SUELLEN BARBARA FERREIRA GALVINO

GENOME STUDIES OF BRAZILIAN ISOLATES OF *Potato virus Y* (PVY)

LAVRAS-MG
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Tese apresentada a Universidade Federal de Lavras como parte das exigências do programa de Pós-Graduação em Fitopatologia para obtenção do titulo de Doutor.

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RESUMO

O *Potato virus Y* existe, atualmente, como um complexo de estirpes e isolados recombinantes que tornam essa espécie um dos vírus mais importantes nos campos produtores de batata de todo o mundo. Devido à enorme lacuna de informações a respeito da composição das populações de PVY distribuídas pelo Brasil, neste trabalho buscou-se estudar os diversos tipos de estirpes e seus recombinantes genéticos presentes no país, as suas possíveis origens e comportamento sob as condições brasileiras. Trinta e nove isolados de PVY, coletados no período de 1985 a 2009, foram estudados quanto às suas características biológicas, sorológicas e moleculares, tendo três deles sido submetidos ao sequenciamento completo de seus genomas e à análise de recombinações gênicas. Com base nas datas de coleta e classificação das estirpes, às quais os isolados estudados pertencem, inferiu-se uma linha histórica da introdução da estirpe necrótica do PVY no país, considerando-se que ela tem sido a responsável por enormes mudanças na epidemiologia desse vírus nos campos brasileiros de produção de batata. O sequenciamento completo dos genomas dos três isolados PVY-AGA, PVY-MON e PVY-AST mostrou um novo genoma recombinante para os dois primeiros, cujos parentais identificados foram PVY^{NTN} e o PVY-NE-11, provando, assim, que a diversidade dos isolados brasileiros de PVY é muito maior do que se suspeitava e excede à encontrada nos países europeus e norte-americanos. Na caracterização sorológica, biológica e molecular, observou-se que as estirpes recombinantes $PVY^{N\text{-}Wi}$ ou $PVY^{N:O}$ e a PVY^{NTN} são dominantes sobre as estirpes não recombinantes PVY^{O} e PVY^{N}. Três isolados estudados, PVY-AST, SGS-MO e MU-AGA, mostraram um padrão sorológico atípico (MAb2-/1F5+/SASA-N), nunca descrito anteriormente para isolados de outras regiões do mundo. Outro isolado, o MAF-VOY, mostrou sorologia PVY^{O}-O5, já descrito nos Estados Unidos para isolados da estirpe comum (PVY^{O}), apesar de ter sido caracterizado como $PVY^{N\text{-}Wi}$. Isso demonstra a grande variabilidade do PVY no Brasil e a dificuldade encontrada para o controle desse vírus por meio da utilização de cultivares resistentes.

Palavras-Chave: PVY, virus, batata, estrutura genômica, recombinação.

ABSTRACT

Potato virus Y (PVY) exists as a complex of strains with multiple types of recombinant genomes. In potato, PVY reduces yield and quality of tubers which makes PVY one of the most important viruses in potato production around the world. Due to a huge information gap about the PVY strain composition and distribution in Brazil, this study aimed to investigate the various strain types in Brazil, their genome structure, possible origins and circulation under Brazilian conditions. Thirty-nine PVY isolates, collected between 1985 and 2009, were typed biologically, serologically, and molecularly. Three of them had their whole genome sequenced and were subjected to a recombination analysis. Furthermore, through tracking the dates of collection of these PVY isolates, an attempt to reconstruct a history of the introduction of recombinant strains into the country was made, considering that these recombinant strains were responsible for dramatic changes in the epidemiology of the virus in Brazilian potato fields. The complete sequencing of PVY-AGA, PVY-MON and PVY-AST showed a novel recombinant genome for PVY-AGA and PVY-MON, whose recombinant parents were identified as PVY^{NTN} and PVY-NE-11, thus proving that the diversity of PVY isolates in Brazil exceeds that found in Europe and North America. The serological, biological and molecular characterization showed that the recombinant strains PVY^{N-Wi}/$PVY^{N:O}$ and PVY^{NTN} are currently dominant over the non-recombinant strains PVY^{O} and PVY^{N}. Three other isolates, PVY-AST, SGS-MO e MU-AGA, presented an unusual serological pattern (MAb2-/1F5 / SASA-N) that had never been reported before for any PVY isolate around the world. The MAF-VOY isolate was PVY^{O}-O5 serotype, which was already described in United States for ordinary strains (PVY^{O}), regardless had being characterized as PVY^{N-Wi} in molecular tests. Taken together, all these results demonstrate the great variability of PVY in Brazil, making difficulty the virus control through resistant potato cultivars.

Keywords: PVY, virus, potato, genomic structure, recombination.

SUMARIO

PRIMEIRA PARTE

1 INTRODUÇÃO

As perdas causadas atualmente pelo *Potato virus Y* (PVY) nos campos brasileiros produtores de batata, quer sejam para consumo ou para semente, têm sido consideradas bastante significativas, com tendência a se tornar ainda maiores nos próximos anos, devido à alta diversidade intraespecífica relatada para tal vírus. Esse prognóstico se deve à constatação de que isolados recombinantes das estirpes desse vírus, os quais já ocorrem há algum tempo na Europa e América do Norte, têm sido introduzidos no Brasil via batata semente importada, pois não existe um mecanismo que permita a detecção, nessa semente, dos variantes genéticos do PVY, que se tornam cada vez mais numerosos e difíceis de serem identificados.

Dentre as propriedades adquiridas por esses recombinantes ou variantes, pode-se citar um aumento na capacidade de disseminação no campo, verificada para os isolados de PVY^{N-Wi}, a habilidade para quebrar a resistência de cultivares de batata, verificada em isolados de $PVY^{N:O}$ e, em alguns casos, a indução de sintomas mais drásticos. Como o clima do Brasil é muito diferente do europeu e do americano, além do fato de a batata ser cultivada três vezes ao ano, nem sempre se conhece o comportamento desses recombinantes nos campos brasileiros de produção.

Recentemente, três isolados muito agressivos de PVY, causando sintomas de broto crespo em plantas de batata, encontrados nos estados de Minas Gerais e São Paulo, foram estudados no Departamento de Fitopatologia da Universidade Federal de Lavras (DFP/UFLA) e classificados como $NA\text{-}PVY^{N/NTN}$. Entretanto, apesar de induzirem sintomas semelhantes em plantas de batata, dois deles apresentaram características

genômicas distintas, mostrando uma alta identidade com um isolado norte-americano denominado, por Lorenzen et al. (2008), de NE-11. Além desses isolados, a suspeita de que o PVY^{N-Wi} já se encontra presente no Brasil desde 1995 foi confirmada por Figueira et al. (2009), com o emprego dos *primers* descritos por Glais, Tribodet e Kerlan (2005) e por Lorenzen et al. (2006a).

Os estudos realizados têm evidenciado que, muitas vezes, a variabilidade genética dos vírus pode estar associada à origem geográfica de cada isolado (MANGRAUTHIA et al., 2009). Assim sendo, seria interessante realizar um estudo mais detalhado dos isolados brasileiros de PVY aqui presentes, visando a investigação de suas origens geográficas e genéticas, dos pontos de recombinação presentes em seu genoma e a elucidação das sequências genômicas responsáveis pelo comportamento atípico destes no campo.

A comparação de sequências genômicas completas de espécies, estirpes ou patótipos virais têm sido bastante empregada, para estudos de recombinação genética entre isolados (GLAIS; TRIBODET; KERLAN, 2002; HU et al., 2009a, 2009b; LORENZEN et al., 2006b, 2008; MANGRAUTHIA et al., 2009; NIE; SINGH, 2002a, 2002b, 2003). A mudança de um único ou de poucos nucleotídeos nos genomas dos isolados recombinantes pode levar a efeitos diretos sobre as suas propriedades biológicas, sorológicas e moleculares (ATREYA; ATREYA; PIRONE, 1991; IVANOVA; SCHLESINGER, 1993; MASUTA et al., 1999). Considerando-se que a recombinação pode ser mais vantajosa para vírus de RNA do que a mutação, pois a primeira cria genótipos mais estáveis e adaptados aos ambientes de forma mais rápida e eficiente do que a segunda (ROOSSINCK, 1997), a introdução de novos isolados de PVY no Brasil pode levar à formação de novas recombinações destes com os isolados

brasileiros, onerando ainda mais os bataticultores e dificultando o trabalho de melhoristas que buscam fontes de resistência a essa virose.

Buscando elucidar a atual situação do PVY no Brasil, o presente trabalho foi realizado com o objetivo de estudar a diversidade da população desse vírus no país ao longo de 24 anos, utilizando isolados originalmente coletados em campos produtores de batata entre os anos de 1985 e 2009 e mantidos na Coleção de Vírus do Laboratório de Virologia da Universidade Federal de Lavras. Trinta e nove isolados de PVY foram caracterizados sorológica, biológica e molecularmente, sendo que três deles, por estarem associados ao sintoma atípico de broto crespo em batata, tiveram seus genomas completos sequenciados e submetidos à analise de recombinação genômica, visando à caracterização detalhada destes.

2 REFERENCIAL TEÓRICO

A batata (*Solanum tuberosum*) tem grande papel na nutrição humana, mas, apesar disso, o consumo dessa hortaliça é desequilibrado comparando-se diferentes regiões do mundo. Em alguns países, o consumo por habitante chega a 200 kg anuais, enquanto em outros é praticamente zero. Essa cultura é uma das principais em diversos países da América do Sul, sendo o Peru o maior produtor, com mais de 270 mil hectares plantados. O Brasil ocupa a 21º lugar no ranking mundial, com 100 mil hectares de plantio, cerca de 5 mil produtores e produção de 2 a 3 milhões de toneladas/ano. Apesar de a produção nacional se manter estável nos últimos trinta anos, o número de produtores e a área plantada reduziram-se muito nas últimas décadas (ANUÁRIO DA AGRICULTURA BRASILEIRA - AGRIANUAL, 2011).

Dentre os patógenos que afetam a cultura, o PVY tem se tornado, progressivamente, um dos vírus mais importantes para a bataticultura

nacional e mundial, sendo responsável por prejuízos similares ou superiores aos dos vírus de grande importância no passado, como o *Potato leafroll virus* (PLRV) (FIGUEIRA, 1999). Isso porque novas estirpes, de rápida disseminação no campo, têm sido introduzidas no país, por meio de importações de batata semente.

Membro tipo do gênero *Potyvirus*, família *Potyviridae*, o PVY possui um genoma constituído por um RNA de fita simples infectivo de, aproximadamente 9,7 Kb. Sua partícula é alongada, filamentosa e flexível, com dimensões de 730 nm de comprimento e 11 nm de diâmetro (BOKX; HUTTINGA, 1981; REGENMORTEL, 2000; SHUKLA; WARD; BRUNT, 1994). O seu RNA genômico possui uma única ORF que codifica uma poliproteína, posteriormente clivada em 10 proteínas funcionais, sendo elas, P_1, HC-PRO, P_3, $6K_1$, CI, $6K_2$, VPG, NIa, NIb, e CP (MURPHY et al., 1995).

Um aspecto importante das proteínas sintetizadas por esse vírus é o seu caráter multifuncional. Praticamente todas as proteínas cujas funções já foram determinadas atuam em mais de um processo no ciclo da infecção (SHUKLA; WARD; BRUNT, 1994). A proteína P1, localizada na porção amino terminal da poliproteína, possui propriedade de protease, que é responsável pela clivagem de seu terminal carboxílico ligado a HC-Pro (VERCHOT; KOONIN; CARRINGTON, 1991), é também mencionada como fator acessório para a amplificação do genoma viral. A proteína HC-Pro (Helper Component-Proteinase) é um componente auxiliar para transmissão por afídeo e também atua como protease. Entretanto, mais três funções já foram atribuídas à HC-Pro: fator acessório de amplificação do genoma (KASSCHAU; CARRINGTON, 1995; KASSCHAU; CRONIN; CARRINGTON, 1997; KERLAN, 2006; ROJAS et al., 1997), transmissão via semente (JOHANSEN et al., 1996) e inibidora da resposta de defesa da

planta (PRUSS et al., 1997; SHI et al., 1997; VANCE et al., 1995). A proteína P3 atua na replicação e na patogenicidade (SHUKLA; WARD; BRUNT, 1994). A proteína CI é responsável pela formação das inclusões citoplasmáticas (tipo catavento), possui função de RNA helicase, atividade de ATPase e atua tanto na replicação quanto no movimento célula-a-célula (ARBATOVA; LEHTRO; PEHU, 1998; MURPHY; JÄRLFORS; SHAW, 1991; ROBERTS et al.,1998; SHAND et al., 2009). As proteínas $6k_1$ e $6k_2$ não têm suas funções bem definidas. Apesar disso, sugere-se a ligação delas ao complexo viral de replicação do genoma (KERLAN, 2006). A proteína NIa (*Nuclear inclusion A*) é a protease responsável pela maioria das clivagens feitas na poliproteína, com exceção de P1/HC-PRO e HC-PRO/P3 (DOUGHERTY et al., 1988). A proteína NIb (*Nuclear Inclusion b*) é a RNA polimerase dependente do RNA (RdRp) do PVY e é responsável pelo reconhecimento das sequências regulatórias do RNA viral e pela interação com fatores do hospedeiro (KERLAN, 2006; SHUKLA; WARD; BRUNT, 1994). A proteína capsidial (CP), além de fazer parte do capsídeo, está envolvida na transmissão por afídeos, no movimento célula a célula, no movimento a longa distância e na indução de sintomas.

Além dessas proteínas funcionais, existem ainda as regiões 5' NTR e 3'NTR, das quais a primeira atua como promotora da tradução e replicação viral e a segunda apenas como promotora da replicação (ATREYA; ATREYA; PIRONE, 1991; SHUKLA; FRENKEI; WARD, 1991). Encontra-se associada covalentemente à extremidade 5' do genoma a proteína VPg (*viral protein genome linked*), uma protease do tipo cisteína, que atua na transmissão por afídeos e no movimento a curtas e longas distâncias (KERLAN, 2006).

As plantas infectadas com PVY reagem com uma série de sintomas bastante variáveis, refletindo a interação vírus-planta-ambiente e a grande

variabilidade genética que ocorre dentro dessa espécie (BOKX; PIRON, 1977; CHICK ALI; MAOKA; NATSUAKI, 2007, 2008; GLAIS; TRIBODET; KERLAN, 2002; HU et al., 2009b; KARASEV et al., 2010, 2011; KERLAN et al., 2011; KERLAN; LE ROMANCER, 1999; LE ROMANCER; NEDELLEC, 1997; LORENZEN et al., 2006b; MCDONALD; KRISTJANSON, 1993; NIE; SINGH, 2003; RAMIREZ-RODRIGUEZ et al., 2009; ROBLES-HERNANDEZ et al., 2010; SINGH et al., 2008). Os isolados de PVY que infectam a batata historicamente têm sido divididos em três principais estirpes de acordo com os sintomas induzidos em *Nicotiana tabacum*, *Physalis floridana* e batata. A estirpe comum, PVY^{O}, induz mosaico leve a intenso em plantas de fumo (*N. tabacum*.) e batata (*S. tuberosum*), e desfolha em *Physalis floridana* (SANCHES; GROGAN, 1970); a estirpe necrótica (PVY^{N}) causa necrose das nervuras nas plantas de fumo, não provoca desfolha em *Physalis* e em batata causa mosaico e/ou moteamento foliar leve e ocasionalmente necrose de nervuras (BALME-SINIBALDI et al., 2006) e a estirpe PVY^{C} é causadora de necrose sistêmica "striple-streak" em cultivares de batata que possuem o gene *Nc* (BOKX; HUTTINGA, 1981). Esta última é a estirpe com distribuição geográfica mais restrita, talvez devido ao fato de alguns de seus isolados não serem transmitidos via afídeo vetor, limitando sua ocorrência (BLANCO-URGOITI et al., 1998; ELLIS; STACE-SMITH; VILLEERS, 1997; HOOKER, 1981).

Progressivamente, têm-se distinguido as estirpes de PVY com base na reação de hipersensibilidade (HR) induzida em cultivares de batata portadoras de gene de resistência, chamados Ny_{tbr}, *Nc* e *Nz* (KERLAN, 2006; SINGH et al., 2008). A estirpe PVY^{O} é então definida por induzir HR em plantas portadoras do gene *Ny* e a estirpe PVY^{C} em plantas portadoras do gene *Nc*. Em contrapartida, a estirpe PVY^{N} é caracterizada por superar esses

dois genes de resistência conhecidos em batata até o momento. A existência da estirpe chamada PVY^{Z} foi proposta pela reação de HR produzida na presença do gene putativo *Nz* (COCKERHAM, 1970; JONES, 1990; KERLAN et al., 2011; SINGH et al., 2008), não eliciando nenhum dos demais genes em batata e nem causando necrose em fumo. Há ainda a estirpe chamada PVY^{E}, inicialmente chamada de PVY^{ZE} e recentemente renomeada, na atual classificação de estirpes de PVY, publicada em 2008 (SINGH et al., 2008). A PVY^{E} foi classificada separadamente por apresentar a capacidade de superar todos os genes de resistência, inclusive o suposto gene *Nz* presente na cultivar Maris Bard (SINGH, 1998).

Dentro das estirpes já classificadas também existe variabilidade, notadamente na estirpe necrótica PVY^{N}, na qual pelo menos cinco variantes genéticos, chamados isolados recombinantes, já foram descritos (PVY^{NTN}, PVY^{N-WI}, $PVY^{N:O}$, PVY^{N-NA}, PVY^{NE-11}), cada um portando propriedades biológicas, sorológicas e estrutura genômica particulares (Figura 1). A estirpe recombinante PVY^{NTN}, descrita em 1980, é capaz de induzir o aparecimento de anéis necróticos superficiais nos tubérculos de batata, causando o que se denominou de *potato tuber necrotic ringspot disease* (PTNRD) (BECZNER et al., 1984; KERLAN; LE ROMANCER, 1999; KUS, 1995; LE ROMANCER; KERLAN; NEDELLEC, 1994), o seu genoma é composto por segmentos genômicos das estirpes PVY^{O} e PVY^{N} recombinados entre si, podendo conter 3-4 junções de recombinação (JR) localizadas nos genes HC-Pro, NIa e 3'UTR CP (Figura 1) (GLAIS; TRIBODET; KERLAN, 2002; LORENZEN et al., 2006b; NIE; SINGH; SINGH, 2004).

Ate o momento, ainda não se conhece, de fato, qual é a sequência viral ou domínio responsável pelo fenótipo necrótico nos tubérculos, o que dificulta a detecção molecular dos isolados de PVY^{NTN}, principalmente

quando não há desenvolvimento da PTNRD nos tubérculos. Apesar de sempre se associar PTNRD com isolados recombinantes, existem evidências de que esse fenótipo pode ser causado por isolados PVY^{NTN} surgidos por mutações, não sendo, assim, a recombinação um pré-requisito obrigatório para o surgimento de anéis no tubérculo (NIE; SINGH, 2003). Nos últimos anos, houve um grande aumento no número de países que relataram a presença da estirpe necrótica e da variante NTN do vírus Y, porém, pouco se sabe a respeito de suas verdadeiras origens. Apesar de, inicialmente, essas estirpes terem sido descritas com maior frequência na Europa, uma enorme quantidade de isolados delas tem aparecido na América do Norte

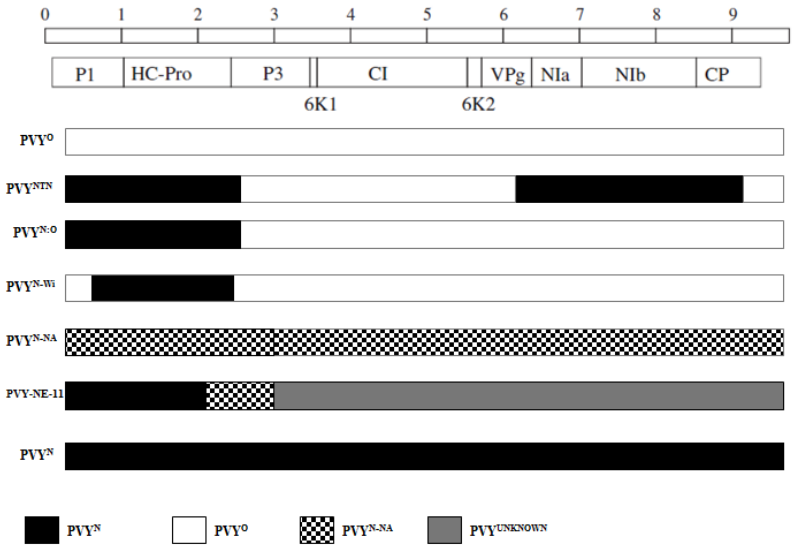

Figura 1 Diagrama da estrutura genômica das estirpes e recombinantes de PVY já descritos.

(LORENZEN et al., 2006b, 2008; NIE; SINGH; SINGH, 2004). Baseando-se nas seqüências da região não-traduzível 5'UTR do gene P1 desses

isolados, dois agrupamentos distintos se formam, sendo um proveniente da Europa e outro da América do Norte (NIE; SINGH, 2002b), porém, não há nenhuma evidência consistente de que as origens parentais dos isolados europeus e dos norte-americanos tenham sido diferentes (LORENZEN et al., 2006b).

A segunda estirpe recombinante de relevância já reportada é a estirpe PVY^{N-Wi}, detectada na cultivar de batata Wilga, em campos da Polônia, esta semelhantemente à estirpe PVY^{NTN} é capaz de, eventualmente, causar necrose nos tubérculos de batata de cultivares sensíveis, agravando ainda mais o problema de detecção da variante PVY^{NTN}. Apesar de frequentemente induzir sintomas mais suaves nas plantas de batata, a PVY^{N-Wi} ocorre em altas concentrações, concorrendo para uma disseminação mais elevada no campo quando comparada aos demais isolados (CHRZANOWSKA, 1991, 1994). Desde sua primeira descrição, ela disseminou-se para várias regiões do mundo, como Espanha, França, Rússia, Finlândia, Alemanha e Brasil (FIGUEIRA et al., 2009; GLAIS; TRIBODET; KERLAN, 2005; MORAES; FIGUEIRA; SANTOS, 1998) e, mais recentemente, para o México (RAMIREZ-RODRIGUEZ et al., 2009). Essa estirpe possui uma característica sorológica diferenciada da maioria dos isolados necróticos devido à sua incapacidade de reagir com anticorpos monoclonais específicos para PVY^{N} e reagir com aqueles específicos para PVY^{O}. Esse sorotipo inesperado se justifica pelo padrão genômico encontrado para esta estirpe. Também composto pela recombinação das estirpes parentais PVY^{N} e PVY^{O}, o PVY^{N-Wi} contém de 1-2 JRs, de forma que a maior parte de seu genoma pertence ao parental PVY^{O} (Figura 1), por isso a reação positiva como o anticorpo monoclonal O – específico (CHRZANOWSKA, 1994).

Apesar de serem as mais expressivas estirpes recombinantes de PVY, os isolados de PVY^{NTN} e PVY^{N-Wi} não são os únicos recombinantes da espécie. Novos isolados recombinantes têm sido frequentemente reportados em diversas partes do mundo, evidenciando a grande habilidade de recombinação e alta taxa de mutação ocorrida nos genomas de PVY.

Dentre as estirpes recombinantes descritas mais recentemente, citam-se: (i) o $PVY^{N:O}$, descrito no Canadá e norte dos Estados Unidos (SINGH et al., 2003) que é idêntico ao PVY^{N-Wi}, quanto às suas características biológicas e sorológicas, porém, em seu genoma, há apenas uma junção de recombinação, enquanto o PVY^{N-Wi} europeu frequentemente possui duas (Figura 1); (ii) o PVY-NE-11, também descrito na América do Norte (LORENZEN et al., 2008), é portador do primeiro genoma recombinante descrito cujos parentais não são o PVY^{O} e o PVY^{N}, e sim uma mistura entre PVY^{N}, PVY^{NA-N} e uma sequência pertencente a um variante ainda desconhecido de PVY (Figura 1) e, finalmente, a última estirpe recombinante descrita antes do presente trabalho é (iii) a estirpe PVY^{NTN-Wi} proposta para isolados da Síria, cujo genoma possui sequências originadas do PVY^{NTN} e PVY^{N-Wi} (CHICK ALI et al., 2010; CHICK ALI; MAOKA; NATSUAKI, 2008).

Nota-se, diante do exposto, que o surgimento de novos isolados recombinantes tem sido cada vez mais comum e, apesar disso, os mecanismos que levam à sua formação ainda não são muito claros. Sabe-se apenas que os processos mais comuns de formação de novos RNAs por recombinação são mediados pela replicase e que as estruturas secundárias do RNA e/ou as regiões ricas em AU afetam esse processo de recombinação entre os genomas de PVY (HU et al., 2009c).

Quanto aos determinantes genéticos da necrose de nervuras em fumo causada por isolados não recombinantes e recombinantes da estirpe PVY^{N},

especula-se que os resíduos K-400 e E-419, encontrados dentro da proteína viral HC-Pro, sejam os responsáveis pelo fenótipo de necrose de nervuras em fumo (TRIBODET et al., 2005), porém, não se sabe se estas regiões são as únicas envolvidas no desenvolvimento desse fenótipo. Hu et al. (2009a), estudando um isolado de PVY^{NTN} (L26) capaz de induzir apenas mosaico foliar e clareamento leve entre as nervuras, sem provocar a necrose típica desse grupo, encontraram, além dos resíduos K-400 e E-419, cinco substituições de bases ao longo do genoma do isolado. No entanto, apenas uma dessas substituições resultou em mudança de aminoácido na proteína HC-Pro (de D-205 por G-205), mudança esta bastante drástica, pois substituiu um aminoácido carregado negativamente (ácido aspártico) por um neutro (glicina). Por esta ter sido a única diferença genética encontrada entre este isolado e vários outros isolados de PVY^{NTN} já estudados, os autores sugeriram que, provavelmente, não exista uma só região genômica determinante do fenótipo de necrose, uma vez que, apesar de os resíduos K-400 e F-419 estarem presentes, o isolado não foi capaz de provocar necrose nas plantas. Casos semelhantes já foram relatados anteriormente, como o isolado Gr99 (*AJ890343*), que possui dois diferentes aminoácidos na HC-Pro, o R-400 e o D-419, que têm sido descritos como responsáveis pela ausência de fenótipo necrótico (SCHUBERT et al., 2007).

As técnicas para identificação e distinção entre as estirpes recombinantes, apesar de terem evoluído consideravelmente na última década, ainda apresentam algumas limitações. As técnicas sorológicas, que utilizam anticorpos policlonais para a identificação e a caracterização de isolados virais, não são suficientes para a distinção da estirpe N e nem dos recombinantes NTN, N:O, N-Wi (wilga) e NE-11. Nestes casos, apenas a utilização de testes sorológicos com anticorpos monoclonais (SHUKLA; WARD; BRUNT, 1994) e as reações de RT-PCR com *primers* específicos

aliadas ao sequenciamento completo dos genomas portadores dos pontos de recombinação característicos podem levar à correta identificação e caracterização dessas estirpes recombinantes (BOOHAM et al., 2002; HU et al., 2009b; LORENZEN et al., 2006a, 2008;SINGH et al., 2008).

Na busca por uma metodologia de diagnose das estirpes que seja rápida, eficiente e não muito dispendiosa, vários autores têm trabalhado com reações de PCR uniplex e multiplex e PCR quantitativo utilizando, na maioria das vezes, *primers* desenhados com base nas sequências recombinantes diferenciais de cada genoma. O desafio, porém, é detectar infecções mistas que, no campo, são relativamente frequentes. Lorenzen et al. (2006a) desenvolveram uma RT-PCR multiplex capaz de diferenciar os varios tipos de estirpes e detectar infecções mistas em plantas ou tubérculos de batata, o que significou uma excelente contribuição para os pesquisadores e produtores de batata semente que necessitam determinar a sanidade de suas amostras. Numerosas técnicas modernas qualitativas e quantitativas têm surgido na intenção de aumentar a especificidade e a sensibilidade da detecção e caracterização de isolados pertencentes aos grupos recombinantes de PVY.

Isolados recombinantes de PVY^{NTN} têm sido discriminados também por meio da técnica de PCR quantitativo (qPCR) ou PCR em tempo real, em alguns países da Europa, explorando a presença de um ponto de recombinação no gene da capa proteica (KOGOVŠEK et al., 2008). Apesar de todas essas técnicas mostrarem-se altamente sensíveis, específicas e passíveis de uso como teste de diagnose em grande escala, incluindo a possibilidade de os testes serem feitos diretamente dos tubérculos, a única dificuldade é gerada pelo fato de que novos variantes continuam a surgir e nem sempre os *primers* desenhados para os isolados que ocorrem em um país são eficientes para detectar novos isolados em outro.

Especula-se que a estirpe necrótica do PVY (PVY^N) teria seu centro de origem localizado na América do Sul, onde o provável primeiro relato dela ocorreu no início da década de 1940 (NÓBREGA; SILBERSCHIMDT, 1944), quando alguns isolados de PVY do Peru, na região andina, foram associados com indução de necrose na cultivar de batata peruana Serrana Negra. Já no Brasil, o primeiro relato oficial do PVY ocorreu na década de 1960, no estado de São Paulo, em um estudo morfológico das partículas virais de diversos isolados utilizando métodos de exsudados ou "dipping" (KITAJIMA; CAMARGO, 1967; KITAJIMA; CARVALHO; COSTA, 1962). Durante os anos seguintes, esse vírus foi objeto de estudos apenas de caráter descritivo e preventivo, buscando-se a descoberta de métodos que fossem capazes de evitar eficientemente a infecção e a disseminação viral (ALEXANDRE; BARRADAS, 1982; BARRADAS; FERRARI, 1987; COSTA; NAGAI; KITAJIMA, 1969). Entretanto, naquela época, o PVY não era considerado um problema significante para os bataticultores em comparação com o vírus *Potato leafroll virus* (PLRV). Porém, a situação no estado de Minas Gerais alertava que, enquanto cerca de 23% dos tubérculos coletados para análise estavam infectados com PLRV, 19,5% estavam com o PVY, evidenciando que a incidência do PVY crescia aceleradamente a cada ano, praticamente se igualando à do PLRV (FIGUEIRA et al., 1985; FIGUEIRA; SOUZA; GASPAR, 1985). No mesmo ano, também no estado de Minas Gerais, reportou-se a existência de isolados de PVY capazes de induzir necrose no fumo em diversas intensidades, desde a típica necrose de nervuras ate o completo colapso da planta (FIGUEIRA et al., 1985; FIGUERIA; SOUZA; GASPAR, 1985). Apesar disso, a estirpe predominante nos campos produtores continuou a ser a PVY^O, por muitos anos, sendo capaz de infectar quase todas as cultivares plantadas durante aquela década (ANDRADE; FIGUEIRA, 1991; FERNANDES;

CARVALHO; MIZUBUTI, 1986; FERNANDES; CARVALHO; ZAMBOLIM, 1986). Durante os anos de 1983 a 1988, a estirpe PVY^{O} foi identificada em 80% das amostras infectadas com PVY de campos nos quais as cultivares Achat, Baraka, Baronesa, Bintje, Granola e Monalisa eram plantadas (ANDRADE; FIGUEIRA, 1992).

A estirpe necrótica do PVY no Brasil passou a ser associada a consideráveis perdas nos campos de batata consumo e batata semente, originados de batata importada apenas em meados de 1994 (FIGUEIRA; PINTO, 1995; SOUZA-DIAS; TRISTÃO, 1997). Suspeita-se que essa estirpe agressiva de PVY^{N} tenha entrado no Brasil por meio de batata semente da cv. Achat importada da Alemanha e disseminou-se tão rapidamente que, já em 1996, vários estados brasileiros, como Rio Grande do Sul, Santa Catarina e São Paulo, reportaram incidências de ate 45% (FIGUEIRA; PINTO; MORAES, 1996). Sob as condições de campo brasileiras, esta estirpe foi capaz de infectar de 60% a 90% das plantas, após duas multiplicações da batata semente originalmente importada (FIGUEIRA; MORAES; PINTO, 1996).

Os dados brasileiros do início da década de 1990 não são suficientes para a classificação correta da referida estirpe necrótica como pertencente ou ao grupo de isolados de PVY^{N} não recombinantes ou a um dos grupos de isolados recombinantes (p. ex. PVY^{NTN}, PVY^{N-WI}, $PVY^{N:O}$, etc). No entanto, sabe-se que a primeira evidência da estirpe recombinante PVY^{N-Wi} no país ocorreu em 1997 (MORAES; FIGUEIRA; SANTOS, 1997, 1998), quando três isolados necróticos não foram reconhecidos por anticorpos monoclonais específicos para PVY^{N} (Agdia Inc. USA) e sim pelo monoclonal específico para PVY^{O}, característica sorológica típica da estirpe Wilga (CHZANOSWSKA, 1994). Similarmente aos isolados de PVY^{N-Wi} europeus, estes foram encontrados em altas concentrações se comparados

com os isolados de PVYN (MORAES et al., 1998; MORAES; FIGUEIRA; SANTOS, 1998). Aproximadamente ao mesmo tempo, a estirpe recombinante PVY^{NTN} também foi reportada, pela primeira vez, no Brasil, em uma primeira geração de tubérculos da cv. Atlantic importada da América do Norte (SOUZA-DIAS et al., 1998).

A mudança na escolha das cultivares de batata plantadas no Brasil foi um dos principais reflexos da entrada dessas estirpes no país, uma vez que praticamente todas elas eram muito suscetíveis aos anéis necróticos induzidos nos tubérculos, tornando-os não comercializáveis, devido à perda de qualidade e ao aspecto visual indesejável. A partir de 1994-95, as cultivares tradicionalmente plantadas, especialmente em Minas Gerais, foram progressivamente trocadas: em 1995, a maioria delas era das cultivares Achat e Baraka; em 1996, 24,3% correspondiam à Achat e 12,3% à Baraka, significando, juntas, 36,6% do total plantado. Em 1997, a quantidade plantada dessas cultivares reduziu-se, drasticamente, para 15,7% de Achat e 2% de Baraka apenas. Em contrapartida, as áreas plantadas com outras cultivares, como Atlantic e Monalisa, aumentaram durante esse período (FIGUEIRA et al., 1998; SOUZA-DIAS; SAWAZAKI, 2004), porém, não duraram por muito tempo, especialmente após a disseminação rápida dos recombinantes NTN. Em poucos anos, a cv Monalisa foi quase totalmente substituída pela cv. Ágata, devido ao fato de esta apresentar certa tolerância a certos isolados de PVY no campo, não mostrando sintomas visíveis, apesar de estar infectada (RAMALHO et al., 2010; SANTOS et al., 2010). Esta situação, apesar de confortável para o produtor, pode ser extremamente perigosa, pois tais plantas tornam-se fonte de disseminação silenciosa do vírus e geram uma falsa ideia de resistência, desencorajando o produtor a realizar as devidas medidas preventivas e as análises exigidas pelo Ministério da Agricultura nos tubérculos produzidos.

Uma análise sistemática das estirpes e recombinantes que compõem a população de PVY no Brasil ainda não havia sido feita até o presente trabalho, tendo o mais recente levantamento da presença dessas estirpes no país sido realizado em 2005-2006. Em todos os sete estados brasileiros amostrados, a estirpe PVY^{NTN} foi predominante em comparação a PVY^{O} e PVY^{N}, sendo cerca de oito vezes mais frequente que estas (ÁVILA et al., 2009). Entretanto, nenhuma informação sobre a estirpe PVY^{N-Wi}, sabidamente presente no país desde meados de 1990, foi mencionada neste trabalho, provavelmente devido à metodologia de RT-PCR usada e a não caracterização sorológica dos isolados. Portanto, há ainda uma lacuna de informações a serem preenchidas sobre a diversidade de isolados de PVY que circulam no Brasil, suas origens e vias de entrada no país, além de inferências acerca da evolução sofrida por esses isolados sob a influência do clima e do sistema de produção de batata brasileiros.

3 CONSIDERAÇÕES FINAIS

Os resultados obtidos nesse trabalho mostraram que a diversidade dos isolados recombinantes de PVY que ocorrem no Brasil é muito maior que a esperada e, de forma geral, excede à diversidade destes já relatada na Europa e na América do Norte.

Isolados indutores do severo sintoma de 'broto crespo' em plantas de batata nos campos produtores de Minas Gerais e São Paulo mostraram-se portadores de uma nova estrutura genômica, a qual nunca havia sido relatada anteriormente. Possivelmente, isolados similares e esses aqui classificados como novos recombinantes já estão disseminados por outras regiões do país e sendo identificados apenas como isolados necróticos mais agressivos, devido à incapacidade de distinção destes com o uso apenas das metodologias de RT-PCR e sorologia disponíveis na literatura até o momento. A utilização de um novo *primer* desenhado nesse trabalho será de grande valia para a distinção destes isolados dentre todos os isolados necróticos que circulam no campo. Além disso, a detecção do novo recombinante brasileiro mostra que os isolados de PVY adaptaram-se bem ao clima tropical e estão em constante processo evolutivo. Provavelmente, isso está sendo favorecido pelo fato de se cultivar batata por três períodos sucessivos no Brasil, aumentando as chances de recombinação entre estirpes.

A partir dos resultados obtidos para os demais isolados estudados, coletados originalmente entre os anos de 1985 a 2009, foi possível recriar uma linha histórica da introdução das diferentes estirpes do PVY no Brasil, mostrando as suas implicações e seus efeitos sobre o sistema de produção de batata no país.

Outro resultado evidente é uma tendência de predominância de alguns isolados, como o PVY^{N-Wi}, sobre os demais, indicando uma maior capacidade competitiva no campo. Dando prosseguimento ao presente trabalho, seria importante monitorar esse comportamento das estirpes no campo e continuar caracterizando os isolados que forem sendo encontrados, com características distintas, para acompanhar o processo de evolução genética do PVY no Brasil.

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SEGUNDA PARTE

ARTIGOS CIENTÍFICOS PUBLICADOS E SUBMETIDOS AOS PERIÓDICOS PLANT PATHOLOGY E PLANT DISEASE, RESPECTIVAMENTE

ARTIGO 1 A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY^{E}

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A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY^{E}

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Abstract

Potato virus Y (PVY) strains are defined based on hypersensitive resistance (HR) response in potato indicators, and some PVY isolates represent recombinants between different PVY strains. Two PVY isolates collected in Brazil, PVY-AGA and PVY-MON, were identified with a novel type of genomic pattern, as recombinants between two novel parent genomes, PVY^{NTN} and PVY-NE11. New recombinants had an ordinary PVY^{NTN} genome structure for approximately 6.7-kb from the 5'- end of the genome, and the 3'-terminal 3.0-kb segment had two fragments of NE-11-like sequence separated by another small NTN-like fragment. Only one of the two isolates, PVY-AGA, induced vein necrotic reaction in tobacco. Both PVY-AGA and PVY-MON isolates did not induce HR in potato cultivars carrying *Ny*, *Nc*, or (putative) *Nz* genes, and thus were able to overcome all known resistance genes to PVY. Biological responses in potato indicators and tobacco define PVY-MON as an isolate from the PVY^{E} strain. To distinguish PVY-AGA and PVY-MON from other PVY^{NTN} isolates, a RT-PCR test was developed utilizing new specific primers from the capsid protein gene area and producing a characteristic 955-bp band. Serological profiling of these PVY isolates with three monoclonal antibodies revealed an unusual reactivity, where one of the two commercial N-specific monoclonal antibodies did not recognize PVY-AGA. Ability of these new PVY recombinants to overcome resistance genes in potato producing mild or no symptoms, combined with lack of serological reactivity towards at least one PVY^{N}-specific antibody may present a significant threat posed by these isolates to seed potato production areas.

Introduction

Potato virus Y (PVY) has emerged in the past decade as a serious threat to potato production worldwide, decreasing potato yield and affecting tuber

quality (Blanchard et al., 2008; Gray et al., 2010; Kerlan, 2006). Spread of recombinant strains of PVY inducing potato tuber necrotic ringspot disease (PTNRD) in susceptible potato cultivars (Beczner et al., 1984; Boonham et al., 2002; Chikh Ali et al., 2007; Crosslin et al., 2002; Glais et al., 2002; Karasev et al., 2008; Lorenzen et al., 2006a; Piche et al., 2004; Schubert et al., 2007) contributed to this emergence. One particular recombinant PVY isolate was associated with tuber necrosis in susceptible potato cultivars, and named PVY^{NTN} (Beczner et al., 1984; Kerlan et al., 1999; Kerlan, 2006). PVY is a type member of genus *Potyvirus*, family *Potyviridae*, having a single-stranded, positive-sense RNA genome of about 9.7-kb with a covalently-linked VPg protein at the 5'-end and a poly(A) tail at the 3'-end. The virus encodes a large single polyprotein which is processed into ten mature protein products by three virus-specific proteases (Fauquet et al., 2005). PVY particles contain single species of 267-aa capsid protein (CP).

PVY exists as a complex of strains or isolates which can be distinguished based on their biology in potato indicators or tobacco, whole genome sequences and/or serological reactivity (Kerlan, 2006; Singh et al., 2008). Based on the hypersensitive response (HR) in potato cultivars with known genetic background, PVY^{O} strain was defined as inducing HR in the presence of *Ny* gene, PVY^{C} strain as inducing HR in the presence of *Nc* gene, and PVY^{N} strain as overcoming all known *N* genes; existence of a PVY^{Z} strain was postulated based on a HR in the presence of a putative *Nz* gene (Cockerham, 1970; Jones, 1990; Kerlan et al., 1999; Singh et al., 2008). Whole genome sequences have been determined for PVY^{O} and PVY^{N} strains, both differ by about 8% along their genomes. In addition to these O and N parental genomes, multiple PVY recombinants were found, with genomes composed of segments from O and N sequences, and containing 1-4 recombinant junctions (RJs). The diversity of these O/N recombinants is found relatively limited (Hu et al., 2009a;

Lorenzen et al., 2006a; Ogawa et al., 2008), with only 9 distinct patterns identified recently (Hu et al., 2009a). The two most common types of PVY recombinants are PVY^{NTN}, with 3-4 RJs, and PVY^{N-Wi}, with 1-2 RJs (Glais et al., 2002; Lorenzen et al., 2006a; Nie et al., 2004; Schubert et al., 2007). A novel recombinant type was described by Lorenzen et al. (2008), named NE-11, exemplifying a recombination event between a PVY^{N} sequence and a yet unknown PVY variant. Most of recombinant strains of PVY, including PVY^{NTN} and PVY^{N-Wi}, induce characteristic vein necrotic reaction in tobacco, similar to the PVY^{N} strain, while PVY^{O} strain induces only mosaic and vein clearing (Kerlan, 2006; Singh et al., 2008). Because of this characteristic vein-necrotic reaction, tobacco is often used to distinguish between O and N types of PVY strains (Singh et al., 2008), although recently certain PVY^{NTN} isolates were found incapable to induce vein necrosis in tobacco (Hu et al., 2009b; Robles-Hernandez et al., 2010).

In Brazil, a PVY^{NTN} recombinant strain was reported for the first time in 1997, in seed potato tubers imported from North America (Souza-Dias et al., 1998). Introduction of recombinant strains of PVY dramatically changed the PVY epidemiology in most potato producing States in Brazil (Figueira et al., 2009). Currently, approximately 80% of mosaic symptoms found in production fields in seven Brazil states are induced by PVY^{NTN} isolates, whereas PVY^{O} and PVY^{N} strains are much less frequent (Avila et al., 2007). The origin of the majority of PVY recombinants found in Brazil can be traced to Europe or North America, from where they were likely introduced with shipments of seed potato. However, the composition of the recombinant strains in Brazil have not yet been studied on a systematic basis, and contribution of mutations and new recombination events occurring in Brazil has not been determined. Among the Brazilian isolates reported to date there are some with high similarities with

PVY^{O} and PVY^{N} isolates and also recombinants similar to PVY^{NTN} (Inoue-Nagata et al., 2001; Sawazaki et al., 2009).

In 2007 and 2008 growing seasons, unusually severe symptoms of shoot yellowing and stunting were observed in several potato fields in Minas Gerais and São Paulo States, these included cultivars Asterix, Mona Lisa, and Agata. Three field isolates were collected in 2008, and named PVY-AST, PVY-MON, and PVY-AGA, according to the potato cultivar where each one was collected. All three isolates were characterized biologically, on indicator plants, as well as on a set of indicator potato cultivars with a known genetic background, and also molecularly, using RT-PCR, serology, and whole genome sequencing. Based on combinations of these studied features, all three isolates represent new types of PVY recombinants, with unusual serological, biological, and molecular characteristics, which make their detection and differentiation a challenge. Here, we describe detailed characterization of PVY-AST, PVY-MON, and PVY-AGA, a new recombinant genome structure for PVY-MON and PVY-AGA, and development of a simple RT-PCR based differentiation assay for these two new recombinant PVY genomes.

Material and Methods

Virus Isolates: Origin and Maintenance

The three Brazilian PVY isolates studied were collected from two separate regions, the isolates referred to as PVY-AST and PVY-MON from Vargem Grande do Sul, São Paulo State, whereas the PVY-AGA isolate was from Ipuiuna, Minas Gerais State. PVY-AGA, PVY-AST and PVY-MON were isolated from potato cultivars Agata, Asterix and Monalisa, respectively, which showed severe symptoms of shoot yellowing and stunting. The infected potato plants were collected in the field and transplanted to pots in an insect-proof

greenhouse, tested serologically for four potato viruses (PVY, *Potato leafroll virus*, *Potato virus S*, and *Potato virus X*) and confirmed to be infected only with PVY. Tubers obtained from these originally infected potato plants were stocked and had been periodically multiplied to maintain the original inoculum. These three PVY isolates were also maintained in tobacco, *Nicotiana tabacum* cv Burley, and periodically tested by RT-PCR and ELISA with several monoclonal antibodies as described previously (Hu et al., 2009b; Karasev et al., 2010). No PVY strain mixtures were detected using either strain-specific TAS-ELISA (Karasev et al., 2010) or the standard RT-PCR assay according to Lorenzen et al., (2006b). The standard PVY isolates Tb60 and Oz (PVY^{O}), ID269 (PVY^{O}-O5), Mont (PVY^{N}), 423-3, N4 and L26 (all PVY^{NTN}) and PVY- NE-11 used for comparisons were from the University of Idaho Plant Virology Laboratory collection (Hu et al., 2009b; Karasev et al., 2010; Lorenzen et al., 2008).

Biological characterization on indicator plants and on potato indicators

To investigate the pathotype of Brazilian isolates, the following indicator plants were used: *C. amaranticolor* and three cultivars of *Nicotiana tabacum, cvs*. Burley, Samsun and Xanthi. All plants were mechanically inoculated with sap from PVY-infected tobacco at 4-6 leaf stage as described previously (Hu et al., 2009b). All plants were grown in an insect-proof greenhouse before and after inoculations. Three potato cultivars with known genetic background were used to type the PVY isolates - Desiree, Maris Bard and King Edward. These potato indicators were maintained as virus-free tissue-culture lines at the Potato Tissue Culture Laboratory at the University of Idaho and inoculated mechanically at the six- to ten-leaf stage. The symptom expression of the PVY strains in the cultivars Desiree (expressing the Ny_{tbr} gene), King Edward (expressing the *Nc* gene) and Maris Bard (expressing the Ny_{tbr}, *Nc* and *Nz* genes) is summarized in Table 1 (Jones, 1990; Singh et al.,

2008). Inoculated potato plants were grown in growth chambers under artificial light provided by fluorescent and incandescent lamps with 18h day/6h night cycle and maintained at 23-26°C. The symptom observations started 4-5 days after inoculation and were carried out for 6-8 weeks. To determine the capacity of isolates to induce PTNRD under experimental conditions, the potato cultivar Yukon Gold was used as described previously (Hu et al., 2009b).

Serological analysis

The serological reactivity of the three PVY isolates was tested in a triple antibody sandwich (TAS) ELISA format, as described by Karasev et al. (2010). In addition to a polyclonal antiserum (Karasev et al., 2010), three strain-specific monoclonal antibodies were used, MAb2 recognizing PVY^{O}, $PVY^{N:O}$, and PVY^{C} strains (McDonald and Kristjansson, 1993; Agdia, Elkhart, IN), 1F5 recognizing PVY^{N}, PVY^{O}-O5, and PVY^{NTN} (Ellis et al., 1996; Karasev et al., 2010; Agdia, Elkhart, IN), and SASA-N recognizing PVY^{N}, and PVY^{NTN} (Scottish Agriculture Science Agency, Edinburgh, Scotland).

Cloning and Sequencing, Sequence Analysis

To generate whole genome sequences of the three Brazilian isolates, five primer pairs were designed with the resulting five PCR products covering the entire PVY genome and overlapping for about 100 nucleotides between the fragments (Appendix I). Total RNA was extracted from PVY-infected tobacco tissue and reverse transcriptase (RT) PCR reactions were performed in two separated steps using M-MLV-RT and GoTaq Flexi DNA Polymerase (both from Promega) according to instructions of the manufacturer. The first-strand cDNA was synthesized using reverse primers of each primers pair designed and the reaction was conducted at 42^{O}C for 60 minutes followed by 5 minutes at 95^{O}C and immediate incubation on ice for at least 5 minutes. For the PCR, the

cycling protocol was 2 minutes at 95^{O}C, followed by 30 cycles of 95^{O}C for 1 minute, 58-65^{O}C for 1 minute, 72^{O}C for 2-4 minutes depending on a fragment size, and a final extension of 72^{O}C for 10 minutes. PCR fragments were visualized using 0.7% agarose gels stained with GelRed (Biotium). The five PCR fragments amplified for each isolate were purified using Wizard SV Gel and PCR Clean Up System kit (Promega), cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* (DH5αc). Clones containing each specific insert were sequenced using initially T7 or SP6 universal primers, and followed with PVY-specific primer sequencing. The CLUSTALW2 with default settings was used to align the complete genome sequences of three Brazilian PVY isolates with several other PVY isolates representative of all PVY strains from GenBank. The RDP3 package (Martin et al., 2005) was used to detect recombination in the whole genomes and default parameters were used for all programs implemented (RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, 3SEQ, and SISCAN).

RT-PCR and differentiating primers

To classify these three isolates, three primers described by Boonham et al. (2002) were initially used (O-9295R, O-8687 and N-8687F), this combination allows to separate isolates belonging to PVY^{N} from PVY^{NTN} by the amplification of fragments with 280 and 609bp, respectively. One primer pair specific to PVY$^{N\text{-}Wi}$ was also used, described by Glais et al. (2005) (W2253F/W6343R), and two primer pairs specific to a new recombinant genome PVY-NE11 described by Lorenzen et al. (2008) (NE(N)1979F/NE(NA)2627R and NE(11)8049F/NE(11)9026R). To confirm initial typing and to check for possible mixed infections a multiplex assay described by Lorenzen et al. (2006b) was used. An additional forward primer

was designed based on comparative analysis of PVY-AGA, PVY-MON, PVY-AST, and NE-11 genomes (AGA_8,032F: 5'GAAATCGACAGCTCGTGTGTA3') located between positions 8,032-8,052 in the NIb gene region, and used to distinguish PVY-AGA and PVY-MON, from PVY-AST, PVY- NE-11 and other ordinary PVY^{NTN} isolates.

Results

Biological characterization in tobacco and Chenopodium

Two of the three isolates, PVY-AGA and PVY-AST, induced vein necrosis in addition to mosaic, vein clearing, and stunting in tobacco plants cvs Burley, Samsun, and Xanthi, however, no vein necrosis was induced by PVY-MON. There were no local or systemic symptoms induced in *C. amaranticolor* (Table 2). Based on *Chenopodium* reactions all three isolates should be classified as belonging to the PVY^{N} group, due to their inability to induce local lesions in *C. amaranticolor*. However, reactions in tobacco would confirm this assignment only for PVY-AGA and PVY-AST, while PVY-MON would be assigned as a PVY^{O} isolate due to inability to induce vein necrosis in tobacco (Table 2).

Serological Analysis

All three Brazilian isolates failed to react to the monoclonal antibody MAb2, similarly to other PVY^{N} and PVY^{NTN} isolates, e.g. Mont and L26, used as controls. As expected for an N-type isolate, PVY-MON was recognized by both PVY^{N} specific monoclonal antibodies 1F5 and SASA-N, but the other two isolates, PVY-AGA and PVY-AST, reacted only with the 1F5 antibody, and were not detected when SASA-N monoclonal antibody was used (Table 3). This

serological pattern - MAb2-negative, 1F5-positive, and SASA-N-negative is very unusual, and has not been reported before.

Table 2 Symptoms induced by PVY-AGA, PVY-AST and PVY-MON in different cultivars of *Nicotiana tabacum* and in *Chenopodium amaranticolor*

Hosts Plants	Cultivars	Isolate		
		PVY-AGA	PVY-AST	PVY-MON
Nicotiana tabacum	Burley	Mo++;vn;st	Mo++;vn;st	Mo+; st
	Samsun	Vcl;vn;st	Vcl;vn;st	Vcl
	Xanthi	Vcl;vn;st	Vcl;vn;st	Vcl
C. amaranticolor	-	NS	NS	NS

NS- no visible symptoms; Mo- mosaic; vn- vein necrosis; st-stunting; Vcl-vein clearing; Intensity of symptoms: faint but visible (+); intermediate (++) and severe (+++).

Sequence analysis and phylogenies

The PVY-MON, PVY-AGA and PVY-AST whole genome sequences were determined to be 9,702, 9,702, and 9,703 nucleotides, respectively, excluding the poly(A) tail. The corresponding sequences have been deposited in the GenBank database under the accession numbers JF928458, JF928459, and JF928460, respectively. An initial BLAST analysis of the CP gene sequences for the three Brazilian isolates indicated that the closest match for PVY-AST was among PVY^{NTN} strains, but for PVY-MON and especially PVY-AGA the closest match was isolate NE-11 (Lorenzen et al., 2008). Given the unusual serological reactivity characteristic of all three isolates, and typical PVY^{NTN} pattern (see below) revealed by the standard RT-PCR profiling (Lorenzen et al., 2006b), a new recombinant genome structure for all or some of the three isolates was suspected.

Table 3 Serological reactivity of PVY isolates and different monoclonal antibodies tested in TAS-ELISA.

Isolates (strain)	TAS-ELISA			
	PAb[1]	MAb2[2]	1F5	SASA-N
PVY-AGA	+	-	+	-
PVY-AST	+	-	+	-
PVY-MON	+	-	+	+
Tb-60(O)	+	+	-	-
ID269 (O5)	+	+	+	-
Mont (N)	+	-	+	+
L26 (NTN)	+	-	+	+
PVY-NE-11	+	-	+	+

[1] Polyclonal antibody: PAb UID8 reacts with all PVY strains (Karasev et al., 2010); [2]Monoclonal antibodies: MAb2 specific to PVY^{O} or PVY^{C}, 1F5 specific to PVY^{O}-O5 or $PVY^{N/NTN}$ and SASA-N specific to $PVY^{N/NTN}$

Application of the RDP3 package to the analysis of the genome sequences of PVY-AST, PVY-MON, and PVY-AGA, suggested that one of the three, PVY-AST, was a typical PVY^{NTN} recombinant, with PVY^{O} and PVY^{N} genomes easily identified as its parents. However, the other two genomes, PVY-MON and PVY-AGA, were similar to a typical PVY^{NTN} strain only for the first 6,754 5'-terminal nucleotides, with the remaining 3'-terminal section, nucleotides 6,755-9,703, representing a different recombinant type, where PVY^{O} and PVY^{N} sequences could not be considered parents.

In order to illustrate this discrepancy, three phylogenetic trees were generated, using either whole genomes or partial sequences (Fig. 1, a-c). When a set of full-length PVY genomes including the three genomes sequenced in this paper was used to build the phylogenetic tree, several distinct clusters were

observed (Fig. 1a). The PVY-MON and PVY-AGA isolates grouped in a separate clade, between N/NA-N and NTN lineages. PVY-AST, on the other hand, clustered within the NTN clade very close to other PVY^{NTN} isolates such as 423-3, Hun-NTN and others. In contrast to this whole genome tree, in the phylogenetic tree built for a genomic segment 1-6,754 nt, all three Brazilian isolates tightly clustered within the NTN clade (Fig. 1b). But the most revealing was the third tree, built for the genomic segment 6,755-9,703 nt, where PVY-AGA and PVY-MON isolates clustered in a separate clade close to PVY-NE-11 (Figure 1c). All these clusterings were highly significant and were supported by 100% bootstrap values (Fig. 1, a-c).

Detailed analysis of the whole genome sequences for the three isolates using the RDP3 program showed that PVY-AST had the same three recombination junctions in HC-Pro/P3, CI/NIa and CP/3UTR regions as ordinary PVY^{NTN} isolates. On other hand, PVY-AGA and PVY-MON genomes were identified as new recombinants, for which PVY^{NTN} and PVY-NE-11 sequences should represent two parent genomes. Assuming PVY^{NTN} and PVY-NE-11 as parental genomes for PVY-MON and PVY-AGA, these novel recombinant genomes contained three recombination breakpoints, two of them identical in both isolates and located at positions 6,754 and 7,642, respectively, within the NIa and NIb genes. The third breakpoint was defined by the RDP3 in two slightly different positions, at nt 8,118 for PVY-AGA, and at nt 8,115 for PVY-MON (Table 4). The two new PVY recombinants have most of the genome, nt 1-6,754, identical to an ordinary PVY^{NTN} strain, and another small region, nt 7,643 – 8,115 or nt 7,643-8,118, also derived from PVY^{NTN}. The two remaining segments of the new genome are derived from PVY-NE-11 parental sequences located between nt 6,754-7,642 and nt 8,115-9,703 or nt 8,118-9,703 (Fig. 2).

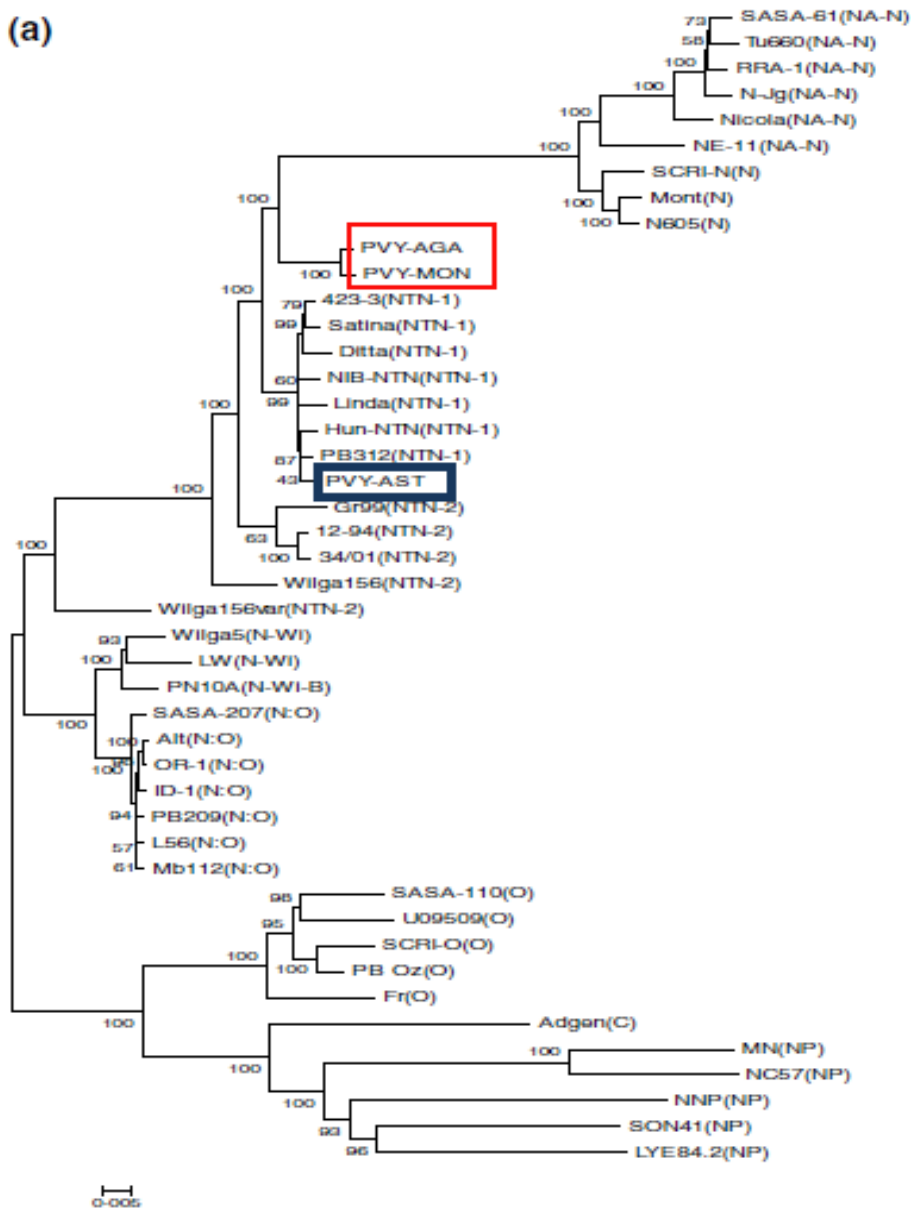

Figure 1 continued…

(b)

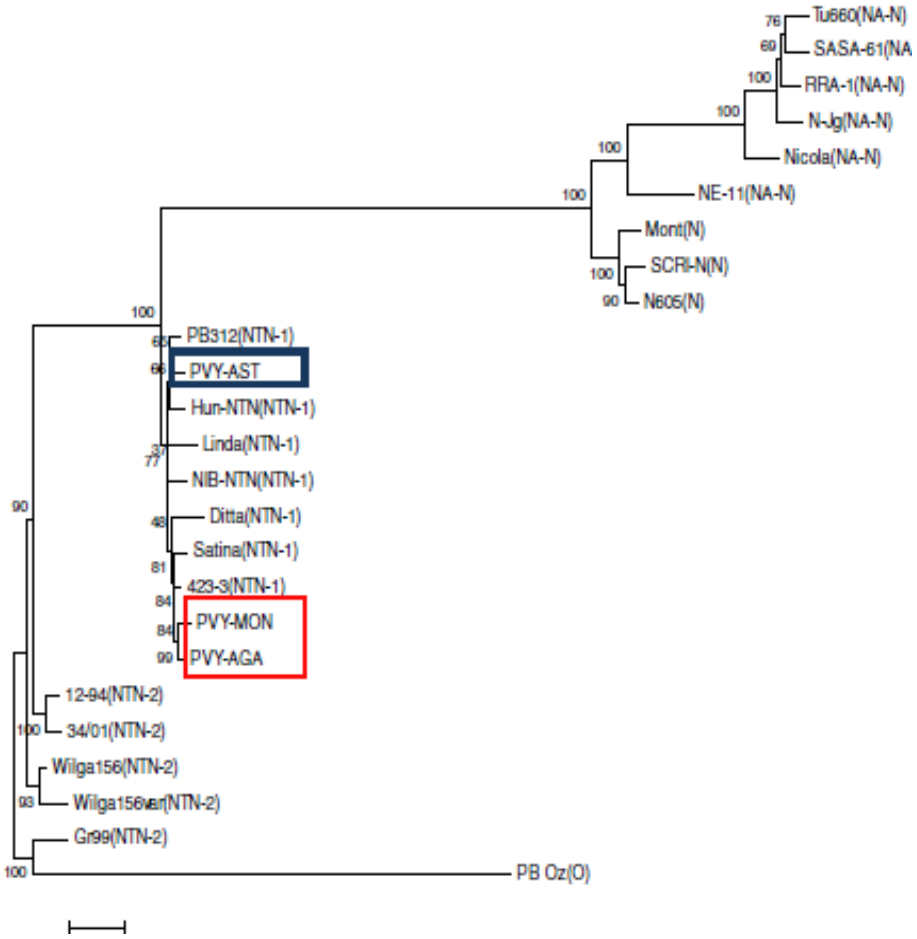

Fig. 1 continued…

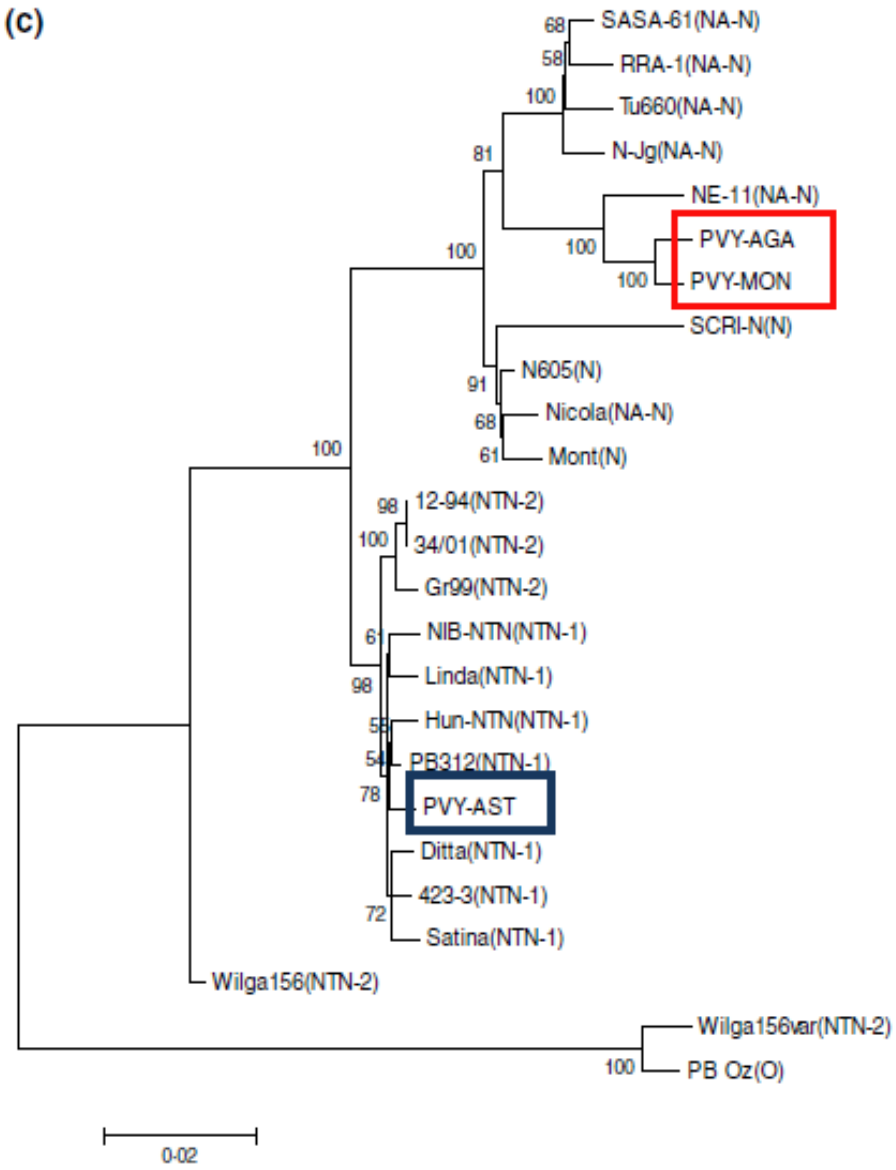

Figure 1 Cladograms of nucleotide sequences of *Potato virus Y* (PVY) isolates. The phylogenetic trees were constructed based on **(a)** whole genome of each isolate; **(b)** genomic segment from 1 to 6754 nt and **(c)** genomic segment from 6755 to 9703 nt

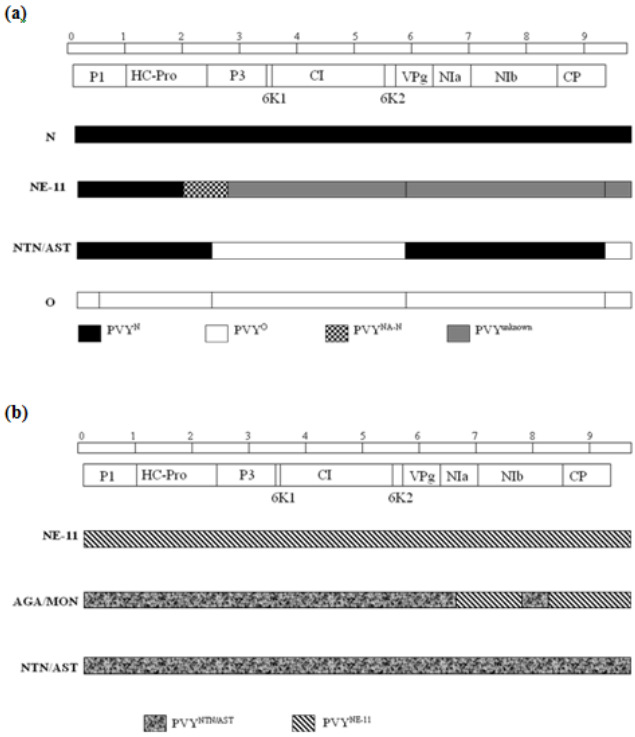

Figure 2 Schematic representation of the PVY recombinants, different types of shading correspond to the same origin of the PVY sequences. **(a)** Comparison of the NTN recombinant genome structure to parental N and O types, and to the NE-11 genome. **(b)** Comparison of the AGA/MON recombinant genome (PVY^{E}) to parental NE-11 and NTN

RT-PCR and differentiating primers

All three Brazilian isolates, PVY-AST, PVY-MON, and PVY-AGA, showed typical PVY^{NTN} profile in the RT-PCR assay performed with the combination of three primers suggested by Boonham et al. (2002), resulting in amplification of a 609-bp fragment. When applied to these same three isolates, the multiplex RT-PCR assay described by Lorenzen at al. (2006b) resulted in amplification of two characteristic fragments typical of PVY^{NTN} (181 and 452

bp). PVY^{N-Wi} specific primers described by Glais et al. (2005) did not produce any bands in RT-PCR when applied to these three Brazilian isolates. An attempt was made to use primers specific to PVY-NE-11 sequence described in Lorenzen et al. (2008). A 648-bp fragment was amplified from all three Brazilian isolates when the first primer pair was used, designed to probe the PVY^{N} / PVY^{NA-N} recombination breakpoint present in PVY-NE-11 genome type. Apparently, this primer pair was not useful for distinguishing the new recombinants from either NE-11 or from PVY^{NTN}. The second primer pair was originally designed to probe a recombinant breakpoint in the CP gene region of PVY-NE-11 (Lorenzen et al., 2008), and was able to amplify a 978-bp fragment only for PVY-NE-11, PVY-AGA, and PVY-MON isolates. Apparently, this second pair was useful to distinguish both PVY-AGA and PVY-MON from PVY^{NTN}, but not from PVY-NE-11.

Since all currently available differentiating PVY-specific primers could not correctly distinguish PVY-AGA and PVY-MON from either PVY^{NTN} or PVY-NE-11 isolates, a new primer was designed, named AGA_8032F, to probe the recombinant junction #3 in both PVY-AGA and PVY-MON genomes (Fig. 3).

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PVY-AGA     8004 CTCTCATCAAGGAGTGCGTTGAGTTTGAAGAAATCGACAGCTCGTGTGTATTCTTTGTCA 8063
PVY-MON     8004 CTCTCATCAAGGAGTGCGTTGAGTTTGAAGAAATCGACAGCACGTGTGTATTCTTTGTCA 8063
NE-11       8003 CTCTCATTAAGGAGTGCGTTGAGTTTGAAGAAATCGACAGCACGTGTGCGTTCTTTGTTA 8062
PVY-AST     8004 CTCTCATTAAGGAGTGCGTTGAGTTTGAAGAAATCGACAGCACGTGTGTATTCTTTGTTA 8063
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Figure 3 Alignment of nucleotide sequences of three PVY Brazilians isolates and NE-11. The region marked in yellow representing the annealing position of the new primer AGA_8,032 ($^{5'}$8,032 - 8,052$^{3'}$) designed to distinguish among PVY-AGA, PVY-MON, PVY-AST, NE-11 and regular PVY^{NTN} isolates.

Using this new primer AGA_8032F in combination with the NE(11)9026R primer described by Lorenzen et al. (2008), it was possible to distinguish PVY-AGA and PVY-MON from PVY^{NTN} isolates PVY-AST, N4, and 423.3, and also from PVY-NE-11 (Fig. 4) in a two-step assay. In the first step, a standard RT-PCR typing was used (Lorenzen et al., 2006b), to distinguish PVY^{NTN} isolates producing two, 181 and 452 bp, bands from PVY-NE-11 producing one, 328-bp, band (Fig. 4, lower panel). In the second step, primer AGA_8032F was used together with NE(11)9026R primer (Lorenzen et al., 2008) to generate a 995-bp amplicon specific only to PVY-AGA and PVY-MON (Fig. 4, upper panel).

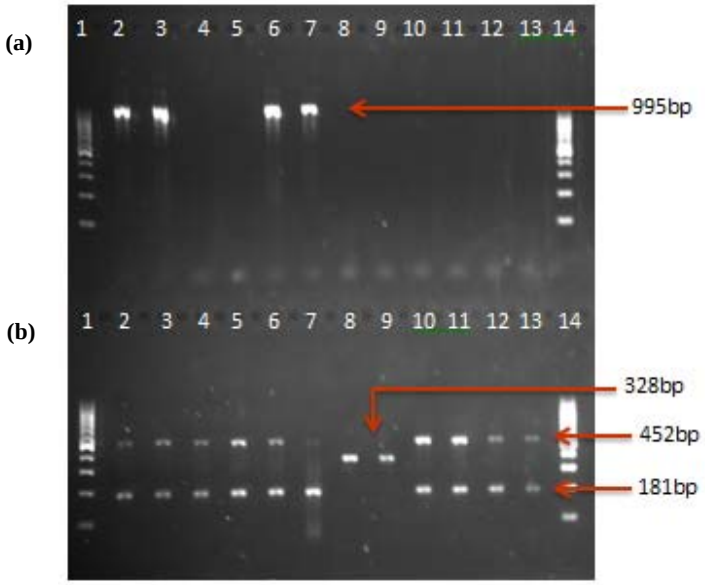

Figure 4 Typing of PVY Brazilian isolates by **(a)** RT-PCR using the primer pair AGA_8032F and NE_9026R (described in ref. 31) and **(b)** multiplex RT-PCR described by Lorenzen et al. (30). The samples were loaded in the following order: (1) and (14) a 100-bp standard ladder; (2) and (3) PVY-AGA; (4) and (5) PVY-AST; (6) and (7) PVY-MON; (8) and (9) NE-11; (10) and (11) N4; (12) and (13) 423-3. Each sample tested represents a separate individual PVY-infected tobacco plant.

Biological characterization in potato indicators

Isolates PVY-MON, PVY-AGA, and PVY-AST induced clear mosaic symptoms on the cultivar King Edward carrying the *Nc* gene, but no HR response, while all three did not induce any visible local or systemic symptoms in the cultivar Desiree carrying the *Ny* gene. In both cultivars, virus could be easily found in upper, systemically infected leaves using RT-PCR or TAS-ELISA with either polyclonal or monoclonal antibodies. These two responses in cultivars King Edward and Desiree suggested that neither isolate belonged to PVY^{C} or PVY^{O} genetic strain groups, respectively.

In the cultivar Maris Bard, carrying three resistance genes, *Nc, Ny,* and *Nz*, PVY-AST isolate induced prominent mosaic with severe crinkling in systemically infected leaves (Fig. 5, A), while PVY-AGA and PVY-MON induced a quite different reaction characterized by a faint mosaic turning into distinct chlorotic spots associated with moderate crinkling (Fig. 5, B). Only for PVY-AST it followed with a clear systemic HR reaction in the lower and middle systemically infected leaves around 4-6 weeks post-inoculation, resulting in massive leaf-drop symptoms (Fig. 5, C), associated with necrotic points and vein necrosis on upper leaves. No HR symptoms were observed in the cultivar Maris Bard inoculated with PVY-AGA and PVY-MON during more than 8 weeks post-inoculation (Table 1; Fig. 5). Taken together, these data indicated that, unlike PVY-AGA and PVY-MON, the typical PVY^{NTN} isolate PVY-AST triggers the HR reaction by interacting with the putative gene *Nz* (Jones, 1990; Singh et al., 2008). Given the symptoms induced in potato indicators, i.e. no HR response due to any of the three resistance genes, and the lack of vein necrotic symptoms in tobacco, the isolate PVY-MON can be defined as belonging to the PVY^{E} strain according to the most recent PVY strain classification (Kerlan et al., 1999; Singh et al., 2008).

Figure 5 Systemic infection induced in the potato cultivar Maris Bard by PVY-AST and PVY-MON isolates. **(a)** necrotic points in upper leaves caused by PVY-AST, **(b)** mosaic turning into chlorotic spots caused by PVY-MON and **(c)** systemic HR response in plants inoculated with PVY-AST but not with PVY-MON isolate, six weeks post-inoculation.

All three isolates, PVY-AST, PVY-AGA, and PVY-MON, induced PTNRD when tested on a susceptible potato cultivar Yukon Gold (Fig. 6). Four weeks after harvesting, tubers collected from infected potato plants showed distinct raised, circular PTNRD lesions typical of other PVY^{NTN} isolates (Gray et al., 2010; Hu et al., 2009b).

Table 1 Symptoms induced by PVY-AGA, PVY-AST and PVY-MON in three cultivars of *Solanum tuberosum.*

Cultivars	Isolate		
	PVY-AGA	PVY-AST	PVY-MON
Desiree (*Ny:nc:nz*)	NS	NS	NS
King Edward (*ny:Nc:nz*)	Mo+	Mo+; crk+++	Mo+
Maris Bard (*Ny:Nc:Nz*)	Mo+++; crk+	Mo+++; Syst HR	Mo+++; crk+

NS- no visible symptoms; Mo- mosaic; crk- crinkling; syst HR-systemic hypersensitive resistance response. Intensity of symptoms: faint (+); intermediate (++) and severe (+++).

Five tubers out of five collected from two plants infected with PVY-MON, two tubers out of two collected from two plants infected with PVY-AGA displayed these characteristic symptoms of PTNRD; three out of three tubers collected from two plants infected with PVY-AST also displayed PTNRD.

Discussion

Since the occurrrence of novel PVY isolates in Europe in the early 1980s shown later to display recombinant genomes (Glais et al., 1998), PVY recombinants spread to most potato producing regions of the world, including the United States (Crosslin et al., 2002; Karasev et al., 2008; Lorenzen et al., 2006a; Piche et al., 2004), Brazil (Figueira et al., 2009; Souza-Dias et al., 1998), and Japan (Chikh Ali et al., 2007; Ogawa et al., 2008).

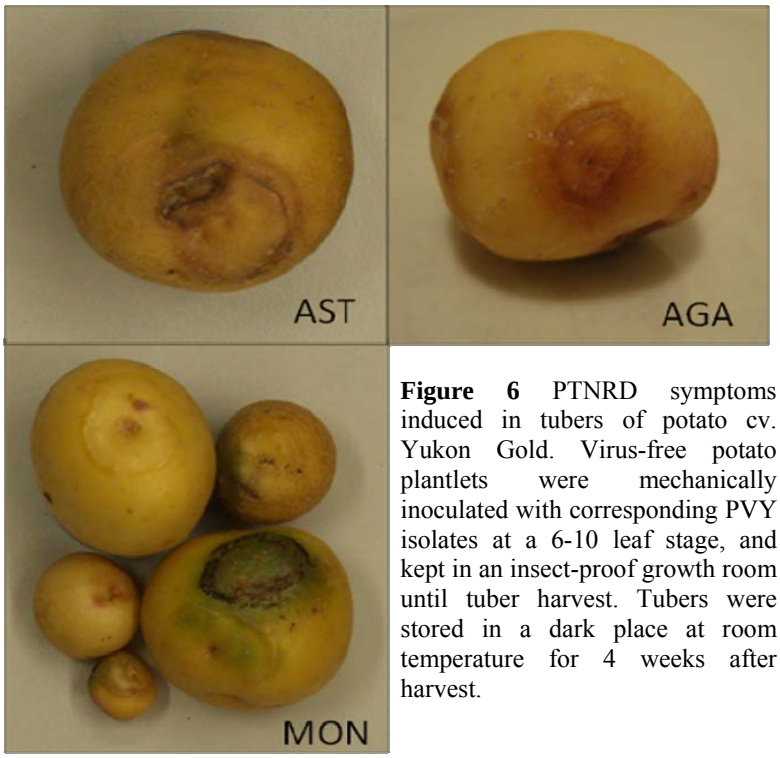

Figure 6 PTNRD symptoms induced in tubers of potato cv. Yukon Gold. Virus-free potato plantlets were mechanically inoculated with corresponding PVY isolates at a 6-10 leaf stage, and kept in an insect-proof growth room until tuber harvest. Tubers were stored in a dark place at room temperature for 4 weeks after harvest.

In Europe, recombinant PVY strains are now dominant, having completely displaced non-recombinant PVY^O and PVY^N strains (Blanchard et al., 2008). A similar situation was recently reported in North Africa (Djilani-Khouadja et al., 2010). Possible factors facilitating the spread of recombinant strains may be introduction of new potato cultivars expressing mild symptoms to PVY infection, selection of PVY strains inducing mild or no symptoms in widely accepted potato cultivars, or selection of isolates which fail to react to the most prominent commercial antibodies. It is important to note that no single PVY detection method can guarantee correct diagnosis and typing of a PVY isolate. For PVY, an additional risk factor is a great diversity of the PVY strain complex providing a huge pool of virus genomes for selection of the best fit

variant in a particular environment. Increased trade in and movement of seed potato between different countries also contribute to the risk of such spread. Indeed, the first interception of the PVY^{NTN} in Brazil was recorded in a potato seed lot acquired from Canada (Souza-Dias et al., 1998).

With all the diversity of PVY strains revealed at the moment, two characteristic features had emerged: a) relatively limited number of recombinant patterns found (Hu et al., 2009a; Lorenzen et al., 2006a; Ogawa et al., 2008); and b) parents identified for most recombinants mainly as PVY^{O} and PVY^{N} genomes (Chikh Ali et al., 2010; Glais et al., 2002; Hu et al., 2009a; Lorenzen et al., 2006a; Nie et al., 2004; Schubert et al., 2007). One distinct exception from this O/N recombinant rule is a novel recombinant named NE-11 (Lorenzen et al., 2008), where one of the parents was identified as a PVY^{N} genome and another as a yet unknown genome, neither of N or O type (Lorenzen et al., 2008). In this work, we demonstrate that PVY genomes identified as recombinant, e.g. PVY^{NTN} and PVY-NE11, may themselves serve as parents in additional recombination events producing new recombinant PVY genomes with new and unique biological, serological, and molecular properties.

This novel type of recombination pattern was clearly demonstrated by sequence analyses using various computer programs for recombination detection, and was visually presented on Fig. 1 (a-c). The newly found recombination structure characteristic of PVY-AGA and PVY-MON required specific methods to distinguish these two isolates from both parental genomes, i.e. PVY^{NTN} and NE-11. New primer AGA_8032F allowed us such differentiation in two steps – first, through conventional RT-PCR using method of Lorenzen et al. (2006b) identifying the NTN genome structure, and second, through an additional RT-PCR step with two primers, our new AGA_8032F and a previously reported primer NE(11)9026R. The parental PVY^{NTN} as well as PVY-AGA and PVY-MON isolates would produce two characteristic bands

during the first step, 181 and 452 bp, while only PVY-AGA and PVY-MON would produce a characteristic 995 bp band during the second RT-PCR step. The second parent, isolate NE-11, would produce only one characteristic 328 bp band during the first step, and no bands during the second RT-PCR step (Fig. 4).

The three Brazilian PVY isolates characterized, PVY-AST, PVY-AGA, and PVY-MON, may be considered an example of the evolution of PVY through new recombinant events creating new types of the PVY genome, like PVY-AGA and PVY-MON (Figs. 1 and 2; Table 4). At the same time, the same three PVY isolates may be also considered examples of the PVY evolution through point mutations leading to changes in serology, creating PVY isolates, PVY-AST and PVY-AGA that fail to react to a PVY^N-specific monoclonal antibody SASA-N (Table 3). This serological pattern - MAb2-negative, 1F5-positive, and SASA-N-negative – had never been reported before for any PVY isolate, and represents a challenge from a regulatory and phytosanitary standpoint. Both monoclonal PVY^N-specific antibodies have been extensively used by quarantine services in regulating international potato trade (Karasev et al., trade (Karasev et al., 2010). Based on the combination of biological reactions on indicator potato cultivars (no HR with any known *N* gene), and reaction in tobacco (no vein necrosis) PVY-MON isolate can be classified as belonging to the PVY^E strain based on criteria proposed by Singh et al., 2008 (Kerlan et al., 1999; Singh et al., 2008). This report, thus, is the first identification of a molecular make-up of the PVY^E strain which previously was only defined through genetic interactions with resistance genes in potato and tobacco (Kerlan et al., 1999; Singh et al., 2008). The ability of the two described recombinant isolates, PVY-AGA and PVY-MON (or PVY^E) to overcome all three known resistance genes in potato, *Ny*, *Nc*, and *Nz*, is especially important, since both of them have the ability to induce PTNRD in susceptible potato cultivars. The incidence and distribution of PVY^E recombinants in Brazil and

elsewhere remains to be determined, given that our RT-PCR based assay can reliably distinguish between PVY^{NTN} and PVY^{E} (Fig. 4).

Since the three isolates studied produced different symptoms in tobacco (Table 2) and potato indicators carrying the putative *Nz* gene (Table 1), an attempt can be made to narrow down the PVY genome regions responsible for vein necrosis in tobacco, on one hand, and responsible for eliciting the potato HR response triggered in the *Nz* gene background, on the other hand. Specifically, since PVY-MON did not induce vein necrotic phenotype in tobacco, we looked at the HC-Pro region known to be involved in vein necrotic reaction in tobacco (Hu et al., 2009b; Tribodet et al., 2005). The two characteristic amino acid residues, K-400 and E-419, necessary for vein necrotic phenotype induction in tobacco (Tribodet et al., 2005) are present in all three isolates, PVY-AST, PVY-AGA, and PVY-MON (not shown). The third amino acid residue, D-205, associated with VN induction in tobacco (Hu et al., 2009b) is also present in all three isolates (not shown). Therefore, we concluded that other regions in the HC-Pro or other PVY genes may also be involved in VN induction by these novel PVY recombinants.

Based on induction of the HR by PVY-AST in potato cultivar Maris Bard, a PVY^{NTN} recombinant, and lack of HR when Maris Bard is infected with PVY-MON (=PVY^{E}), a preliminary conclusion may be made on a location of the PVY genome determinant responsible for triggering HR by the putative *Nz* gene. Given the recombinant structure of the three isolates (Fig. 2), such a determinant may be located somewhere downstream of the nucleotide 6,754, i.e. within the region encoding NIa, NIb, and capsid proteins. On the other hand, since all three isolates, PVY-AGA, PVY-MON and PVY-AST, did induce PTNRD in Yukon Gold, a genetic determinant of this trait would be expected to reside in the virus genome region common for both isolates, between nt 1 to 6,754, and be distinct from the determinant responsible for the HR response

towards the *Nz* gene. However, conclusions on genetic determinants responsible for specific symptoms in tobacco and potato should be considered only preliminary, and will need confirmations through reverse genetics experiments.

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ARTIGO 2 Molecular typing of *Potato virus Y* isolates from Brazil reveals a history of introduction of recombinant strains

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Molecular typing of *Potato virus Y* isolates from Brazil reveals a history of introduction of recombinant strains

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Abstract

In Brazil, *Potato virus Y* (PVY) currently presents a significant problem for potato production, reducing tuber yield and quality. Recombinant, tuber necrotic isolates of PVY had been reported to occur in the country, but no systematic study of the PVY isolate diversity was conducted so far. Here, a panel of 36 PVY isolates, collected in Brazil from potato between 1985 and 2009, was subjected to molecular and serological typing using RT-PCR and a series of PVY^{O} and PVY^{N}-specific monoclonal antibodies. The data collected were combined with biological characterization of the same isolates in tobacco. Of the 36 isolates tested, three were typed as PVY^{O}, ten $PVY^{N:O/N-Wi}$, twenty-one PVY^{NTN}, and two "unusual" or inconclusive. Two PVY^{O} isolates were collected in mid-1980s, and the third PVY^{O} isolate was collected in 1999; no PVY^{O} isolate was collected since then. Of the ten isolates from the recombinant $PVY^{N:O/N-Wi}$ strain group, one isolate was found to have an unusual serological profile identical to the non-recombinant PVY^{O}-O5 strain group. Of the most prolific PVY^{NTN} group tested in this work, one recombinant PVY^{NTN} isolate was initially collected as early as 1996, and since approximately 1999, PVY^{NTN} seems dominant among PVY strain groups circulating in potato in Brazil. The twenty one tested PVY^{NTN} isolates included one isolate that did not induce vein necrosis in tobacco and two isolates with an unusual serological profile, i.e. displaying negative reactivity to one commercial PVY^{N}-specific monoclonal antibody. Taken together, these data suggest that the switch from PVY^{O} to recombinant strains of PVY occurred in Brazil in the mid-1990s, approximately at the same time as in Europe. The two recombinant strains, PVY^{NTN} and $PVY^{N:O}$, now are apparently dominant in Brazilian potato crop. Overall, the

diversity of recombinant PVY strains in Brazil exceeds PVY diversity observed currently in Europe and in North America.

Introduction

Potato virus Y (PVY) is one of the most important pathogens in potato worldwide (27, 21). It affects potato tuber yield (41) and quality, inducing potato tuber necrotic ringspot disease (PTNRD) which makes tubers of susceptible cultivars unmarketable as fresh potato (3). PVY belongs to the genus *Potyvirus*, family *Potyviridae*, and under natural conditions is transmitted by more than 50 species of aphids in a non-persistent manner (cf. 27).

Difficulties in managing PVY in potato and other crops susceptible to PVY, like pepper, tomato, and tobacco, stem to a large extent from a huge diversity in PVY strains, and multiple recombinant virus genomes. Five genetic strain groups of PVY were defined based on hypersensitive response (HR) reactions in a set of potato indicators carrying different *N* resistance genes, and reactions in tobacco, these were PVY^{O}, PVY^{C}, PVY^{N}, PVY^{Z}, and PVY^{E} (9, 24, 45). In addition to these genetic strains of PVY, multiple recombinants were found carrying segments of PVY^{O} and PVY^{N} genomes in various combinations. The most common recombinants found in Europe, North America, and Japan were $PVY^{N:O}$ (one recombinant junction, [RJ]), PVY^{N-Wi} (two RJs), and PVY^{NTN} (3 or 4 RJs) (19, 22). Additional recombinants and variants were reported, like PVY^{NA-N} and PVY-NE-11 (30, 31). Since about early 1980s, a transition was observed in Europe and elsewhere, from predominantly non-recombinant strains like PVY^{O}, PVY^{C}, and PVY^{N}, to recombinant variants like PVY^{NTN} and PVY^{N-Wi}/$PVY^{N:O}$, often associated with PTNRD syndrome (3, 29, 21). Changes in PVY strain composition had important consequences for potato industry in all countries affected by PVY, leading to changes in potato certification systems,

and complete removal from production of potato cultivars susceptible to PTNRD.

To distinguish PVY strain groups and variants, several approaches can be utilized. In addition to the potato indicator test, reactions in tobacco are often used to broadly divide PVY isolates into those inducing vein necrosis, and those inducing only mosaic and vein clearing. Isolates inducing vein necrosis in tobacco generally belong to PVY^{N} strain group, or have recombinant genomes, like PVY^{NTN}, PVY^{N-Wi}, or $PVY^{N:O}$. Nevertheless, recently, PVY^{Z} and PVY^{E} strain groups were demonstrated to have recombinant genomes, and some isolates from these strain groups were found non-necrotic in tobacco (23, 28, 18). Two laboratory methods are also used to distinguish PVY strain groups and variants, these are serology with PVY^{N} an PVY^{O}-specific monoclonal antibodies (MAbs) (11, 26), and reverse-transcription polymerase chain reaction (RT-PCR) methodology utilizing nucleotide polymorphism characteristic of specific PVY strains and recombinant variants (5, 32, 7). Tremendous diversity in PVY strains and rapid evolution of the virus through recombination and mutations created an interesting situation where no single method of PVY strain differentiation is sufficient to type a field isolate of PVY, and multiple methods are necessary to confidently assign such an isolate to a particular strain group (21).

In South America, PVY was first described in the early 1940's, however, it was not considered a problem for potato production at that time (40). Since then, PVY gradually became one of the most important potato viruses in many South American countries, including Brazil. During 1985-87, PVY incidence in the south of Minas Gerais State nearly equaled the *Potato leafroll virus* (PLRV) incidence which was the major potato virus in 1980s, and at the same time some PVY isolates inducing vein necrosis in tobacco were described (15, 16). A few years later, these tobacco necrotic isolates were also found in Brazilian seed potato multiplied from imported seed potatoes (12, 47). These

necrotic PVY isolates apparently spread quickly, and in 1996 several Brazilian States reported problems in potato related to PVY. It is not clear if any of the isolates causing vein necrosis in tobacco, described during this early PVY history in Brazil, belonged to the recombinant PVY strains or the non-recombinant PVY^{N} strain. Hence, the first official evidence of the presence of PVY^{N-Wi} and PVY^{NTN} recombinant strains in Brazil was reported by 2 different laboratories, independently, in 1997, and both in potato seed increases originated from seed potatoes imported from Europe or North America (35, 36, 37, 46). Currently, the PVY^{NTN} and PVY^{N-Wi} have been reported the predominant PVY recombinant strains in potato in almost all Brazilian States (1, 43, 17, 6, 42). Nevertheless, the molecular, serological, and biological diversity among the PVY isolates circulating in Brazil in potato crops was not studied on a systematic basis up until very recently (18).

A recent initial systematic study of just three recombinant PVY isolates collected from potato in 2008 demonstrated the occurrence of a rare PVY^{E} genetic strain in Brazil, and unusual serological profile and/or recombinant structure characteristic of PVY^{E} and PVY^{NTN} isolates circulating in Brazilian potato (18). These initial findings suggested a substantial diversity among potato PVY isolates in Brazil, and circulation of rare and unusual types of PVY recombinants, and prompted our study of a larger set of thirty six PVY isolates collected from field potato and maintained in the Plant Virology Laboratory in the Federal University of Lavras, Minas Gerais, Brazil. Here, we describe the diversity of potato PVY isolates having circulated in Brazil over a span of 24 years, from 1985 to 2009, using a combination of biological, serological, and molecular techniques. The data obtained allowed us to re-create an approximate history of changes in PVY strain composition in potato in Brazil between 1985 and 2009.

Materials and Methods

Virus isolates: origin and maintenance

The 36 Brazilian PVY isolates studied were collected between 1985 and 2009. Locations of the initial field collections and potato cultivars where these isolates originated are listed in Table 1. The infected potato plants were collected in the field and transplanted to pots in an insect-proof greenhouse, tested serologically for four potato viruses (PVY, *Potato leafroll virus*, *Potato virus S*, and *Potato virus X*) and confirmed to be infected only with PVY. Tubers obtained from these originally infected potato plants were stocked and had been periodically multiplied to maintain the original inoculum. All 36 PVY isolates were also maintained in tobacco, *Nicotiana tabacum* cv Burley, and periodically tested by RT-PCR and ELISA with several monoclonal antibodies as described previously (23, 26). Potential PVY strain mixtures were detected using either strain-specific TAS-ELISA (26) or the standard RT-PCR assay according to Lorenzen et al. (32). The standard PVY isolates Tb60 and Oz (PVY^{O}), ID269 (PVY^{O}-O5), Mont (PVY^{N}), 423-3, N4 and L26 (all PVY^{NTN}) and PVY- NE-11 used for comparisons were from the University of Idaho Plant Virology Laboratory collection (23, 26, 31).

Biological characterization in tobacco

To investigate the pathotype of Brazilian isolates, tobacco cvs. Burley and Samsun were used. All plants were mechanically inoculated with sap from PVY-infected tobacco at a 4-6 leaf stage as described previously (23). Control PVY isolates from the laboratory collection were always included into the experiment. Visual scoring of PVY symptoms in tobacco commenced 5 days post-inoculation, and continued for 4-5 weeks. All plants were grown in an

insect-proof greenhouse before and after inoculations under natural light supplemented by fluorescent and incandescent lamps with a day-and-night cycle of 18 and 6 h, respectively, and maintained at 24-26^{o}C/day and 16-18^{o}C/night throughout the experiment.

Table 1. List of PVY isolates studied, grouped according to the year of collection. Potato cultivars where the isolate was collected from and location of the initial collection are also listed.

Isolate ID	Yr collected	Cultivar	Location (city/state)
YO-ANT25	1985	Achat	Maria da Fe /Minas Gerais
PVY-FORTE	1996	Baraka	Maria da Fe /Minas Gerais
YN-UFLA#1	1987	Unknown	Southern Minas Gerais
YN-UFLA#2	1987	Unknown	Southern Minas Gerais
YN-IBIA	1997	Monalisa	Ibiá /Minas Gerais
Y-BR	1996	Achat	Itajubá/Minas Gerais
YN-SP22	1999	Jaette Bintje	Jose Furtado/ São Paulo
YN-VELOX	1999	Velox	Três Corações/Minas Gerais
YO-SP08	1999	Bintje	São Paulo
PVY^{N}-MG	1999	Bintje	Tiros/ Minas Gerais
Y-BR2	2000	Achat	Itajubá/Minas Gerais
SGS-AG	2007	Agata	São Gonçalo Sapucaí/Minas Gerais
ALF-VI	2007	Vivaldi	Alfenas/Minas Gerais
ARA-MO	2007	Monalisa	Araxá/Minas Gerais
TAP-AG	2007	Agata	Tapira/Minas Gerais
COR-AG	2007	Agata	Coromandel/Minas Gerais
IPU-MA	2007	Markies	Ipuiúna/Minas Gerais
LAV-CL	2007	Unknown	Lavras/ Minas Gerais
MFE-AG	2007	Agata	Maria da Fe/Minas Gerais
SGS-MO	2008	Monalisa	São Gonçalo Sapucaí/Minas Gerais
MF-AST	2008	Asterix	Maria da Fe/Minas Gerais
PA2-AGA	2008	Agata	Pouso Alegre/Minas Gerais
LA-MON	2008	Monalisa	Lavras/Minas Gerais
ES-AGA	2008	Agata	Estiva/Minas Gerais
SA-AST	2008	Asterix	Senador Amaral/ Minas Gerais
MU-AGA	2008	Agata	Muzambinho/Minas Gerais
LUI-AGA	2009	Agata	Luiziana/Paraná
PEN-AG2	2009	Agata	Pedrinópolis/Minas Gerais
PED-AX	2009	Asterix	Pedralva/Minas Gerais
MAF-CA	2009	Caesar	Maria da Fe/Minas Gerais
PED-CA	2009	Caesar	Pedralva/Minas Gerais
CRI-AX	2009	Asterix	Cristina/Minas Gerais
MAF-VOY	2009	Voyager	Maria da Fe/Minas Gerais
DOV-AG	2009	Agata	Dom Viçoso/Minas Gerais
PED-AG	2009	Agata	Pedralva/Minas Gerais
MAF-VIV	2009	Vivaldi	Maria da Fe/Minas Gerais

Serological analysis

The serological reactivity of PVY isolates was tested in a triple antibody sandwich (TAS) ELISA format, as described by Karasev et al. (26). All tests included control PVY isolates from the laboratory collection, displaying distinct serological patterns characteristic of PVY^{O}, PVY^{N}, and PVY^{O}-O5 strain groups. In addition to a polyclonal antiserum (26), three strain-specific monoclonal antibodies were used, MAb2 recognizing PVY^{O}, $PVY^{N\text{-}Wi/N:O}$ and PVY^{C} (33; Agdia, Elkhart, IN), 1F5 recognizing PVY^{N}, PVY^{O}-O5, and PVY^{NTN} (11; 26; Agdia, Elkhart, IN), and SASA-N recognizing PVY^{N} and PVY^{NTN} (Scottish Agriculture Science Agency, Edinburgh, Scotland).

RT-PCR and differentiating primers

To classify all 36 isolates, and to check for possible mixed infections, a multiplex assay described by Lorenzen et al. (32) was used. In this assay, eight primers are combined in a single multiplex RT-PCR test to differentiate PVY^{O}, PVY^{N}, $PVY^{N:O}$, PVY^{NTN}, and $PVY^{NA\text{-}N}$, or mixtures of those. An additional primer pair was used to differentiate PVY^{E} genome from PVY- NE-11 and ordinary PVY^{NTN} isolates as described elsewhere (18). Virus RNA extraction and the multiplex RT-PCR assay were performed as described (32). About 100 mg of tobacco leaf tissue was homogenized using a mini-pestle in an Eppendorf tube containing 400 µl of fresh extraction buffer (Dellaporta I: 100mM Tris pH8.0, 50mM EDTA, 500mM NaCl and 10mM 2-mercapto-ethanol), then 52.8 ul of 10% sodium dedecyl sulfate (SDS), followed by vortexing and incubating at 65^{o}C for 10 min. After the addition of 128 µl of 5M KOAc, the mixture was vortexed, and subjected to low-speed centrifugation for 10 min. The resulting supernatant was carefully removed and nucleic acids precipitated with isopropanol. The pellet was washed twice in 70% ethanol, air-dried, and resuspended in 80 µl of DEPC-treated water. Reverse transcription was

performed using 1 µl of the total nucleic acid extract in a 15-µl reaction volume that contained 5x first-strand buffer (Invitrogen, CA), 1 mM (each) dNTP, 0.12 µM oligo-dT primer mix, 6 units RNase Out Ribonuclease Inhibitor (Invitrogen), and 60 units of SSII reverse transcriptase (Invitrogen).

Results

Biological characterization in tobacco

All 36 isolates were tested in two tobacco cultivars, Burley and Samsun, following mechanical inoculation at the 4-6 leaf stage. Symptoms were consistent between cvs Burley and Samsun, and both the type of symptoms and timing of their appearance were very similar for each isolate. Eight of the isolates tested induced vein clearing, various . degree of mosaic and leaf mottling, but no vein necrosis (Table 2; Fig. 1). On the other hand, twenty nine of the isolates induced vein necrotic reaction in both tobacco cultivars, sometimes visible in just 5-7 days post-inoculation (dpi), in addition to the vein clearing and mosaic (Table 2; Fig. 1). This vein necrosis generally coincided with stunting, sometimes very severe, and often with stem necrosis. If a younger plant was inoculated, at a 4-leaf stage, it could be completely necrotized and dead at about 10 dpi. Each plant was scored daily for visual symptoms starting at 5 dpi, and later tested by TAS-ELISA and RT-PCR in order to confirm the PVY infection status, and to type the PVY isolate infecting the specific plant. In each experiment, four plants per isolate were inoculated, and testing was repeated three times for each isolate. No inconsistencies or cross-contaminations were identified for more than 360 inoculated tobacco plants. In just a few cases inoculations failed and these plants were excluded from further experiments.

Serology of the Brazilian isolates

Serological typing of the Brazilian isolates under study is summarized in Table 2. Of the 36 isolates analyzed, 14 bound the PVY^{O}-specific antibody Mab2 and were thus typed as having the PVY^{O} serotype. Twenty two isolates reacted positively to the PVY^{N}-specific MAb 1F5, and nineteen of these were also positive against another PVY^{N}-specific MAb SASA-N. One of the 1F5-positive isolates, MAF-VOY, was also positive against PVY^{O}-specific Mab2, and negative against MAb SASA-N, it was thus typed as having a characteristic PVY^{O}-O5 serotype (26).

Two other 1F5-positive isolates, SGS-MO and MU-AGA, did not bind the second PVY^{N}-specific antibody SASA-N, while being Mab2-negative; this identified both isolates as having a characteristic PVY-AST serotype described elsewhere (18). In summary, fourteen isolates were typed as having PVY^{O} serotype, one was typed as PVY^{O}-O5, and twenty one isolates as having broadly defined PVY^{N} serotype, i.e. Mab2-negative, 1F5-positive, SASA-N-positive or negative (Table 2).

Molecular typing by RT-PCR

The 36 isolates were typed using the multiplex assay developed by Lorenzen et al. (26) which can distinguish between main recombinant and non-recombinant potato PVY strain groups, and their mixtures. The summary of this RT-PCR typing is presented in Table 2. Three isolates were typed as non-recombinant PVY^{O} type, one as $PVY^{NA\text{-}N}$ type, ten as recombinant $PVY^{N:O}$ type, twenty one as recombinant PVY^{NTN} type, and one, Y-BR, as an unusual type (Fig. 2). This unusual RT-PCR profile included only one of the two bands characteristic of PVY^{O}, and the $PVY^{NA\text{-}N}$ band; in this case, the possibility of mixed infection was excluded based on the clear PVY^{O} serological typing for this isolate. To help further type this isolate, Y-BR, we used individual primer

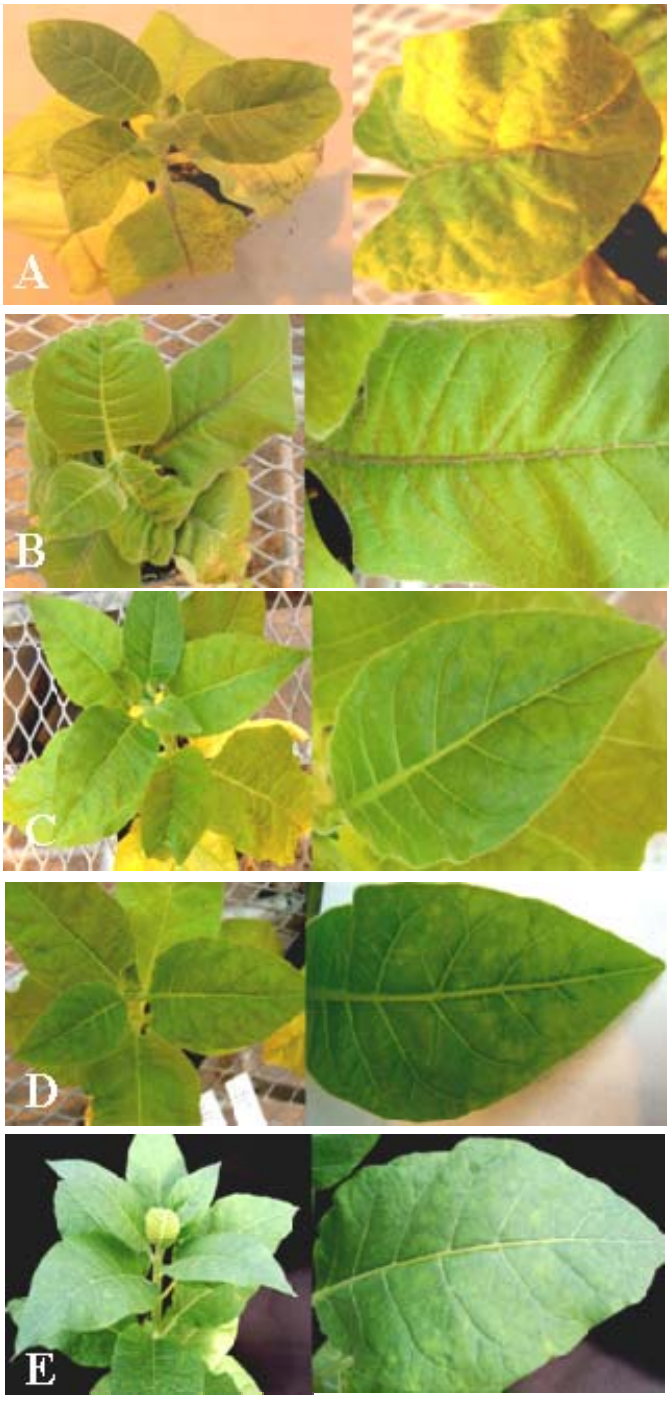

Figure 1 Symptoms induced by five Brazilian isolates in tobacco (cv. Samsun). **A,** MAF-VOY (PVYN:O-O5); **B**, TAP-AG (PVYNTN); **C**, YO-SP08 (PVYO); **D**, LAV-CL (PVYN:O - minus); **E**, MF-AST (PVYZ-NTN).

Table 2 Summary of biological, serological, and molecular typing of the 36 Brazilian isolates studied

	Tobacco[a)]	Serology[b)]				
Isolate ID	**symptoms**	MAb2	1F5	SASA-N	**RT-PCR[c)]**	**Final call**
YO-ANT25	M	+	-	-	O	O
PVY-FORTE	VN	-	+	+	NTN	NTN
YN-UFLA#1	SM	+	-	-	NA-N	?
YN-UFLA#2	FM	+	-	-	O	O
YN-IBIA	VN	-	+	+	NTN	NTN
Y-BR	SM	+	-	-	NA-N/O	?
YN-SP22	VN	-	+	+	NTN	NTN
YN-VELOX	VN	-	+	+	NTN	NTN
YO-SP08	M	+	-	-	O	O
PVY^{N}-MG	VN	-	+	+	NTN	NTN
Y-BR2	VN	+	-	-	N:O	N:O
SGS-AG	VN	+	-	-	N:O	N:O
ALF-VI	VN	-	+	+	NTN	NTN
ARA-MO	VN	-	+	+	NTN	NTN
TAP-AG	VN	-	+	+	NTN	NTN
COR-AG	VN	-	+	+	NTN	NTN
IPU-MA	VN	-	+	+	NTN	NTN
LAV-CL	FM	+	-	-	N:O	N:O
MFE-AG	VN	+	-	-	N:O	N:O
SGS-MO	VN	-	+	-	NTN	NTN/AST
MF-AST	M	-	+	+	NTN	NTN/Z
PA2-AGA	VN	-	+	+	NTN	NTN
LA-MON	VN	-	+	+	NTN	NTN
ES-AGA	VN	-	+	+	NTN	NTN
SA-AST	VN	-	+	+	NTN	NTN
MU-AGA	VN	-	+	-	NTN	NTN/AST
LUI-AGA	VN	-	+	+	NTN	NTN
PEN-AG2	VN	-	+	+	NTN	NTN
PED-AX	VN	+	-	-	N:O	N:O
MAF-CA	VN	+	-	-	N:O	N:O
PED-CA	VN	+	-	-	N:O	N:O
CRI-AX	VN	-	+	+	NTN	NTN
MAF-VOY	VN	+	+	-	N:O	N:O/O5
DOV-AG	VN	+	-	-	N:O	N:O
PED-AG	VN	-	+	+	NTN	NTN
MAF-VIV	VN	+	-	-	N:O	N:O

[a)] Tobacco reaction in cvs Burley and Samsun; M – mosaic/vein clearing, VN – vein necrosis; Intensity of mosaic reaction: F – faint, M – regular, S – severe; [b)] Serological reactivity in TAS-ELISA when the listed monoclonal antibodies were used as detecting antibodies; [c)] RT-PCR typing was conducted according to Lorenzen et al., 2006b.

pairs designed to type PVY^{O} and PVY^{NA-N}, in four different PCR assays (26). In the first assay, the isolate was tested using a single primer pair specific to PVY^{NA-N} (n_5707+A6032m) which consistently amplified a characteristic NA-N band (328-bp), and in the second assay, two specific PVY^{O} primer pairs (S5585m+o6266c and o2172+o2439) were used separately, with only o2172+o2439 primers generating one band (267-bp) characteristic of PVY^{O}. Thus, the primer pair responsible for amplification of the larger band, 689-bp (S5585m+o6266c) characteristic of the PVY^{O} sequence, consistently failed to produce the respective amplicon in RT-PCR.

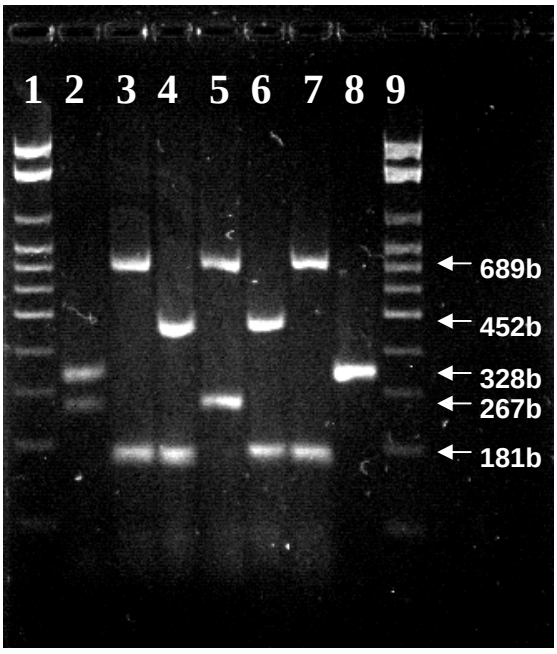

Figure 2 Molecular typing of seven representative Brazilian PVY isolates using multiplex RT-PCR (Lorenzen et al., 2006). From left to right: 100bp molecular size ladder, Y-BR ("unusual" PVYNA-N/O pattern), MAF-VOY (PVYN:O-O5), MF-AST (PVYZ-NTN), YO-SP08(PVYO), CRI-AX (PVYNTN), MAF-VIV (PVYN:O), YN-UFLA#1 (PVYNA-N), 100bp molecular size ladder.

Discussion

Brazil is one of the largest producers of ware potato in South America, however, it is currently dependent on seed potato importation necessary for subsequent multiplication and propagation in certified seed potato fields. Large volumes of imported potato seed may certainly carry a risk of introduction of potato pathogens, including the new recombinant PVY strains. The main sources of seed potatoes imported by Brazilians producers are located in Europe, Canada, and United States (13, 14) where a wide range of PVY types had been reported since 1980s (3, 8, 34, 20, 29, 19, 38, 39, 30, 44, 2, 22). Recently, it was shown that the PVY isolates in Brazil were dominated by recombinant strains PVY^{NTN} and $PVY^{N\text{-}Wi}$ (1), similarly to what was reported in Europe and North Africa (4, 10). In particular, the PVY^{NTN} was found the most frequent among these recombinant strains, in at least seven different States of Brazil (1). Galvino-Costa et al. (18) reported on a rare PVY^{E} strain found in two States in Brazil, and an unusual serological profile characteristic of two isolates from PVY^{E} and PVY^{NTN} strains. However, presence of other PVY strains and variants, like $PVY^{N:O}$, PVY^{Z}, PVY^{O}-O5, and $PVY^{NA\text{-}N}$ as well as the general PVY diversity among isolates circulating in Brazilian potato have not been studied so far.

In order to address the diversity of PVY isolates circulating in potato in Brazil and trace the history of PVY strain composition changes, we systematically characterized the set of 36 potato PVY isolates collected from seed potato fields in three States of Brazil during the span of 24 years, between 1985 and 2009. All 36 isolates were tested for their symptoms in tobacco, subjected to serological profiling using three monoclonal antibodies specific to PVY^{O} or PVY^{N} serotypes, and molecularly typed using the standard, multiplex RT-PCR protocol of Lorenzen et al. (32). No non-recombinant PVY^{N} strain

isolates were found among the 36 isolates in the collection, and only three non-recombinant isolates belonging to the ordinary strain PVY^{O} were identified (Tables 1 and 2). Interestingly, all three PVY^{O} isolates were collected between 1985 and 1999, and no PVY^{O} isolate was collected after 1999. The first recombinant isolate, PVY-FORTE, collected originally in 1996 from potato cultivar Baraka, had typical PVY^{NTN} features, i.e. PVY^{N} serotype, two characteristic recombinant junctions in its genome identified by RT-PCR, and induced vein necrosis in tobacco (Table 2). Four additional PVY^{NTN} isolates (YN-IBIA, YN-SP22, YN-VELOX, and PVY^{N}-MG) were collected between 1997 and 1999 from four different potato cultivars, suggesting a sudden influx of PVY^{NTN} isolates into seed potato system in Brazil in late 1990s. Another recombinant PVY type, $PVY^{N:O/N-Wi}$, was first collected later, only in 2000 (isolate Y-BR2).

Two of the isolates from these early collections conducted between 1987 and 1996 (YN-UFLA#1 and Y-BR) seem to be of an unusual PVY type, simultaneously displaying features typical for PVY^{NA-N} and PVY^{O} (Table 2; Fig. 2). Both isolates induced severe mosaic and crinkling symptoms in tobacco but no vein necrosis was observed, both reacted only with the MAb2 monoclonal antibody showing serotype PVY^{O}, however the RT-PCR pattern in the multiplex assay according to Lorenzen et al. (32) revealed a single, 328-bp amplicon characteristic of PVY^{NA-N}. In the case of Y-BR isolate, this 328-bp amplicon was present in addition to the 267-bp amplicon characteristic of the PVY^{O} type (Table 2; Fig. 2). All possibilities of mixtures were tested: a) of the seven different monoclonal antibodies including PVY^{N}, PVY^{O}, and $PVY^{O/C}$ specific MAbs tested, only the O-specific antibodies reacted with both YN-UFLA#1 and Y-BR; and b) a series of RT-PCR assays using the primer pairs specific to PVY^{NA-N} and PVY^{O} types were conducted separately (not in a multiplex format) always producing the same bands amplified in a multiplex

assay. Isolates YN-UFLA#1 and Y-BR have been currently assigned an "unusual" status, and the final classification of these two isolates will await for the whole genome sequencing and subsequent recombination analysis.

A large group of twenty five PVY isolates collected between 2007 and 2009, contained only the PVY^{NTN} and $PVY^{N:O/N-Wi}$ recombinant strains, with PVY^{NTN} being almost twice more frequent than $PVY^{N:O/N-Wi}$ strain (Table 2). Among the $PVY^{N:O/N-Wi}$ isolates recognized only by the O–specific MAb, one isolate, LAV-CL, induced only mosaic and vein clearing (Fig. 1). Although this isolate did not induce vein necrosis in tobacco, it had a typical N:O profile amplified in the RT-PCR multiplex assay (32), which indicated it belonged to a relatively rare PVY strain group called "N:O-minus" (21). Such an isolate could be easily misidentified as an ordinary PVY^{O} if only biological and serological diagnostic tests had been taken into account. One recombinant isolate, MAF-VOY, displayed a typical N:O band pattern in the multiplex RT-PCR assay and induced vein necrosis in tobacco (Table 2; Fig. 1), but had a recently described serotype PVY^{O}-O5, e.g. reacting to MAb2 and 1F5 but not to SASA-N (Table 2; 26). Up until now, this specific serotype, PVY^{O}-O5, had never been reported outside the United States (25, 26). Isolate MAF-VOY is especially unusual because this is the first documented case where PVY^{O}-O5 serotype is associated with a recombinant PVY genome, $PVY^{N:O/N-Wi}$ in this case (Fig. 2), while all previous reports found this serological marker exclusively in non-recombinant, PVY^{O}-type genomes (25, 26).

Of the 21 recombinant isolates typed as PVY^{NTN}, twenty induced severe vein necrosis in tobacco. One isolate, MF-AST, did not induce vein necrosis in tobacco (Fig. 1) and was thus provisionally classified as PVY^{Z}-NTN due to a typical PVY^{NTN} band pattern in RT-PCR (Fig. 2), and a typical PVY^{N} serotype (Table 2). This assignment is based on a recent characterization of the molecular make-up of the PVY^{Z} strain group, which apparently comprises recombinant

PVY^{NTN} isolates which do not induce vein necrosis in tobacco (23, 28). Two other PVY^{NTN} isolates, SGS-MO and MU-AGA, shared an unusual serotype recently described for a recombinant PVY^{NTN} isolate PVY-AST from Brazil (MAb2-negative/1F5-positive/ SASA-N-negative) (18) indicating this serotype may be relatively common in the PVY isolates circulating in Brazil.

If the entire set of the PVY isolates collected over the years in Brazil and maintained at the University of Lavras collection (Tables 1 and 2) is analyzed from a historical perspective, we can conclude that an influx of recombinant PVY strains in the mid-to-late 1990s completely changed composition of PVY strains circulating in potato in Brazil, and eventually displaced non-recombinant PVY^{O} type with recombinant PVY^{NTN} and $PVY^{N\text{-}Wi}$ types. It is likely that the switch from PVY^{O} to recombinant strains of PVY occurred in Brazil in the mid-1990s, almost at the same time as in Europe (4). If only the isolates collected most recently (2007-2009) are analyzed, a new, on-going switch in isolate composition, from PVY^{NTN} to $PVY^{N\text{-}Wi}$, may be hypothesized. Currently, the PVY^{NTN} is considered prevalent in several local Brazilian PVY populations (1), however, among the ten isolates collected by us in 2009, only four belonged to PVY^{NTN} recombinant strain group while the other six belonged to $PVY^{N:O/N\text{-}Wi}$ (Table 2). That is the first time $PVY^{N\text{-}Wi}$ was found more frequently than PVY^{NTN} in potato in Brazil, although the number of samples is certainly not sufficient to formulate a definite conclusion about the current epidemiologic distribution of PVY strains in the country. This study should be considered only preliminary indication that the PVY strains and recombinants populations in Brazil are not static and continue to evolve over time. It appears that for some reason, the population of $PVY^{N\text{-}Wi}$, which has long been known to enter the country in mi-1990s, started to outperform PVY^{NTN} isolates.

The introduction of recombinant strains in Brazil led to many changes in the potato production system, both seed and ware potato, it especially affected

the choice of cultivars, shifting it to less susceptible to PTNRD. One of the unintended consequences of this was the introduction and spread of cv. Agata, due to its failure to show foliar and tuber symptoms when infected by the majority of PVY strains and recombinants. Although the official report of necrotic recombinant strains had been published in late 1990s (46, 35, 36) these recombinant isolates could have been present in Brazil a few years before that, as exemplified by the isolate PVY-FORTE collected in 1996. The principal route of entry for all these PVY strains and recombinants into Brazil has likely been via seed potato importation, and it still is an open door for new recombinants of PVY to enter and evolve under the favorable conditions of the tropical potato production system in Brazil.

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