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ORIGINAL ARTICLE

Characterization and genetic variability of coat protein genes of *Apple chlorotic leaf spot virus* isolates from southern Brazil

Osmar Nickel[1] · **Fabio N. Silva**[2] · **Thor V. M. Fajardo**[1] · **Eduardo S. Gorayeb**[2]

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Abstract *Apple chlorotic leaf spot virus* (ACLSV) infects temperate rosaceous fruit trees worldwide and causes a wide range of diseases that are economically highly damaging. This study was carried out to analyze genetic variability of regional ACLSV isolates and compare it with ACLSV isolates from other parts of the world. Nineteen amplicons of ACLSV, corresponding to the coat protein (CP) gene of isolates from apple, plum, and nectarine, from two states in southern Brazil, have been analyzed for genetic variation and compared phylogenetically among themselves and with other sequences available in GenBank. Sequences identities among complete CP genes of these isolates ranged from 87.5 to 100% and 92.2 to 100% at the nucleotide (nt) and the deduced amino acid (daa) levels, respectively. In the comparison with isolates from Asia, Europe and North America, identities were 68.4 to 100% and 72.5 to 100% at nt and daa levels, respectively. Phylogenetic trees based on nucleotide sequences showed that these isolates grouped into two clusters, cluster 1 containing apple isolates and cluster 2 comprising apple, plum and nectarine isolates. Most Brazilian isolates showed conserved signatures (Ser^{40}, Leu^{59}, Tyr^{75}, Thr^{130} and Leu^{184}) in their CPs, which place them with type B6 isolates. However, some Brazilian isolates were found to be variants of type B6. These analyzes indicated that Brazilian isolates had lower genetic variability compared to isolates from China, India and Japan and that the CP genes were under negative selection. The greatest diversity of nucleotides was observed in the central portion of the CP gene, represented predominantly by synonymous substitutions. One natural recombinant was detected among ACLSV isolates from Brazil.

Keywords ACLSV · Molecular characterization · Variability · Phylogeny · Recombination · Selection

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✉ Osmar Nickel
osmar.nickel@embrapa.br

[1] Embrapa Uva e Vinho, Bento Gonçalves, RS 95701-008, Brazil

[2] Programa de Pós-graduação em Produção Vegetal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Lages, SC 88520-000, Brazil

Introduction

Apple chlorotic leaf spot virus (ACLSV) occurs worldwide in several fruit tree species, including apple, pear, quince, cherry, peach, plum, apricot and almond. Although latent in most commercial varieties, it causes a wide range of disorders. In sensitive cultivars of *Malus*, symptoms may include asymmetric leaf distortion, leaf drop, chlorotic leaf spots, ring and line patterns on foliage, stunting, bark necrosis and xylem pitting. In sensitive cultivars of *Prunus*, symptoms include graft incompatibility, necrosis and early decline of peach, plum pseudopox and internal necrosis of fruits, premature fruit fall, cracking and splitting of bark. Usually, severity of symptoms is a function of a viral strain's virulence, the plant species/variety and the presence of mixed infections (Németh 1986; Jelkmann and Kunze 1995; Hadidi and Barba 2011). Incidence of latent infections in apple may reduce quality and yield of crop in nurseries and orchards up to 30% (Cembali et al. 2003). In southern Brazil, the virus is generally associated with scion-rootstock incompatibilities and decline of apple plants grafted on Maruba kaido (*Malus prunifolia* var. ringo) rootstocks (Nickel et al. 2001), most commonly in

mixed infection with other latent viruses, a disfunction similar to apple topworking disease (Yanase et al. 1990). Heavy economic losses have occurred as a consequence of the common use of this highly virus-sensitive rootstock in the main apple producing areas.

ACLSV, the type species of the genus *Trichovirus*, family *Betaflexiviridae*, possesses filamentous, flexuous particles of 680–780 × 12 nm, containing a single-stranded, positive sense RNA, about 7.5 kb nt in length (German et al. 1990; Yaegashi et al. 2011), excluding the polyadenylated tail at its 3′ end. The virus genome contains three overlapping open reading frames (ORFs). ORF 3 encodes the coat protein (CP) of 21.5 kDa (Sato et al. 1993; Yoshikawa 2001). Studies on the characterization of genomic regions as well as the determination of genetic variability are important for understanding how viruses evolve, for the prediction of epidemics, for recommending control measures and for the development of reliable tools for diagnosis. There are no studies using the descriptors of genetic variability of ACLSV isolates. The few studies available in the literature assess local/regional populations for nucleotide and amino acid variation based on sequence comparisons and phylogeny (Rana et al. 2010; Gadiou et al. 2010). The N-terminal region of the ACLSV CP gene is considered to be among the most variable regions of ACLSV (German-Retana et al. 1997; Gadiou et al. 2010).

We undertook an analysis of genetic variability of the CP genes of Brazilian ACLSV isolates from apples, plum and nectarine in order to increase knowledge on their variability and qualify the support for diagnosis, molecular characterization and disease control.

Material and methods

Plant material, virus isolates, nucleic acid extraction, RT-PCR, cloning and sequencing

Apple production in Brazil is concentrated in hubs of high altitude (817 to 1360 m) in three southern states, Rio Grande do Sul (RS) (Vacaria, Latitude 28°50′83″ S, Longitude 50°94′ 23″ W; Veranópolis, 28°94′27″ S, 51°55′07″ W), Santa Catarina (SC) (Caçador, 26°77′65″ S, 51°01′25″ W; São Joaquim, 28°29′25″ S, 49°93′76″ W) and Paraná (PR), (Lapa, 25°46′ 11″ S, 49°42′57″ W; Palmas, 26°29′03″ S, 51°59′26″ W).

Nineteen different plant samples were collected in commercial orchards and research stations in SC and RS. Data on the characterized regional ACLSV isolates, GenBank accession numbers and host plants used in this study are listed in Table 1. Total nucleic acids were extracted from bark scrapings and young leaves. Samples were powdered in liquid nitrogen, macerated in the presence of grinding buffer (4 M guanidine hydrochloride, 0.2 M NaOAc pH 5.2, 25 mM EDTA pH 8.0, 1.0 M KOAc and 2.5% *w*/*v* PVP-40) (Mackenzie et al. 1997) and adsorbed to silica particles (Rott and Jelkmann 2001). First strand cDNA synthesis reactions were carried out with Moloney murine leukemia virus reverse transcriptase (Invitrogen, 200 U/μl), using an oligo-dT primer or reverse primer CL7365r (5′CTAAATGCAAAGAT CAGTCGAC 3′). PCR thermocycling (PTC100, MJ Research Inc.) was carried out under conditions described previously (Silva et al. 2008), using primers CL7365r and CL6784s (5′ ATGGCAGCAGTTCTGAATTTG 3′).

Amplified bands of the expected size were analyzed in agarose gels, purified using the Wizard SV Gel and PCR Clean Up System (Promega), and ligated into the cloning vector pGEM-T Easy (Promega) following the manufacturer's instructions. Ligation reactions were used to transform strain DH5α of *Escherichia coli* cells by thermal shock at 42 °C, for 90 s. Selected recombinant clones were cultivated in LB medium containing 100 μg/ml ampicillin (Russel and Sambrook 2001), purified using the Wizard Plus SV Miniprep DNA Purification System (Promega) and sequenced by the Sanger method (Sanger and Coulson 1975). Sequencing was carried out at Embrapa Recursos Genéticos e Biotecnologia (Brasília, DF).

Nucleotide alignments and phylogenetic relationships

Multiple nucleotide sequence alignments were performed using the Muscle algorithm in MEGA 6.0 (Tamura et al. 2013). Phylogenetic relationships were determined using the neighbor joining method implemented in MEGA 6.0 using the Kimura 2-parameter nucleotide substitution model with discrete gamma distribution (G) and bootstrap support from 2000 replicates.

Coat protein genetic variability, recombination and selection analyses

Datasets for analyses of genetic variability were constructed with at least 10 isolates (Brazil, China, India and Japan). An additional, "world" dataset comprising isolates from Brazil (19), China (126), France (2), Germany (1), India (30), Japan (10) Latvia (12 clones from the same plant), Poland (1) Slovakia (2), Taiwan (2) and the United States (1) was included in the analyses (Table 1; Supplementary Table S1). Molecular variability descriptors [total number of segregating sites (S), number of nucleotide differences between sequences (K), nucleotide diversity (π), haplotype number (H), haplotype diversity (Hd) and Watterson's estimator for the population-scaled mutation rate] were estimated using DnaSP software v. 5.10 (Rozas et al. 2003). The mean pairwise number of nucleotide differences per site (π) was calculated using a sliding window of 100 bases, with a step size of 25 bases across the ACLSV CP coding region. The CP

Table 1 Data on full coat protein gene sequences of Brazilian isolates of *Apple chlorotic leaf spot virus* characterized in this study

Host/cultivar	Collection site (Municipality/ state	Host	Isolate name	Genbank accession number
Malus domestica cv. unknown	Epagri[a], São Joaquim, SC	Apple	BR1	EF138602
M. domestica cv. Fuji	Orchard, São Joaquim, SC	Apple	M220	KT183386
M. domestica cv. Macfree	Epagri, Caçador, SC	Apple	M283	KX668476
M. domestica cv. Nova Easygrow	Epagri, Caçador, SC	Apple	M281	KX668477
M. domestica cv. Fuji	Orchard, São Joaquim, SC	Apple	M222	KX668478
M. domestica cv. Fuji	Orchard, São Joaquim, SC	Apple	M228	KX668479
M. domestica cv. Fuji	Orchard, São Joaquim, SC	Apple	M230	KX668480
M. domestica cv. Fuji	Orchard, São Joaquim, SC	Apple	M232	KX668481
M. domestica cv. Fuji Select	Orchard, Vacaria, RS	Apple	M184	KX668482
M. domestica cv. Fuji Suprema	Epagri, Caçador, SC	Apple	M267	KX668483
Prunus persica var. nucipersica cv. Armking	Orchard, Vacaria, RS	Nectarine	NBR2	KX668484
M. domestica cv. Cripps Pink	Orchard, Vacaria, RS	Apple	M075	KX668485
M. domestica cv. Fuji Mishima	Orchard, Vacaria, RS	Apple	M270	KX668486
M. domestica cv. Fuji Precoce	Epagri, São Joaquim, SC	Apple	M210	KX668487
M. domestica cv. Golden Delicious	Epagri, São Joaquim, SC	Apple	M177	KY009917
M. domestica cv. Red Delicious	Epagri, São Joaquim, SC	Apple	M176	KY009918
P. salicina cv. Polli Rosa	Fepagro[b], Veranópolis, RS	Plum	PR1	KY009919
M. domestica cv. Maxi Gala	Orchard, Vacaria, RS	Apple	MG8B	KY009920
M. domestica cv. Fuji Kiku	Orchard, Vacaria, RS	Apple	M266	KY315922

[a] Santa Catarina State Agricultural Research Agency, SC, Brazil;

[b] Rio Grande do Sul State Agricultural Research Foundation, RS, Brazil

gene molecular variability of ACLSV was evaluated using five previously described datasets.

Site-specific selection pressures were analyzed using four algorithms, Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Random Effects Likelihood (REL) and Partitioning for Robust Inference of Selection (PARRIS), within the HyPhy software implemented in the Datamonkey server (www.datamonkey.org) with default conditions. The nucleotide substitution model of Hasegawa Kishino-Yano (HKY) was used for all datasets, except the Chinese dataset (Tamura-Nei model). To avoid the effect of recombination events on selection analysis, recombination analysis was performed using the program RDP v.4.77 (Martin et al. 2015) and the GARD method (available at the Datamonkey server). In recombination analysis using the program RDP, only recombination events detected by at least three of the methods available in the program were considered reliable.

Results and discussion

The genetic diversity of ACLSV isolates was studied by sequencing and aligning nt CP sequences of cDNA clones of 19 southern Brazilian isolates. All CP sequences contained 582 nucleotides (nt) and 193 deduced amino acid (daa) residues (Table 1). The regions of highest genetic variation in the ACLSV CP appear to vary considerably. They have been observed in the N-terminal (German et al. 1990) and the middle portion of the CP gene (Rana et al. 2010; Chen et al. 2013). Nucleotide diversity (π) was analyzed throughout the length of the coat protein gene of ACLSV (Fig. 1). The tendency for π values was similar in the five datasets analyzed, differing only in the absolute values of π (Fig. 1). The ACLSV CP showed the greatest nucleotide diversity at position 200, and showed lower nucleotide diversity close to its 5′-terminal region (position 75) in the five datasets analyzed (Fig. 1). It is also possible to observe a decrease in the π value from position 425 towards the 3′-terminal region. In this study, variability of amino acids (non-synonymous substitutions) was concentrated in the N-terminal and middle regions of the CP (amino acids 2 to 114), which accumulate 17 positions with aa variations, while the C-terminal ends were substantially less variable (two amino acid variations, at positions 122 and 188). This is similar to the report by Al Rwahnih et al. (2004), where variability (represented by non-synonymous substitutions) was high in the N-terminal, with the C-terminal being significantly less divergent. When we analyzed the isolates from different countries, including the Brazilian dataset, we verified the same pattern. Nucleotide differences at non-synonymous sites are concentrated in the 5′-terminal region (corresponding to the N-terminal region of the protein)

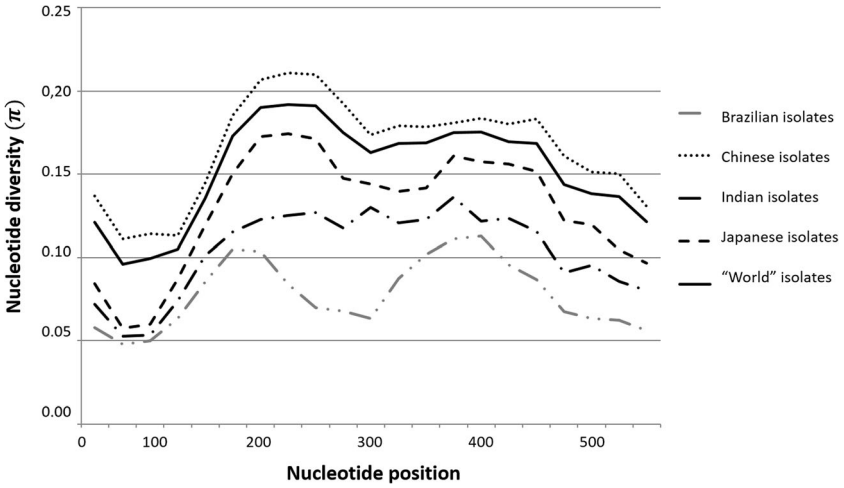

Fig. 1 Mean pairwise number of nucleotide differences per site (nucleotide diversity, π) calculated on a sliding window across the coat protein gene of *Apple chlorotic leaf spot virus* isolates

and in the central portion of the CP gene, although greater diversity of nucleotides relative to the synonymous sites was observed in the 3′-terminal region (corresponding to the C-terminal region of the protein). Even though the 3′-terminal region has the highest average value of π (Fig. 2), these changes in the nucleotide sequence did not result in amino acid changes. These results suggest a strong negative selection acting on this region of the ACLSV CP.

Complete genome nucleotide sequences of 13 ACLSV isolates have been previously published, including isolates P205, A4, MO-5 from apple and B6 from apple and hawthorn (Sato et al. 1993; Yaegashi et al. 2007; Guo et al. 2016), P863 and PBM1 from plum (German et al. 1990; Jelkmann 1996) and Ta Tao 5, Z1 and Z3 from peach (Marini et al. 2008; Niu et al. 2012). Based on amino acid sequences, Japanese ACLSV CPs were reported to cluster into types P205 and B6, according to covariation of two combinations of five highly conserved aa sets: Ala^{40}, Val^{59}, Phe^{75}, Ser^{130} and Met^{184} for isolates P205 and A4, and Ser^{40}, Leu^{59}, Tyr^{75}, Thr^{130} and Leu^{184} for isolates B6 and MO-5 (Yaegashi et al. 2007). These authors also showed that co-variable aa positions 40 and 75 were critical for effective viral replication. Other multiple aa alignments showed another covariation in the ACLSV CP at position 79, among 55 Chinese apple ACLSV isolates (Chen et al. 2013). Brazilian isolates showed conserved signatures of type B6 isolates at positions 40, 59, 75, 130 and 184, that places them with type B6 isolates, except for isolates M220 (apple) and PR1 (plum), which appear to be variants of the type B6 isolates. Isolate M220 possesses one single P205-type amino acid residue change, Leu^{59} to Val^{59}. Isolate PR1 appears as a newly recognized unique variation event, which shows a Glu^{79} to Gly^{79} variation, deviating from Chinese isolates (Chen et al. 2013) as well as from B6 type from Japan. Additionally, isolates M177 and M266 were uniquely co-variable, showing Glu to Lys at position 111. The functional role of these variations remains to be elucidated.

Phylogenetic analysis of the nineteen ACLSV sequences [18 ACLSV isolates from this study and one isolate (BR1) from a previous report (Silva et al. 2008)] and representative foreign isolates revealed their clustering into two groups based on nucleotide sequences (Fig. 3); daa sequence analysis

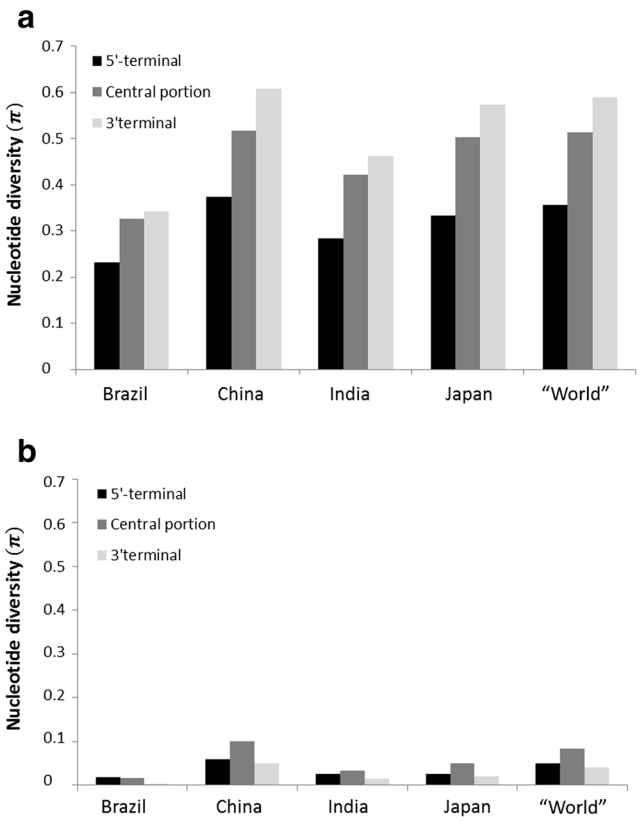

Fig. 2 Mean pairwise number of nucleotide differences per site (nucleotide diversity, π) at synonymous (**a**) and non-synonymous (**b**) sites calculated for three regions (5′-terminal, central and 3′-terminal) of the coat protein gene of *Apple chlorotic leaf spot virus* isolates from Brazil, China, India, Japan and "world"

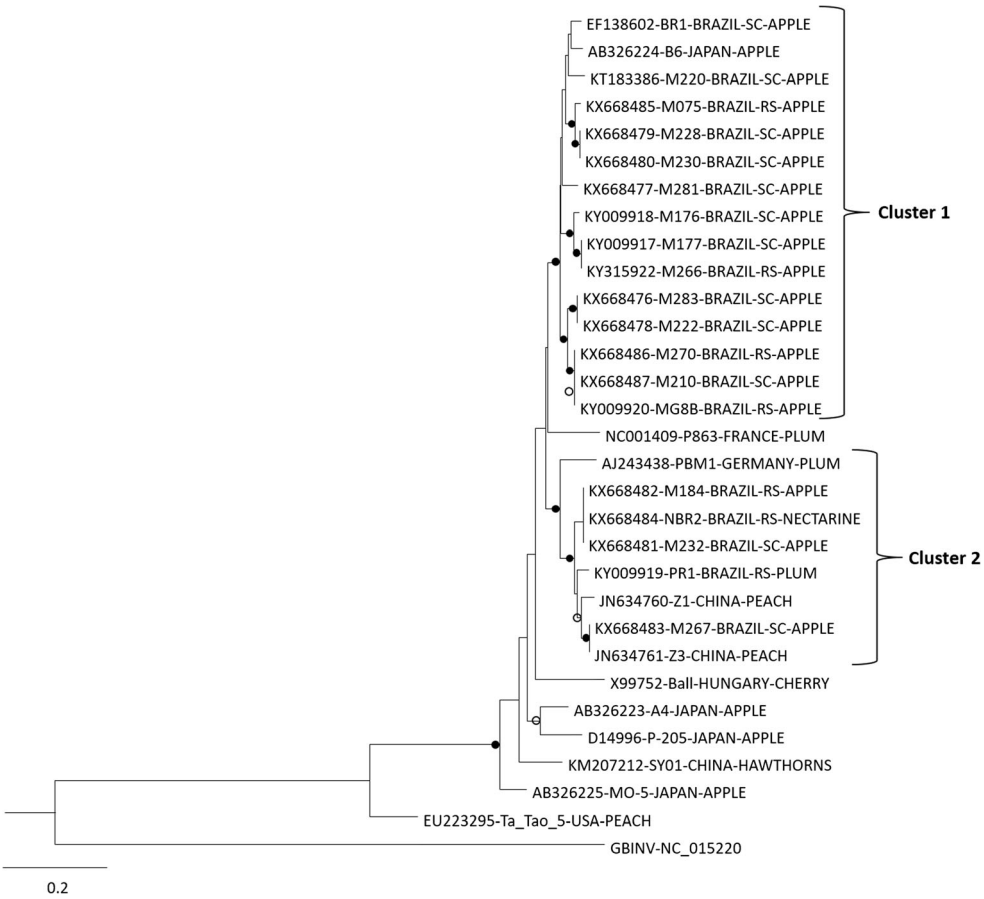

Fig. 3 Phylogenetic tree based on the alignment of coat protein genes of *Apple chlorotic leaf spot virus* isolates from Brazil and representative foreign isolates. The tree was constructed by the neighbor joining method implemented in MEGA 6.0 program using the Kimura 2-parameter nucleotide substitution model with discrete gamma distribution (G) and bootstrap support of 2000 replications. Nodes with bootstrap values equal or higher than 80% are indicated by filled circles, and those with values lower than 80% and higher than 50% by empty circles. Names of isolates, location, access number and hosts are indicated. *Grapevine berry inner necrosis virus* (GBINV) was used as outgroup. Bar indicates number of substitutions per site.

confirmed this grouping (data not shown). Cluster 1 contains apple isolates, while cluster 2 includes apple (M184, M232 and M267), plum (PR1 and PBM1) and *Prunus* (Z1, Z3 and NBR2) isolates, in accordance with the highest nt divergence values in this group (12.3 to 13.2%). Phylogenetic analyses confirmed Brazilian isolates in cluster 1 clustering with B6-type from Japan, while isolates in cluster 2 grouped together with Chinese isolates Z1 and Z3 from peach and German isolate PBM1 from plum (Fig. 3). Peach isolates have been previously classified into types Z1 and Ta Tao 5 (Niu et al. 2012).

To better characterize the genetic variability of the different isolates analyzed in this study, the descriptors of variability were determined (Table 2). Descriptors for the dataset consisting of Chinese, Japanese and "world" isolates indicated higher genetic variability than those from the

Table 2 Genetic variability of *Apple chlorotic leaf spot virus* coat protein genes from Brazil, China, India, Japan and "world" isolates

ACLSV isolates[a]	Number of isolates	CP lengh (nt)	S[b]	K[c]	π[d]	H[e]	Hd[f]	θ-W[g]
Brazil	19	582	130	44.754	0.07690 ± 0.00542	13	0.959	0.0639
China	126	582	330	95.971	0.16490 ± 0.00796	121	0.999	0.1048
India	30	582	162	60.421	0.10382 ± 0.00391	19	0.959	0.0703
Japan	10	582	173	74.711	0.12837 ± 0.00974	10	1.000	0.1051
"World"	206	582	350	88.442	0,15,196 ± 0.00605	172	0.997	0.1019

[a] Five datasets of ACLSV sequences were analyzed: The datasets comprise isolates of Brazil, China, India, Japan and "world"

[b] Total number of segregating sites

[c] Mean number of nucleotide differences between sequences

[d] Nucleotide diversity

[e] Haplotype number

[f] Haplotype diversity

[g] Watterson's estimate of the population mutation rate based on the total number of segregating sites

Table 3 Selection analysis of coat protein genes of Brazilian and foreign isolates of *Apple chlorotic leaf spot virus*

ACLSV isolates[a]	Number of isolates	dN/dS	SLAC[b]		FEL[c]		REL[d]		PARRIS[e]
			PS[f]	NS	PS	NS	PS	NS	PS
Brazil	18	0.0559	–	33	1	64	1	95	–
China	124	0.0775	1	150	1	154	nd	nd	nd
India	29	0.0405	–	66	–	94	–	58	–
Japan	10	0.0571	–	58	1	97	1	79	–

[a] Recombinant ACLSV isolates M176 (KY009918 - Brazil), YC-WR-2 (KC404877 - China), YT-3-1 (KC404872 - China) and India 20 (AM498047 - India) were excluded from the selection analysis

dN/dS – non-synonymous to synonymous substitution ratios

[b] Single likelihood ancestor counting

[c] Fixed effects likelihood

[d] Random effects likelihood

[e] Partitioning for robust inference of selection

[f] PS/NS number of positive (PS) and negative (NS) selection sites; −, no site under selection; nd, not determined due to high number of sequences

dataset comprising only Brazilian or Indian isolates (Table 2). The higher genetic variability is represented by a higher number of segregating sites (S), nucleotide diversity (π), haplotype number (H) and haplotype diversity (Hd) (Table 2). Nucleotide diversity (π) in the Chinese dataset was the highest (0.16490 ± 0.00796) observed within the datasets analyzed. The dataset comprising ACLSV Brazilian isolates had a π value of 0.07690 ± 0.00542, indicating lower genetic variability than the isolates from other countries. The π value detected in ACLSV (for five datasets) is in accordance with π values found in different viral species by several authors (Garcia-Arenal et al. 2001; Lima et al. 2013, 2017; Zanardo et al. 2014; Fajardo et al. 2017). The Watterson's estimator for the population-scaled mutation rate (θ-W) for Brazilian and Indian ACLSV isolates was in the order of 10^{-2}, and for Chinese, Japanese and "world" isolates was in the order of 10^{-1} (Table 2), meaning that Chinese and Japanese isolates are more variable due to the greater number of mutations. The θ-W values of 10^{-1} are lower when compared with other coding regions and other viral species described in the literature (Fajardo et al. 2017; Lima et al. 2013; Moura et al. 2018; Rocha et al. 2013; Zanardo et al. 2014).

In the selection analysis, datasets comprising Brazilian, Chinese, Indian and Japanese isolates were used, which represent a population of ACLSV. Since recombination events may affect the selection analysis, the Brazilian isolate M176, found to be a recombinant by three methods available in the RDP4 software, was excluded from site-specific selection analysis (Supplementary Table S2). This is the first report on the occurrence of a recombinant ACLSV isolate from Brazil. Further recombination events were found in the CP genes of Chinese and Indian isolates (Rana et al. 2010; Chen et al. 2013), demonstrating that this mechanism contributes to the evolution of ACLSV CP genes. Recombinants from Chinese and Indian isolates were also excluded from the selection analyses. No recombination event was found in the dataset comprising Japanese isolates (data not shown).

The CP gene of ACLSV Brazilian isolates showed dN/dS ratios (non-synonymous/synonymous substitutions ratios) of 0.0559 (lower than 1.0), indicating negative selection (Table 3). Isolates from the other evaluated countries also presented dN/dS ratios lower than 1, with the CP of Chinese isolates presenting the highest value of dN/dS, featuring them as less restrictive to changes. At one site, codon 37, a positive selection in Brazilian and Chinese isolates was detected (Table 3). In this position, six Brazilian isolates (M075, M220, M222, M270, M281 and M283) possessed Thr; one isolate (M267) possessed Met; and the remaining contained Ile. The same amino acids were observed at position 37 of Chinese isolates; isolates LN-1-1, SMX-3-3 and TS-4-2 also showed Met at this position. In the Japanese dataset, position 60 was observed to be under positive selection with variation in aa Leu, Met, Gln, Ser, Thr and Val. The codon AUG in viral RNA sequences (corresponding to aa Met) at position 37 could indicate an internal ribosome entry site (IRES). IRES are able to mediate internal entry of the 40S ribosomal subunit on viral messenger RNAs upstream of a translation initiation codon (Bonnal et al. 2003). If the first AUG is in an unfavorable context, 40S subunits may bypass it and initiate RNA synthesis at downstream AUG codons (Ivanov et al. 1997; Martínez-Salas et al. 2008). Although the AUG codon was found within the CP coding region of some isolates from Brazil, China and Japan, viral IRES Prediction System (Hong et al. 2013) did not find an IRES structure in the respective sequences in this study (data not shown). For Indian isolates, sites under positive selection were not detected (Table 3). The positive selection found at positions 37 and 60 may

indicate an adaptive selection for these sites in the CP gene of Brazilian, Chinese and Japanese ACLSV isolates.

The number of sites under negative selection varies with the method employed and datasets used (Table 3; Supplementary Table 3). Selection pressures are important for expression of functional features of viral structures (Garcia-Arenal et al. 2001). Amino acids that are relevant for the assembly and stabilization of virus particles are conserved in tobamoviruses (Altschuh et al. 1987). Viral proteins are multifunctional and may be involved in other processes such as replication, cell-to-cell, and long distance movement and transmission of viruses (Garcia-Arenal et al. 2001). Accordingly, negative selection is predominant in the coding regions of viral proteins. The results presented on characteristics of CP genes of ACLSV are consistent with the fact that the viral structural genes are under negative selection and are constrained regions (Chare and Holmes 2004; Zanardo et al. 2014). To our knowledge, this is the first report on complete CP genetic variability and phylogenetic relationships of ACLSV isolates occurring in *Malus* and *Prunus* spp. in southern Brazil and the first detection of regional recombinant isolates and comparative analyzes with CP genes of ACLSV isolates from other apple growing regions.

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