dwarf virus* - OYDV(JN127342), *plum pox virus* – PPV(AB576080), *soybean mosaic virus* – SMV (FJ376388), *sweet potato feathery mottle virus* – SPFMV (AB509453) e *zucchini yellow mosaic virus* – ZYMV (NC003224).

Resultados e discussão

Foram obtidos e analisados dois fragmentos genômicos do SYSV, sendo o primeiro com 1,6Kb, correspondente ao terminal 3', amplificado com os *primers* universais para o gênero *Potyvirus* (Ha et al., 2002) e o segundo, com 682 nucleotídeos, amplificados com os *primers* degenerados para a região CI, proteína relacionada à formação de inclusões cilíndricas típicas de *Potyvirus* (Shukla et al., 1994, Riechmann et al., 1992).

A análise do fragmento de 1,6 kb revelou que esta região inclui parte da proteína NIb (nuclear inclusion b), que é uma RNA-dependente de RNA polymerase (RdRp) (Hong and Hunt, 1996), toda a capa proteica seguida pela região 3' UTR (Untranslated region) e cauda poliA.

Membros do gênero *Potyvirus* possuem uma longa ORF, traduzida em uma única grande proteína que é processada em 10 proteínas funcionais: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa, que contêm os domínios VPg e NIa-Pro, NIb e capa proteica (CP) (Shukla et al.,1998). Recentemente, uma pequena ORF codificando uma proteína de 25 kDa foi identificada, denominada P3N-PIPO. O

papel preciso desta proteína no ciclo de *Potyvirus* permanece desconhecido, mas tem sido demostrado que o *knock out* da expressão de PIPO é letal para o vírus em plantas (Chung et al., 2008; Wen e Hajimorad, 2010)

A clivagem proteolítica da capa proteica (CP) a partir do C-terminal da poliproteína é realizada pela protease NIa (Shukla et al.,1994), em domínios que variam de acordo com o gênero da família *Potyviridae* (Adams et al., 2005). Analisando a sequência obtida para o SYSV e considerando os possíveis sítios de clivagem entre a NIb e CP dos *Potyvirus*, três são as regiões, localizadas entre os aminoácidos 200 e 216 da sequência gerada para SYSV, que poderiam ser reconhecidas pela NIa. Na posição 200 existe um possível sítio de clivagem E/G que geraria uma capa proteica com 279 aminoácidos. Outro possível sítio seria o Q/N, na posição 207, originando uma capa proteica com 271 aminoácidos e o terceiro sítio de clivagem (Q/G) se encontra na posição 214, que daria origem a uma capa proteica com 264 aminoácidos. O sítio de clivagem Q/N, na posição 207, coincide com os sítios dos demais *Potyvius* empregados para comparação neste trabalho (Figura1). Assim, o provável tamanho para o gene da capa proteica do SYSV é de 271 aminoácidos (813 nucleotídeos).

```
NYSV          DVEQSDITKY---IEAIFEDY-VEGEFSEVFHQSGK----QALDAG-------------- 216
PPV           EASETEIERY---LEAFYDDINDDGESNVVVHQADEREDEEEVDAGKPIVVTAPAATSPI 235
LiMoV         EASQDELERY---LAALDLNV-EQCNTEIVAFQANE-----TLNAG-------------- 214
SPFMV         QPSADDLSEYTRVLNEMYDDSLLQDNDLSVYHQSGNPSEFKDAGANPPAPKPKGPYTAPE 238
OYDV          DASEAELEKY---YKVYMDLENEEVVPKEVRYQAGK----------------------- 211
EAPV          NASLDELQEY---LRVLDFEH-TEGCCESVSLQSSTGKDKEEESKD------------- 220
SMV           NAQTSELQRY---LEVLDFNH-ADDCCESVSLQS--GKEKE------------------ 213
BCMV          KTKIEELAKY---LEVLDFDY-DVGCGESVHLQSGTGQPQPP----------------- 216
ZYMV          GAETSELARY---LQALHQDI-FFEQGDTVMLQSGTQPTAAD----------------- 216
BTMV          SVDENELVKY---WKALAPEE-DDGP-DIVTYQGDEKPSKSS----------------- 215
SYSV          DEDFVYDGRY-----DVEMHFEDKEGVVVLTPQNETAINQGS----------------- 217
                .     .*                 :   *
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Fig 1. Alinhamento da sequência de aminoácidos das regiões da NIb e CP. Os dipeptídeos sublinhados representam os prováveis sítios de clivagem SYSV. O retângulo mostra os sites de clivagem identificados para os demais *Potyvirus* identificados.

Diversos sítios de clivagem têm sido propostos para os *Potyvirus*. Shukla et al. (1994) tomaram como base o sequenciamento da região amino-

terminal da capa proteica de diversos vírus e sugeriram que o domínio de aminoácidos V-X-X-Q/(A, S, G ou V) seria um dos sítios de clivagem vírus específico para *Potyvirus*. Entretanto, dois outros sítios de clivagem têm sido propostos para o *Papaya ringspot virus*, VYHE/S e VFHQ/S, separados por 20 nucleotídeos, mas isso ainda não foi confirmado por análise direta. (Quemeda et al., 1990, Wang et al., 1994).

Para *Zucchini yellow mosaic virus*, o sítio de clivagem parece ter sequência variável e é postulado como V-X-X-Q(E)/S (Wu et al.,1993). Colinet et al. (1998) propuseram o sítio de clivagem NIb/CP para o S*weet potato mild mottle virus* (SPMMV) como sendo VVQ/RE, resultando em uma capa proteica de 275 aminoácidos. Esses autores consideraram, ainda, a possibilidade de haver um outro sítio de clivagem para o SPMMV, constituído pelos aminoácidos VYVE/P, localizado 29 aminoácidos antes do VVQ/RE, uma vez que ambos os sítios propostos são exclusivos de *Potyvirus*. Para outros gêneros da família *Potyviridae*, os sítios são diferentes: LQ/A para *Bymovirus* (Foulds et al., 1993), LQ/M para *Macluravirus* (Badge et al., 1997) e VVHE/A para *Rymovirus* (Schubert et al., 1999).

No fragmento da NIB obtido para o SYSV está localizado o bloco triplo de aminoácidos Gly-Asp-Asp na posição 41 a 43, que é universalmente conservado entre RdRps dos *Potyvirus* (Kamer and Argos, 1984). Mutações neste domínio podem impedir a replicação do vírus na planta (Li and Carrington, 1995).

A região 3' do genoma do SYSV contendo a NIb e SP foi bastante variável, apresentando identidades de nucleotídeos entre 55% a 59%. Maior variabilidade foi observada na região 5' da capa proteica. A maior identidade foi observada entre o SYSV e o PVP e a menor foi entre este e os SMV e LiMoV (Tabela 2). Quando as sequências de outros *Potyvirus* foram comparadas entre si, a maior identidade de nucleotídeos foi de 76% entre EAVP e SMV. A

identidade de aminoácidos variou de 27% a 33% entre o SYSV e os outros *Potyvirus*, sendo a maior identidade entre SYSV e OYDV (Tabela 2).

Analisando apenas parte da região NIb, a maior identidade de nucleotídeos (65%) e similaridade de aminoácidos (61%) da região NIb foi observada entre SYSV e *Glycine virus Y* (Tabela 2), porém, apenas 468 nucleotídeos desta região estão disponíveis no banco de dados, o que impediu a inclusão deste isolado na análise do restante da região 3'. O GVY apresentou alta identidade de nucleotídeos (57% a 62%) com os demais *Potyvirus* utilizados na análise, porém, a similaridade de aminoácidos foi menor e variou de 39% a 46%.

A análise do fragmento com 682 nucleotídeos revelou que ele parte da proteína CI, proteína relacionada à formação de inclusões cilíndricas típicas de *Potyvirus* (Riechmann et al., 1992), iniciando-se aproximadamente após os 250 primeiros nucleotídeos do terminal 5'. O alinhamento desta sequência com outros isolados virais mostrou uma identidade de nucleotídeos que variou de 51% a 60% entre o SYSV e os demais *Potyvirus* utilizados na análise (Tabela 2). A menor identidade foi entre o SYSV e o SPFMV e a maior foi, novamente, entre SYSV e PPV. Entre os demais *Potyvirus* utilizados para comparação, as identidades observadas para essa região ficaram entre 61% (entre OYDV e EAPV/SMV) e 77% (entre BCMV e SMV).

Tabela 2. Identidade (%) das sequências de nucleotídeos (nt) e aminoácidos das regiões sequenciadas do SYMV em relação a outros *Potyvirus* do banco de dados.

Isolate	CI		Região 3'		Nib parcial	
	nt	aa	nt	aa	nt	aa
BCMV	59	45	57	29	56	41
BTMV	63	47	57	29	62	42
EAPV	59	46	56	32	58	40
GVY	-	-	-	-	65	61
LiMoV	56	46	55	29	58	42
NYSV	59	46	58	32	61	47
OYDV	58	47	57	33	59	46
PPV	60	44	59	27	58	41
SMV	59	44	55	31	57	42
SPFMV	51	44	57	30	60	45
ZYMV	57	44	58	30	60	40

A forma flexuosa das partículas e as inclusões em forma de cata-vento (Santos, 2000) observados em folhas de soja infectadas já haviam sugerido que o SYSV seria um *Potyvirus*, o que agora se confirma ao se analisar o fragmento 1,6Kb amplificado com o auxílio de *primers* universais, juntamente com o sequenciamento parcial da região CI. De acordo com os critérios atualmente utilizados para a classificação dos *Potyvirus*, vírus compartilhando entre 76%-77% de identidade de nucleotídeos na região da capa proteica seriam considerados espécies distintas (Adams et al., 2005). Portanto, a análise parcial do genoma do SYSV é suficiente para demonstrar que este se trata de uma nova espécie de *Potyvirus* ainda não descrita, no Brasil e no mundo.

Árvores filogenéticas construídas com base nas sequências de nucleotídeos e aminoácidos da região do terminal 3', CI e sequência parcial da NIb para inclusão do *Potyvirus* GVY se encontram na Figura 2 (A-F).

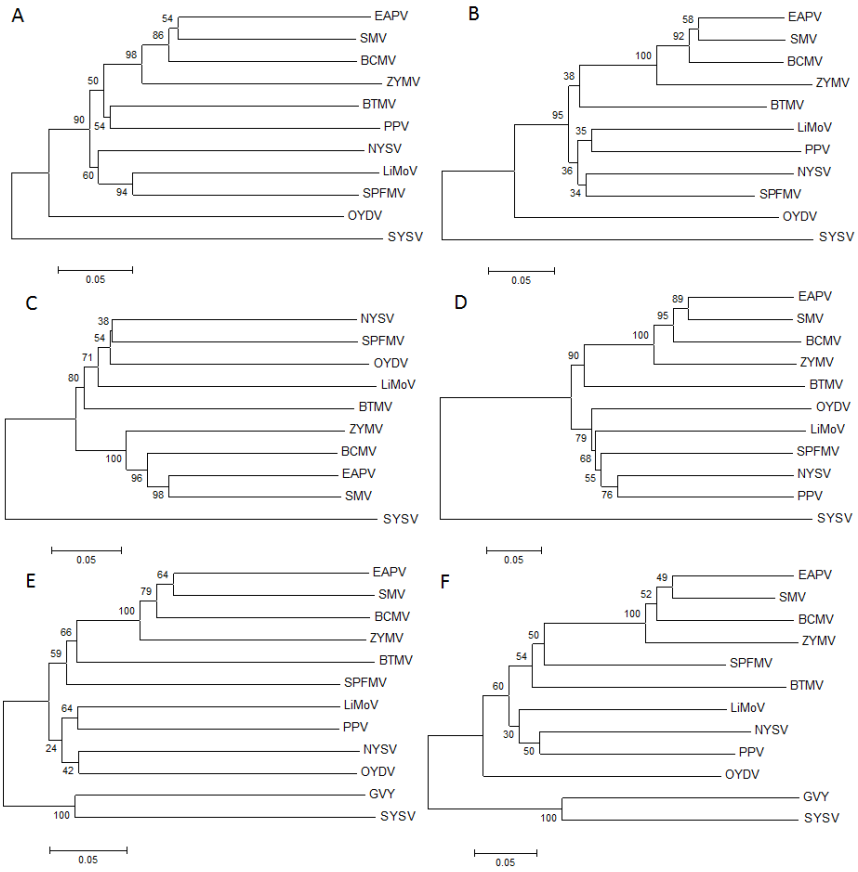

Fig. 2 Análise filogenética da **A**. sequência de nucleotídeos da CI **B**. sequência de aminoácidos da CI **C**. sequência de nucleotídeos da região 3' **D**. sequência de aminoácidos da região 3' **E**. sequência parcial de nucleotídeos NIb, incluindo GVY **F.** sequência de aminoácidos NIb incluindo GVY

As árvores, baseadas na sequência de nucleotídeos (Figura 2A) e aminoácidos da CI (Figura 2B), mostram a separação dos *Potyvirus* analisados em dois grandes grupos, com o SYSV separado em um deles, mostrando uma grande distância genética entre eles.

Assim como para a região CI, a análise filogenética das sequências de nucleotídeos e aminoácidos da região 3', contendo a NIb e a CP, mostrou que o

SYSV não pertence ao mesmo clado dos demais *Potyvirus*, permanecendo novamente separado dos demais. Quando as sequências de nucleotídeos foram analisadas, houve a formação de dois clados, com dois grandes grupos no primeiro deles, sendo um formado por BTMV, ZYMV, BCMV, EAPV e SMV e outro por NYSV, SPFMV, OYDV e LiMoV (Figura 2C). No segundo clado, o SYSV permaneceu novamente isolado. Um agrupamento similar ocorreu na árvore construída com base na sequência de aminoácidos, porém, o BTMV passou a ser agrupado com NYSV, SPFMV, OYDV e LiMoV e não mais com ZYMV, BCMV, EAPV e SMV (Figura 2D).

As árvores construídas com base na sequência parcial de nucleotídeos (Figura 2E) e aminoácidos da NIb (Figura 2F) apresentaram novamente dois clados, sendo ambos divididos em dois subgrupos. No primeiro clado, um subgrupo foi formado pelos vírus EAPV, SMV, BCMV, ZYMV, BTMV e SPFMV e o outro, com os demais *Potyvirus* utilizados neste estudo. No segundo clado, quando se construiu uma árvore empregando-se parte da sequência NIb do GVY, da Austrália, este se agrupou ao SYSV, porém, em um ramo distinto (Figura 2E). Como existe apenas um pequeno fragmento do GVY, correspondente à NIb, disponível no GenBank, não é possível comparar as outras regiões desse vírus com o SYSV. Entretanto, a identidade entre esses dois vírus, para essa região, foi de apenas 61%, descartando uma maior proximidade genética entre eles.

A análise das duas regiões do genoma do SYSV, incluindo suas características biológicas e sorológicas, deixa clara a singularidade da partícula, levando à necessidade de descrição desta nova espécie de *Potyvirus*. Trabalhos futuros são necessários para determinar as variações e as características do genoma desta nova espécie, visando, principalmente, evitar sua introdução e disseminação nos campos de cultivo de soja do Brasil, levando ao aumento de perdas na produção.

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ARTIGO 3: Isolation and characterization of host proteins involved in *Cowpea mosaic virus* movement tubules formed in the plasma membrane

Artigo preparado de acordo com as normas da Revista Journal of General Virology

Isolation and characterization of host proteins involved in *Cowpea mosaic virus* movement tubules formed in the plasma membrane

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Abstract

The systemic infection of plant virus depends on the movement of the virus particles from the infected cell to healthy neighboring cells. The *Cowpea Mosaic virus* moves from cell-to-cell using tubular structures formed by its movement protein. These tubules are tightly surrounded by the host plasma membrane and it is clear the involvement of plant factors in anchoring of the movement structure at the plasma membrane or even as a structural component of the tubules. Due to this evident virus dependence of the host proteins for movement from cell to cell, in this work the tubules formed in protoplasts of cowpea plants (*Vigna unguiculata*) were used to identify possible host factors involved in the virus transport. Therefore, a protocol for tubules isolation was developed and the isolated proteins were analyzed using tandem mass spectrometry (MS/MS). Besides the viral proteins, 19 proteins were identified and characterized as belonging to 11 different groups of host conserved proteins. Among groups of proteins identified, AAA-ATPase, chaperonin HSP 60, chaperonin HSP 70, transketolase, polyubiquitin, dynamin, proteasome complex and translation initiation factor proteins have already been identified as involved in different steps of the virus infection including the movement. The proteins belonging to the group of Asparagine synthetase, NADP-specific isocitrate dehydrogenase and transferase are very important in the cellular pathways but were still not reported as involved in viral movement process. The identification of these new

target proteins could help the understanding of the viral movement process, still not well known for most of the viral families.

Introduction

Over the years the interest to understand how viruses move from cell-to-cell is increasing and the interactions between virus and hosts have been the object of many studies. The virus cell-to-cell movement is determinative in the plant infection and studies have shown that it happens in different ways, depending of the virus family and genus (Schoelz et.al. 2011). Specific proteins encoded by viruses mediate the spread of the particles from cell to cell, via plasmodesmata. These movement proteins modify the plasmodesmata structure and alter the size exclusion limit (Carrington et al., 1996; Lazarowitz & Beachy, 1999). Two basic strategies have been described for the virus movement from cell-to-cell through plasmodesmata: one is the viral nucleic acids trafficking in form of ribonucleoprotein complexes (Heinlein et al., 1995; Kawakami et al., 2004; Wolf et al., 1989) and other is the tubule guided movement of virus particles (Lazarowitz & Beachy, 1999). This second type of virus movement is employed by wide range of plant virus and *Cowpea mosaic virus* (CPMV) has been a model virus in the study of the tubule guided movement, from infected cells to uninfected cells (Kasteel et al., 1993; van Lent et al., 1990; Wellink and Vankammen, 1989; Powels et al., 2002).

CPMV belongs to the family *Comoviridae*, genus *Comovirus* (Murphy et.al.,1995) and exhibit particles with icosahedral symmetry of about 28 nm in diameter. They contain two positive sense RNA molecules, encapsidated separately, both of which are required for infection. Each RNA contains a single open reading frame and is translated in polyproteins that are cleaved to give several intermediate and final processing products. RNA-1 encodes four proteins: the RNA-dependent RNA polymerase and helicase, involved in replication of viral RNAs; 24K proteinase and proteinase cofactor, involved in the polyprotein processing. RNA-2 encodes the 48K movement protein, essential for cell to cell movement and systemic spread, and 58K cofactor, required for replication, and the two coat proteins, large (L) and small (S), that also play a role on cell to cell movement (Sainsbury, et al., 2010)

Imunogold labeling studies had shown the movement protein of CPMV forming tubules in plasmodesmata to enable the transport of mature virions (van Lent., 1991) and the movement protein alone was sufficient to induce these tubular structures (Wellink,et al., 1993) . The tubules are tightly surrounded by the plasma membrane, and even if the tubules are formed in non-host cells, as insect cells (Wellink et al., 1993; Kastel et al., 1996), it was suggested the involvement of conserved host proteins in anchoring of the movement structure at the plasma membrane or maybe as a structural component of the tubules. CPMV movement protein does not interact directly with the surrounding plasma

membrane but an indirect interaction possibly happens between the tubule and the surrounding plasma membrane via a host plasma membrane intrinsic or peripheral protein (Pouwels et al., 2004). Movement binding proteins were identified by affinity chromatography of purified plasma membrane fractions, using immobilized movement protein, such as the subunits H, D and E of v-ATPase and aquaporin, which are conserved membrane proteins (Carvalho, 2003).

Despite the virus dependence of the host proteins is evident, still little is known about the role of host proteins in this process. Based on these observations, the aim of this work was identify and characterize another possible host membrane proteins involved in the cell-to-cell trafficking of virus particles through tubules, using the tubular movement structure, formed by CPMV in protoplasts of *Vigna unguiculata*. Therefore, a protocol for tubules isolation was developed and the isolated proteins were analyzed using tandem mass spectrometry (MS/MS). The identification of these proteins can help the understanding of the viral movement process, still not well known for most of the viral families.

Material and Methods

Plant material and growing conditions

The seeds of cowpea (*Vigna unguiculata*) were germinated in wet vermiculite at 25°C for 3 days in the dark. The pots with germinated seeds were

transferred for other chamber with 14h light per day and 28°C. The plants were watered with Hoagland solution (Hoagland and Arnon, 1950) during seven days.

Protoplasts isolation and virus inoculation

The cowpea protoplasts were prepared from primary leaves fully expanded, 10 days old, basically as described by Hibi et al. (1975). The primary leaves were harvest and the lower epidermis was peeled off and placed in enzyme solution containing 1% Cellulase, 0,25% Macerozyme, 10mM $CaCl_2$ and 0,6M Mannitol. Leaves were incubate in the solution for 3hr at 30°C. The mixture was filtered and the protoplasts were pelleted by centrifugation at 600rpm for 5 min. Protoplasts were washed with washing solution (10mM $CaCl_2$ and 0,6M Mannitol) and centrifuged at 600rpm for 5 min. The protoplasts were resuspended in washing solution and the concentration of protoplasts was determined by hemocytometer.

Each 10^6 protoplasts were inoculated with 10µg of purified CPMV using 500µL of 40% polyethylene glycol (PEG MW 6000) and then resuspend in protoplast incubation medium (0,2mM KH2PO4, 1mM KNO3, 1mM MgSO4, 1µM KI, 1 µM CuSO4, 10mMCaCl2, 0,6M Mannitol and 10 µg/ml gentamicine, pH 5,4). Protoplasts were incubated at 25°C at constant light. The protoplast infection and tubule formation were confirmed by immunofluorescence (van Lent et al., 1991), using the antiserum anti MP- 58K diluted 1:500 in 1% (w/v)

BSA/PBS as primary antibody and goat anti- rabbit Alexa fluor 488, diluted 1:400 in 1% (w/v) BSA/PBS as secondary antibody.

Collecting and Purifying Tubules

After 40h incubation the protoplasts suspension was collected and transferred to a clean glass centrifuge tube. The tube was covered with parafilm and vortex at 1600 rpm for 1 min, and then protoplasts were pelleted by centrifugation at 600 rpm, 5 min. The supernatant was collected and distributed in 1.5mL centrifuge tubes and centrifuged at 15000g for 15min. The supernatant was discarded and the pellet resuspended in 50µL of Microtubule Stabilizing Buffer – MTSB (1M Pipes, 1M MgSO4, 0.2 M EGTA), pH 6.9. In order to have the tubules suspension free of the big aggregates, the suspension was spined during 10sec at 12000rpm. The supernatant was placed in a clean tube and the pellet discarded.

For better tubules purification, they were firstly treated with 0,5% of nonidet NP40, to dissolve the plasma membrane surrounding the movement structure, and after immunoprecipitated with magnetic beads, coated with protein A (Dynabeads Protein A - Invitrogen), previously prepared as manufacture instruction. For each 50 µL of magnetic beads were added 6,6 µL of antibody (α 58) against MP, diluted in 200µL of PBS/ 0,02% Tween 20 (PBST). The mixture was incubated for 30min at 80 rpm and room temperature.

After the incubation, the beads/AB complex was washed twice, by placing the tube on the magnet, discarding the supernatant and adding 200µL of PBST. To avoid the co-elution of antibodies, they were crosslinked to the beads by adding 250µL of 5mM Bis (sulfosuccinimidyl) suberate (BS3) in conjugation buffer (20mM Sodium Phosphate, 0.15M NaCl) and incubated for 30min at room temperature with tilting/rotation. The unreacted crosslinker was blocked by adding 20µL of 100mM Ethanolamine and incubated at room temperature for 15min with tilting/rotation. The cross-linked beads were washed 3 times with 200 µL of PBST by placing the tube on the magnet and the supernatant discarded. After the last wash, sample containing the tubules pre-purified as described previously was added and incubated with the Dynabeads/Ab complex overnight at 4^{o}C with rotation. The supernatant was removed and analyzed to check the presence of remaining tubules. The complex Dynabeads/AB/Tubules was washed 3 times with 200µL of MTSB/0.02% Nonidet NP40, using the magnet to separate the supernatant from the complex. The complex was transferred to a clean tube, avoiding co-elution of proteins bound to the tube wall.

The elution of the tubules bounded to the beads/AB complex was performed by the denaturing method. The SDS sample buffer pre mixed with reducing agent (Laemmli, 1970) was added to the beads, the pellet resuspended and the mixture heated for 10 min at 70^{o}C. The beads were removed using the

magnet and the supernatant collected and place in a clean tube. The presence of tubules proteins in the suspension was checked by 10% SDS polyacrylamide gel. The gels were silver stained using Silver Xpress Kit (Invitrogen) according to the manufacturer's procedure. Viral proteins were detected by immunoblotting in immobilon P membrane probed with a mixture of rabbit antibodies against MP (dilution 1:1000) and anti CPMV coat protein (dilution 1:1000). Secondary antibodies used were goat anti rabbit conjugated with alkaline phosphatase (DAKO). The negative control was performed using magnetic beads protein A coated with rabbit pre-immune serum.

Host protein identification and database search

The purified samples of tubules and the negative control (10 µL of each) were separated in one dimension by SDS polyacrylamide gel electrophoresis. The samples were loaded at low voltage (40V) in a 12% acrylamide gel until all proteins had migrated into the running gel. The gel was stained with Oriole (Bio-rad) stain for 1.5 hours and then rinsed in water. The protein spots were visualized under UV-light and excised from the gel, washed in fresh 50mM Ammonium BiCarbonate (ABC), in-gel reduced and S-alkylated by submerging the pieces in 50mM Dithiotreitol, at 60° for 1 hour followed by incubation in 100mM iodoacetamide at 20ºC in the dark. The fragments were digested in cold

fresh Trypsin solution overnight at 20°C and the supernatant was transferred to a new tube.

The samples were size separated by liquid chromotochraphy and analysed using a LTQ-Orbitrap spectrometer (Thermo electron) to determine mass-to-charge ratios. The MS/MS spectra of the samples, and respective controls, were analysed using the Maxquant software in duplicate (Cox and Mann, 2008). The protein database used for identification of peptides was composed of all known Fabales proteins (downloaded from uniport.org on 20 august 2011), all known CPMV proteins and a list of common contaminants (BSA, trypsin and several keratins).

Results

Collecting and Purifying Tubules

After 40 hours post infection, the protoplasts were plenty of tubules that were efficiently harvested using vortex rotation. The best speed and time to pellet the semi purified tubules were 12000rpm for 10 seconds, because no tubules were detected on the supernatant and the electron microscopy analysis of the suspension of pelleted tubules showed the intact membrane surrounding the tubule (Figure 1A). The use of higher centrifugation speeds in this process resulted in an increasing of large aggregates, containing tubules and cells debris in the pellet.

The further treatment of the semi purified tubules with detergent dissolved the plasma membrane, allowing the correct binding of the antibodies against the movement protein in the next steps of tubules purification. After the treatment with the detergent Nonidet N40, was possible to observe that some proteins were still attached to the tubule wall and the tubule structure remained intact (Figura 1B).

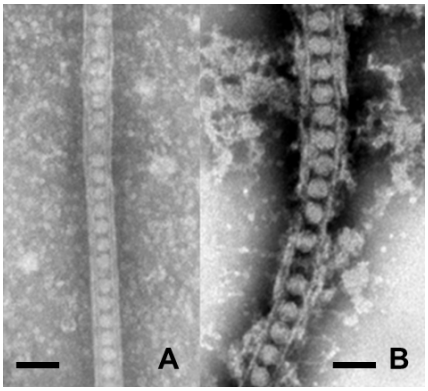

FIGURE 1: Electronmicrographs od transmission of the semipurified tubules. **A.**. Before treatment with detergent Nonidet 40. **B.** After the treatment with detergent Nonidet 40. Bars represent 50 nm.

The next purification step, using magnetic beads conjugated with the antibody anti 58K, allowed the obtainance of tubule suspensions with a good purity degree and without formation of protein or tubules agglomerates. The addition of the crosslinker BS3 to the antibody conjugated magnet beads decreased the tubes yield more than 60%, but was efficient to avoid the contamination of the purified tubules with the antibodies employed. Magnetic beads that were not crosslinked with BS3 were more efficient for

imunoprecipitaton of the tubules, however the eluate was contaminated with antibodies (Figure 2), causing problems on MS/MS analysis.

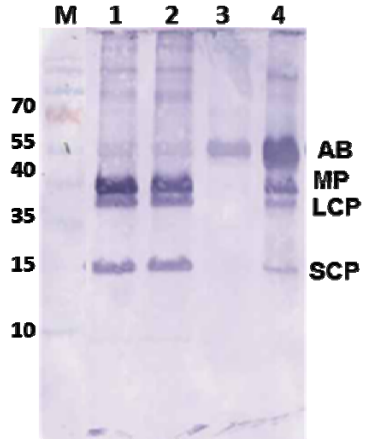

FIGURE 2: Western Blot of the tubules purified by magnetic beads protein A conjugated with immunoglobulin anti MP non crosslinked. M = protein marker, 1 = Input (pre-purified tubules), 2 =pre- purified tubules + 0,5% nonidet NP40; 3 = supernatant after incubation of the magnetic beads / proteinaA / antiMP with input ; 4 = Tubules purified eluted from magnetic beads after heat at 70º C for 10min. (AB = antibodies, MP = movement protein, LCP = large subunit of the coat protein, SCP = coat protein subunit).

The most efficient process for tubules elution was by heating the complex magnetic beads/Protein A/crosslinked antibodies/tubules at 70ºC for 10 minutes, which allows the recovering of the tubule proteins with no antibodies contamination. This temperature was efficient to denature the proteins, breaking the linkage between the antibodies and movement protein but not cleaving the link between protein A and antibody. The western blot analysis of the obtained tubules confirmed the presence of the normal tubules constituents such as MP

and the CP (Figure 3A) and when the silver staining were used, other proteins, besides the MP and CP were identified (Figure 3B).

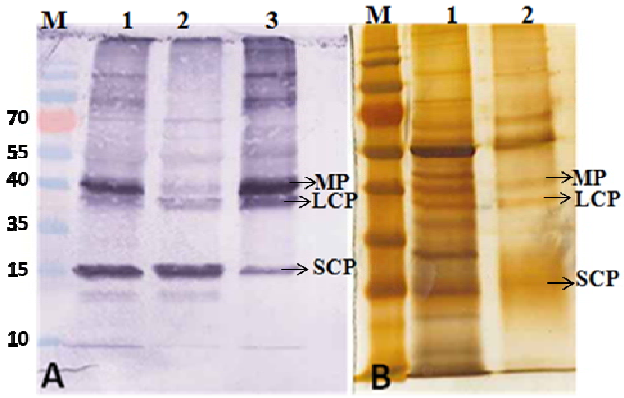

FIGURE 3: **A**- Western blot of tubules purified by magnetic beads conjugated with immunoglobulin anti MP crosslinked with protein A. M = protein marker, 1 = Input (pre-purified tubules), 2 = supernatant after incubation of the magnetic beads protein A / antiMP with input 3 = tubules purified eluated from magnetic beads by heating the complex at 70º C for 10min. **B**-Silver stained gel of (1) pre purified tubules samples and (2) after purification with magnetic beads. The analysis of the gel shows the purity of the sample after the use magnetic beads. (MP = movement protein, LCP = large subunit of the coat protein, SCP = small subunit of coat protein)

Host protein identification and database search

The host protein components, separated from the viral proteins by one-dimensional SDS PAGE electrophoresis and analyzed by MS/MS, are shown in Table 1. Nineteen host proteins were identified, that could be involved in the CPMV transport from cell to cell, belonging to 11 groups of conserved proteins.

Table 1 Host factors that could interact with movement protein of CPMV identified by tandem mass spectrometry (MS/MS)

Protein group	**Protein ID(uniprot):**
AAA-ATPase	B9S0I1
	D7TQP5
	D7L8D2
Chaperonin HSP 60	Q2PEP1
	D7SLM9
	Q9ZTV1
	Q1RSH4
Chaperonin HSP 70	Q2HT97
	B7FL88
Transketolase	B9GPE7
Polyubiquitin	Q6KFS1
Asparagine synthetase	P49092
dynamin	A2Q1P0
NADP-specific isocitrate dehydrogenase	Q7Y0W7
proteasome complex	Q1SL20
transferase	B0FNB2
	Q9AYM4
Translation initiation factor	A2Q5B8
	B7FK44

The first group is composed by three proteins (B9S0I1, D7TQP5 and D7L8D2), identified as AAA ATPases; the second group is composed by four proteins (Q2PEP1, D7SLM9, Q9ZTV1 and Q1RSH4), identified as chaperonin HSP 60; the third group, with two proteins (Q2HT97 and B7FL88), was identified as chaperonin HSP 70 group; the fourth group is formed by one protein (B9GPE7),

classified as Transketolase; the fifth group contain one protein (Q6KFS1), belonging to Polyubiquitin group; the protein of the sixth group (P49092) is an Asparagine synthetase; the seventh group with one protein (A2Q1P0), is a dynamin, generally classified as 'large GTPases'; the eighth group, also with one protein (Q7Y0W7), is analogue to NADP-specific isocitrate dehydrogenase; The nineth group, with one protein (Q1SL20), is analogue to proteasome complex; The tenth group is formed by two proteins (B0FNB2 and Q9AYM4), belongs to the transferases group, and Pyrroline-5-carboxylate synthetase subgroup; the last group, composed by two proteins (A2Q5B8 and B7FK44), belongs to the translation initiation factor protein group.

Discussion

Plant virus cell-to-cell movement depends on a series of mechanisms involving virus and plant factors (Scholthof, 2005). Cell-to-cell spread of CPMV is mediated by movement proteins that are the major component of the tubular structures formed during the virions transport. The molecular pathway, by which CPMV MP promotes cell-to-cell transport, is not completely understood but the tubular structures involved in the cell-to-cell movement, tightly surrounded by the host plasma membrane, suggests a possible involvement of host factors in the process of tubules formation (van Lent et al., 1990; Wellink et al., 1993; Kasteel, et al., 1997). The host components seems be involved not as a structural

component, but anchoring the tubule at the plasma membrane (Kasteel, et al., 1997). Thus, the development of a protocol for isolation of viral movement structures, done in this work, is extremely helpful to carry out studies to better understand this process.

The system CPMV-*Vigna unguiculata* was useful to obtain the movement structures to be isolated, and approximately 40 hours after protoplast infection with CPMV particles, the tubule structures, involved in cell-to-cell movement were ready to be collected and purified. The treatment of the pre purified tubules with detergent was efficient to remove most of the plasma membrane proteins that were not interacting with the movement protein. Therefore, only the plasma membrane proteins interacting with the tubule wall were purified for identification analysis. Without the plasma membrane the antibody could reach the movement protein that forms the tubule wall, allowing the immunoprecipitation of tubules for further purification. The treatment of the structures with nonidet NP40 does not affect the tubule structure and was already described by Kastel et al. (1997).

The use of Magnetic beads protein A improved the purity of the tubules and showed advantages over other protocols, such as those described by Kasteel et al., (1997) and Carvalho (2003). Besides the speed and efficiency to collect a high amount of structures, the covalent binding between the Fc portion of antibody and the protein A ensured that all antibodies used for

immunoprecipitation were active and were effective to capture the movement structures of interest.

Even causing more than 60% decrease in the tubules yield, the use of the BS3 crosslinker was still considered advantageous, because the BS3 is an ester homobifunctional (N-hydroxysuccinimidyl - NHS) and induce an irreversible and not cleavable linkage between the protein A and the antibody. In addition, it is hydro-soluble, which avoided the use of organic solvents in the dilution of the cross linker, and don't interfere in the molecular structure of the tubules. The lower efficiency in immunoprecipitation of the tubules, due to the BS3 action, was compensated by using four times the initial volume of beads/AB for the same volume of tubules suspension used.

The isolation and analysis of movement structures, as described in this work, allowed the identification of several host protein groups that plays important roles in cell pathways. All proteins identified in this work as possibly involved in the movement process were conserved among animal and plant species, and it is in agreement with what was proposed by Kasteel et al. (1996). They reported the tubule formation in non-host and also in insect cells by the expression of the MP using *baculovirus* vector. These authors verified tubule formation when they expressed the MP, via the proper vector, in host and non-host plants and also in insect cells.

Eleven groups of host factors were detected, and no one was described before as involved in the CPMV cell-to-cell movement. The first group includes AAA ATPases, that are involved in different cellular activities such as proteolysis, protein folding, membrane trafficking, cytoskeleton regulation, organelle biogenesis, transcription control and microtubule regulation (Patel and Latterich, 1998; Vale, 2000). One protein of this group was found to be involved in RNA virus replication and interaction with *Tobacco mosaic virus* (TMV) replicase proteins (Abbink et al., 2002). The recruitment of AAA ATPases proteins is needed for the precise assembly of the replicase complex of *Tomato bushy stunt virus*, which might help the virus infection recognition by the host defense (Chen and Lamb, 2008).

The Movement Protein of *Rice dwarf phytoreovirus* (RDV) has ATPase binding activity. The ATPase activity of MP may be important for RDV cell-to-cell movement (Ji et al., 2011). Several MPs of plant virus have ATPase activities. In potexviruses, the ATPase activity of PVX 25 K may be required to provide the driving force to traffic viral RNA through plasmodesmata or to suppress silencing (Howard et al., 2004). Carvalho (2003) obtained four host factors, three of which identified as subunits H, D and E of v-ATPase, and the fourth as aquaporin, using affinity chromatography of purified plasma membrane fractions and MP of CPMV immobilized.

The following groups of proteins identified in this work were the chaperonin HSP 60 and Hsp70, considered as the two major groups of molecular chaperones, belonging to a class of proteins that mediate the general folding and assembly of other proteins, including virion proteins (Hartl et al., 1992; Mayer and Bukau, 2005; Napuli et al., 2000; Alzhanova et al., 2001).

Interaction between HSP 60 and polymerase virus protein were reported for human viruses, such as *Hepatitis B virus* (Park and Jung, 2001) and *Human immunodeficiency virus* (Bartz et al., 1994), but also for the plant virus *Rice yellow mottle virus* (Brizard et al., 2006). Reports of HSP60 interacting with the movement protein of virus are scarce but HSP70 has been shown to be important in cell-to-cell transport of plant closteroviruses (Alzhanova et al., 2001) and the retrovirus *Human immunodeficiency virus* (Gurer et al., 2002). The fact that HSP70s has motor activities that drive protein translocation (Pilon and Schekman, 1999; Voisine et al., 1999) suggest that HSP70 proteins may have the ability to actively translocate viral movement proteins through plasmodesmata pores (Boevink and Oparka, 2005). In addition, HSP70 chaperones are supposed to be regulatory factors that control the fidelity and subcellular location of *Polyomavirus* capsid assembly in vivo (Chromy et al., 2003). For several animal viruses, the interaction with HSP70s appears to be involved in virion assembly that may occur in the nucleus cytoplasm, or endoplasmic reticulum (Liberman et al., 1999).

Other protein identified and purified in this work belongs to the group of transketolase, that acts in the pentose phosphate pathway and catalyses reactions in the Calvin cycle (Lindqvist et al., 1992). It is important in photosynthetic and phenylpropanoid metabolisms (Henkes et al., 2001) and could be involved in plant resistance. The expression genes coding for a transketolase in apricot plants were clearly linked to the susceptible interaction leading to *Plum pox virus* plant invasion (Escalettes et al., 2006) but its role in the virus transport is not established.

The next group, Polyubiquitin, controls many cellular processes, including DNA repair, cell cycle progression, protein trafficking and targeted for protein degradation, virus budding, and receptor endocytosis. An increase of polyubiquitin in plants infected by virus is associated with the infection for a range of viruses (Aranda et al., 1996). An evidence for the activation of the ubiquitin gene in tobacco mosaic virus-infected *Nicotiana sylvestris* protoplasts was reported by Genschik et al. (1992). Ubiquitin has been shown to be associated with the *Tobacco mosaic virus* replicase protein (Gaspar et al., 1990).

Dynamin, another protein identified, was also reported as host factor interacting with viral proteins during the virus-host infection. It facilitates the human *Adenovirus* internalization and infection (Wang et al., 1998) and seems to be dynamin-dependent in the fusion and entry of HIV into host cells (Carter et al., 2011). Dynamin has been proposed to mediate the constriction of coated pits

and the budding of coated vesicles from the plasma membrane (Hinshaw and Schmid, 1995; Takei et al., 1995).

Other group identified was the proteasome complex, which plays an important role in cellular virus defense and usually is involved in degradation of viral mRNAs. Recent work showed that inhibitors of proteasome endopeptidase activities help the multiplication of the HIV in cells (Schwartz et al., 1998). Thus, the enzymatic activities of proteasomes is blocked by viral encoded proteins and the interaction between proteasome and viral protein could be related to the antiviral strategies (Jarrousse et al., 1999). However, no reports about the relation of proteasome and cell-to-cell virus movement could be found.

The last group identified was the Translation initiation factor (Robaglia and Caranta, 2006). Many works relates the evolvement of this group of proteins with the virus infection. The most studied example is the interaction of the viral protein VPg, in the *Potyviruses* that recruit the translational machinery from host plants, eIF4E, eIFiso4E, eIF4G (Wittmann et al., 1997; Yoshii et al., 2004). A specific interaction of eukaryotic translation initiation factor 3 with the 59 nontranslated regions of *Hepatitis virus C* and classical *Swine fever virus* RNAs was also reported (Sizova et al., 1998) and has an important regulatory role in the initiation of translation. Among the eleven groups of proteins that were identified in this work, listed in table1, three of them, Asparagine synthetase, NADP-specific isocitrate dehydrogenase and transferase, play very

important roles in the cell, such as biosyntheses, transport of amino acids and defense. However, no reports about their direct interaction with virus proteins were found.

In this study was described for the first time an efficient method to purify the movement tubules structures induced by CPMV infection of cowpea protoplasts, and this purified tubules were used to identify host proteins by mass spectrometry. Some proteins were identified and sequenced and the functional relevance of these proteins remains to be evaluated, using other methods such as mutagenesis, silencing strategies or yeast two hybrid systems. This method of analysis may help to identify new target proteins that may be useful to find new markers for plant selection or to develop new strategies to interfere in plant virus infection processes, aiming its control.

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3 CONSIDERAÇÕES FINAIS

Neste trabalho, foram realizadas análises da sequência de nucleotídeos e aminoácidos de dois isolados virais detectados no Brasil. O primeiro isolado, denominado BB-AND, foi caracterizado como sendo da estirpe Andina do *Potato virus S* e o segundo, SYSV, foi descrito como sendo uma nova espécie do gênero *Potyvirus*.

A comparação entre as sequências de aminoácidos e nucleotídeos do BB-AND e isolados das diferentes estirpes de PVS mostrou que o BB-AND, além de ser o primeiro isolado de PVS^{A} detectado no Brasil, apresenta características moleculares diferentes, permanecendo sempre separado dos demais isolados descritos. A existência de eventos de recombinação entre PVS^{O} e PVS^{A} foi descrita pela primeira vez e a identificação de um recombinante das duas estirpes representa um aumento do risco de danos nos campos de batata do Brasil e do mundo. Já o estudo da região CI e 3'do genoma SYMV deixa clara sua singularidade, indicando ser essa uma espécie de *Potyvirus* diferente de qualquer outra já descrita no Brasil e no mundo.

Além da caracterização de dois novos isolados virais, o envolvimento de proteínas da membrana plasmática do hospedeiro no movimento célula-a-célula do CPMV foi estudado e possibilitou o desenvolvimento de um protocolo rápido e eficiente para a purificação dos túbulos de movimento e identificação de novas proteínas que podem ajudar no entendimento do processo de transporte viral, ainda pouco conhecido para a maioria das famílias virais.

A identificação e a caracterização de novos vírus, além da compreensão dos mecanismos de interação entre vírus e planta, são importantes e valiosos no desenvolvimento de novos procedimentos para controle, principalmente em países como o Brasil, onde existe alta diversidade de vetores no campo o ano todo, favorecendo a transmissão e a dispersão de vírus dentro e fora das culturas.