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BRIEF REVIEW

Genetic diversity in the 3′-terminal region of papaya ringspot virus (PRSV-W) isolates from watermelon in Oklahoma

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Abstract The 3′-terminal region (1191 nt) containing part of the NIb gene, complete coat protein (CP) and poly-A tail of 64 papaya ringspot virus (PRSV-W) isolates collected during 2008-2009 from watermelon in commercial fields of four different counties of Oklahoma were cloned and sequenced. Nucleotide and amino acid sequence identities ranged from 95.2-100% and 97.1-100%, respectively, among the Oklahoman PRSV-W isolates. Phylogenetic analysis showed that PRSW-W isolates clustered according to the locations where they were collected within Oklahoma, and each cluster contained two subgroups. All subgroups of Oklahoman PRSV-W isolates were on separate branches when compared to 35 known isolates originating from other parts of the world, including the one reported previously from the USA. This study helps in our understanding about the genetic diversity of PRSV-W isolates infecting cucurbits in Oklahoma.

Papaya ringspot virus (PRSV; genus *Potyvirus*, family *Potyviridae*) is economically important due to its worldwide distribution and serious losses in both cucurbit crops and papaya (*Carica papaya* L.) [30]. PRSV has two different strains (W and P) that can be differentiated biologically by their ability to infect different hosts, but not serologically [30]. PRSV-W infects cucurbits but is unable to infect papaya [41]. PRSV-P primarily infects papaya but can also infect cucurbits in an experimental setting; however, it has never been reported to infect cucurbits naturally in the field [13].

PRSV has long, flexuous filamentous particles of 780 x 12 nm. The genome of PRSV consists of a positive-sense ssRNA of approximately 10,000 nucleotides, which is translated into a single large polyprotein and subsequently cleaved into individual functional proteins [9, 32], including a coat protein (CP) of 36 kDa [12, 15, 29]. The core region of the CP of PRSV is highly conserved, while the N terminus is more variable [33].

Populations of both strains (W and P) of PRSV are highly diverse [5, 40]. However, most of the genetic variability studies of PRSV have been limited to the P strain, which has been detected in many countries, including Australia [4], Brazil [22], China [40], India [18, 19], Jamaica and Venezuela [7], Mexico [25, 26, 34], Taiwan [39], Thailand, Vietnam and the Philippines [5], the USA [8], and Venezuela [9]. There is, however, little information on the genetic diversity of PRSV-W isolates in cucurbits. Although PRSV-W is considered to be one of the five most important viruses infecting field-grown vegetables [36], only recently has a comparison of two PRSV-W isolates from Brazil shown the genetic diversity of the W strain [16]. Recently, Olarte Castillo et al. [27] reported a detailed molecular phylogenetic analysis of PRSV isolates from all over the world, but they felt the need to analyze a range of PRSV-W isolates that is still scarce in the literature.

In the USA, the PRSV-P strain was first reported in 1949 from Hawaii [21]. The nucleotide sequences of the 3′-terminal regions of a PRSV-P (HA-isolate) from Hawaii and a PRSV-W (FL-isolate) from Florida have been determined [31]. The HA-P and FL-W strains shared 98.2% nucleotide similarity in the NIb gene and 97.7% in the CP gene.

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The genetic diversity of the PRSV-P strain in papaya in Florida has also been reported [8]. However, to the best of our knowledge, no information is available about the genetic diversity of PRSV-W isolates in the USA. During a recent study of the incidence of viruses infecting cucurbit crops in Oklahoma [1], a high incidence (50%) of PRSV-W was found in watermelon compared to other viruses.

This study is focused on determining the genetic variability in the 3′-terminal region of PRSV-W isolates collected from watermelon in Oklahoma and finding the relationships among these isolates and with other isolates of PRSV reported either in the USA or other parts of the world.

A total of 60 PRSV-W isolates were selected randomly from dot-immunobinding assay (DIBA)–positive symptomatic samples collected during 2008-2009 from various commercial watermelon fields in four different counties (Atoka, Blaine, Jefferson and Tulsa) of Oklahoma [1]. Fifteen samples from each county were selected randomly, and in some cases, more than one sequence was obtained from a given sample (Table 1). Sequences of the complete CP gene for 14 isolates of PRSV-W and 21 isolates of PRSV-P used in comparison were obtained from the GenBank database (Table 2).

Total RNA was extracted from 100 mg of tissue using the TRI-Reagent method (Molecular Research Center Inc., Cincinnati, OH, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with PRSV-W-specific primers as described previously [1] in the presence of *Taq* DNA polymerase (Roche Applied Science, USA) and *Pfu* DNA polymerase (Stratagene, USA).

The PCR product was confirmed on a 1% agarose gel, and the remaining amplified products were purified directly using a QIAquick PCR Purification Kit (QIAGEN, USA). PCR products were cloned using pGEM®-T Easy Vector (Promega, USA), and introduced by transformation into *Escherichia coli* DH5α competent cells (New England BioLabs, USA). Recombinant plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (QIAGEN, USA) and sequenced in both directions using Big-Dye terminator cycle sequencing (Applied Biosystems 3130 Genetic Analyzer, USA) at the core facility of the Department of Biological Science, The University of Tulsa, Oklahoma, USA.

Sequences of all three clones for individual isolates were aligned individually using EditSeq™ and MegAlign™ within the DNASTAR suite of programs (Madison, USA). Consensus nucleotide (nt) and amino acid (aa) sequences of each isolate were obtained using the Clustal X program. Phylogenetic analysis was performed either on the whole 3′-terminal region or on complete CP sequences of all 60 isolates from Oklahoma and 35 isolates from other geographical locations available in the GenBank database, using the MEGA5 program [35] to produce a maximum-likelihood tree using the general time reversible (GTR) model with gamma distribution with 1000 bootstrap replicates. Pairwise sequence identities for both nt and aa sequences were calculated by Clustal V.

The 3′-terminal region (approximately 1.2 kb), which covered part of the NIb gene (309 nt), the complete CP gene (864 nt) and part of the 3′-UTR (18 nt), was successfully amplified from all 60 PRSV-W isolates. Nucleotide sequence analysis revealed that all PRSV-W isolates have the same fragment length, with the exception of 14 isolates (T1, T4, T5, T6, T7, T8, T9, T10, T11a, T11b, T12, T13, T14a, T15) from Tulsa County that contained a deletion of three nt (AAC) at position 286-288 in the NIb gene. The partial NIb encoded 103 aa, while the complete CP gene encoded 287 aa and was similar in size among all other isolates. There was no insertion or deletion in the CP gene of any Oklahoman PRSV-W isolate, but nucleotide substitutions were observed.

A maximum-likelihood phylogenetic tree based on the complete CP nt sequence (Fig. 1) of PRSV-W isolates from Atoka County revealed the existence of two subgroups (I and II). Subgroup I contained 5 isolates while subgroup II had 8 isolates and was supported by a strong bootstrap value (96-100%). Sequences A1a and A1b isolated from the same sample (Table 1) clustered separately into two subgroups (Fig. 1). Nucleotide sequence identity ranged from 95.7 to 96.0% between the subgroups and 99.9-100% within the subgroups, while aa identity ranged from 98.6-100%. There were 18 and 29 group-specific nt substitutions, respectively, in subgroup I and II isolates. All substitutions were evenly distributed in the NIb and CP genes and were not specific to either the N- or C-terminus of the respective gene. When the aa sequences of the Atoka isolates were compared to the published sequences of FL-W, HA-P isolates from USA, and NT-W isolates from Australia, three aa changes were observed in the NIb gene (Supplementary Table 1) and five in the CP gene (Supplementary Table 2) between the two subgroups.

All PRSV-W isolates from Blaine County also formed two subgroups. Subgroup I included 13 isolates, while subgroup II had only two isolates (B5 and B11), which were on separate branches (Fig. 1). Nucleotide sequence identities within and between the subgroups ranged from 99.7 to 100% and from 96.0 to 96.9%, respectively, while amino acid identity ranged from 98.6 to 100%. There were 5 and 18 nt differences in the NIb and CP gene, respectively, between the two subgroups. Similarly, four aa changes were observed in the NIb gene, and only one in the CP gene (Supplementary Tables 1 and 2).

Phylogenetic analysis of PRSV-W isolates from Jefferson County also revealed two subgroups. Subgroup I included 11 isolates, while subgroup II included 5 isolates

Table 1 Locations and names of PRSV-W isolates obtained from watermelon in four counties of Oklahoma

Name of isolate	Year of collection	County	Accession no.	Reference	Name of isolate	Year of collection	County	Accession no.	Reference
A1a*	2008	Atoka	JN132408	This study	J1	2009	Jefferson	JN132439	This study
A1b*	2008	Atoka	JN132409	This study	J2	2009	Jefferson	JN132440	This study
A2	2008	Atoka	JN132410	This study	J3	2009	Jefferson	JN132441	This study
A3	2008	Atoka	JN132411	This study	J4	2009	Jefferson	JN132442	This study
A4	2008	Atoka	JN132412	This study	J5	2009	Jefferson	JN132443	This study
A5	2008	Atoka	JN132413	This study	J6	2009	Jefferson	JN132444	This study
A6	2008	Atoka	JN132414	This study	J7	2009	Jefferson	JN132445	This study
A7	2008	Atoka	JN132415	This study	J8	2009	Jefferson	JN132446	This study
A8	2008	Atoka	JN132416	This study	J9	2009	Jefferson	JN132447	This study
A9	2008	Atoka	JN132417	This study	J10	2009	Jefferson	JN132448	This study
A10	2008	Atoka	JN132418	This study	J11	2009	Jefferson	JN132449	This study
A11	2008	Atoka	JN132419	This study	J12	2009	Jefferson	JN132450	This study
A12	2008	Atoka	JN132420	This study	J13	2009	Jefferson	JN132451	This study
A13	2008	Atoka	JN132421	This study	J14	2009	Jefferson	JN132452	This study
A14	2008	Atoka	JN132422	This study	J15a*	2009	Jefferson	JN132453	This study
A15	2008	Atoka	JN132423	This study	J15b*	2009	Jefferson	JN132454	This study
B1	2008	Blaine	JN132424	This study	T1	2009	Tulsa	JN132455	This study
B2	2008	Blaine	JN132425	This study	T2	2009	Tulsa	JN132456	This study
B3	2008	Blaine	JN132426	This study	T3	2009	Tulsa	JN132457	This study
B4	2008	Blaine	JN132427	This study	T4	2009	Tulsa	JN132458	This study
B5	2008	Blaine	JN132428	This study	T5	2009	Tulsa	JN132459	This study
B6	2008	Blaine	JN132429	This study	T6	2009	Tulsa	JN132460	This study
B7	2008	Blaine	JN132430	This study	T7	2009	Tulsa	JN132461	This study
B8	2008	Blaine	JN132431	This study	T8	2009	Tulsa	JN132462	This study
B9	2008	Blaine	JN132432	This study	T9	2009	Tulsa	JN132463	This study
B10	2008	Blaine	JN132433	This study	T10	2009	Tulsa	JN132464	This study
B11	2008	Blaine	JN132434	This study	T11a*	2009	Tulsa	JN132465	This study
B12	2008	Blaine	JN132435	This study	T11b*	2009	Tulsa	JN132466	This study
B13	2008	Blaine	JN132436	This study	T12	2009	Tulsa	JN132466	This study
B14	2008	Blaine	JN132437	This study	T13	2009	Tulsa	JN132467	This study
B15	2008	Blaine	JN132438	This study	T14	2009	Tulsa	JN132468	This study
					T15a*	2009	Tulsa	JN132469	This study
					T15b*	2009	Tulsa	JN132470	This study

* Isolates were obtained from the same sample

(Fig. 1). The nucleotide sequence identities ranged from 99.3 to 100% between the subgroups, and from 99.9 to 100% within the subgroups. Amino acid sequence identity ranged from 98.9 to 100%. There were one and five nt differences in the NIb and CP gene, respectively, between subgroup I and II isolates. Sequences J15a and J15b were in different subgroups, even though they were isolated from the same sample (Table 1). Two aa changes in the NIb gene (Supplementary Table 1) and one in the CP gene (Supplementary Table 2) were observed.

Analysis of the PRSV-W isolates from Tulsa County showed that they belonged to subgroup I, with the exception of the T2, T3 and T14b isolates. The T2 isolate clustered together with subgroup II isolates from Jefferson County, while the T3 and T14b isolates were on separate branches (Fig. 1). The nt and aa sequence identities ranged from 97.3 to 98.2% and from 99.9 to 100%, respectively, between subgroup I and the three isolates (T2, T3 and T14b). There were 11 and 7 nt substitutions in the NIb and CP gene, respectively, between the subgroup I isolates and the three isolates. In the NIb aa alignment, one aa deletion at position 97 was observed among the subgroups I isolates only (Supplementary Table 1). Sequences T11a and T11b were obtained from the same sample, T11, and similarly, sequences T14a and T14b were both obtained from sample T14 (Table 1), showed some differences either in the NIb

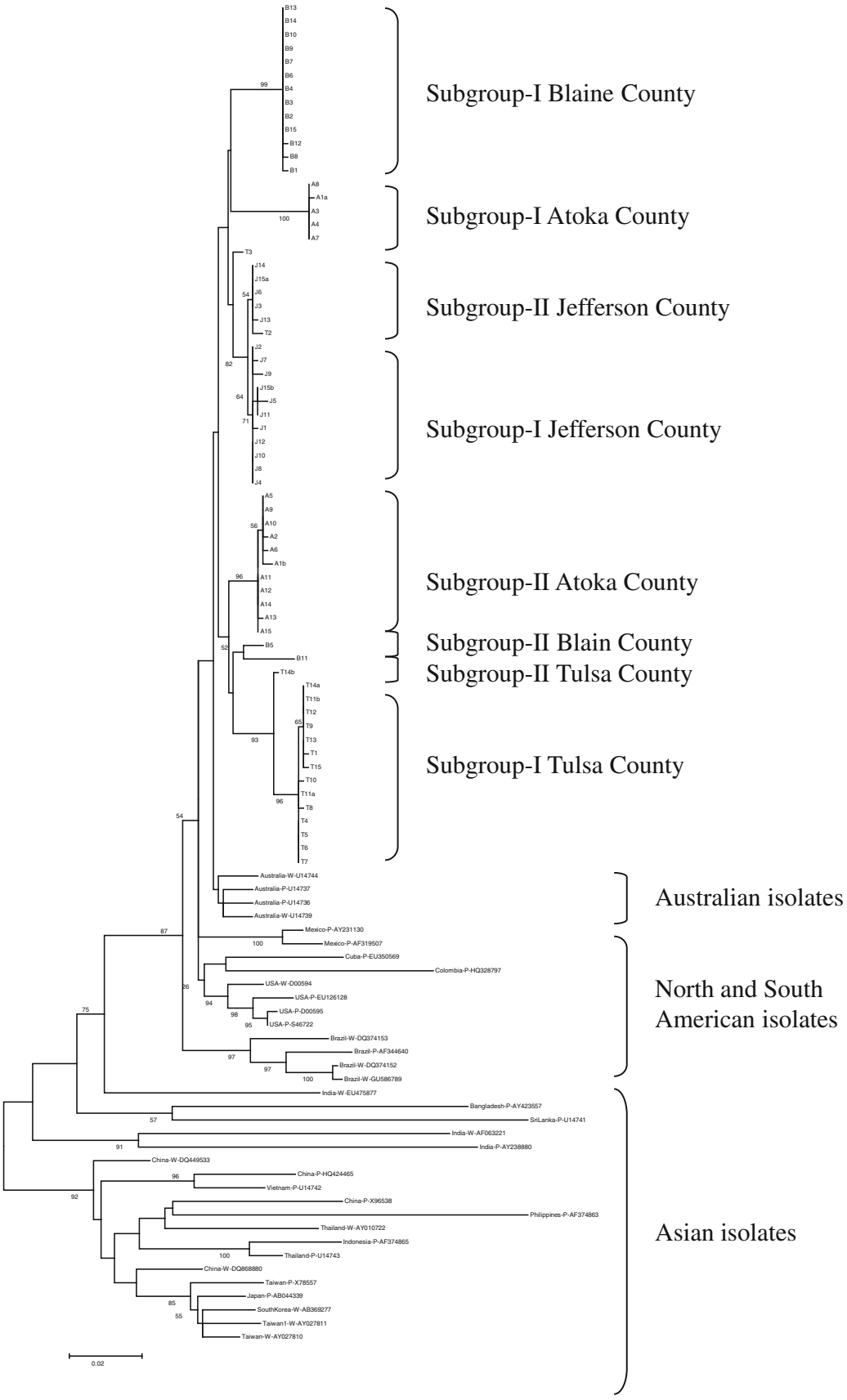
Table 2 Sources of coat protein sequences of papaya ringspot virus (PRSV) isolates from different countries used for sequence comparison in this study as reported in the GenBank database

Country	Isolate name	Strain	Accession no.	Reference
Australia	BD	P	U14736	[4]
Australia	BUN	P	U14737	[4]
Australia	GAT	W	U14739	[4]
Australia	NT	W	U14744	[4]
Bangladesh	BA	P	AY423557	[19]
Brazil	W1	W	DQ374153	[16]
Brazil	WC	W	Q374152	[16]
Brazil	PTRICH	W	GU586789	[17]
Brazil	BA-IT2	P	AF344640	Unpublished
China	HN-1	P	HQ424465	Unpublished
China	BN	W	DQ449533	Unpublished
China	CH99/113	W	DQ868880	Unpublished
China	SM	P	X96538	Unpublished
Colombia	COL-vc29	P	HQ328797	[27]
Cuba	C3	P	EU350569	Unpublished
India	IND	W	AFO63221	[18]
India	AP	P	AY238880	[20]
India	PRSV-W	W	EU475877	[24]
Indonesia	ID	P	AF374865	Unpublished
Japan	JAP	P	AB044339	Unpublished
Mexico	Mex-VrPo	P	AY231130	[26]
Mexico	VrCo-7	P	AF319507	[26]
Philippines	PHP	P	AF374863	Unpublished
Taiwan	YK	P	X78557	[38]
Taiwan	PT	W	AY027811	[39]
Taiwan	CI	W	AY027810	Unpublished
Thailand	TH	P	U14743	[4]
Thailand	PRSVth-W	W	AY010722	[3]
South Korea	PRSV-SK	W	AB369277	Unpublished
Sri Lanka	SL	P	U14741	[4]
USA	FL	W	D00594	[31]
USA	FL	P	D00595	[31]
USA	PG	P	EU126128	Unpublished
USA	HA	P	S46722	[42]
Vietnam	VIET	P	U14742	[4]

or CP aa comparison (Supplementary Tables 1 and 2), and were in different subgroups (Fig. 1).

Nucleotide sequence analysis of the CP gene of Oklahoman isolates showed sub-groupings according to geographical origin of the isolates, as is evident from the phylogenetic tree (Fig. 1). When compared to sequences from previously published PRSV isolates originating from different countries (Table 2), Oklahoman PRSV-W isolates from four different counties grouped together on separate branches. Nucleotide sequence identities (96.4-98.3%) between the CP genes of Oklahoman PRSV-W and Australian W or P isolates were slightly higher than the identities (95.9-96.7%) between Oklahoma isolates and previously known PRSV-FL-W and HA-P isolates from the USA. Similarly, phylogenetic analysis (Fig. 1) clustered Oklahoman PRSV-W and Australian isolates together, but the branch of Australian isolates has a low bootstrap value (26%), and therefore their relationship is not statistically supported. However, it merits further study.

This is the first study to analyze the genetic diversity in a range of PRSV-W isolates that infect cucurbits. Grouping of PRSV-W isolates from four counties of Oklahoma according to their geographic locations suggests that they are indigenous isolates circulating in their respective areas. However, there was no dispersal event or introduction of PRSV-W isolates to other counties. A possible explanation


B13
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Australia-W-U14744
Australia-P-U14737
Australia-P-U14736
Australia-W-U14739
Mexico-P-AY231130
Mexico-P-AF319507
Cuba-P-EU350569
Colombia-P-HQ328797
USA-W-D00594
USA-P-EU126128
USA-P-D00595
USA-P-S46722
Brazil-W-DQ374153
Brazil-P-AF344640
Brazil-W-DQ374152
Brazil-W-GU586789
India-W-EU475877
Bangladesh-P-AY423557
SriLanka-P-U14741
India-W-AF063221
India-P-AY238880
China-W-DQ449533
China-P-HQ424465
Vietnam-P-U14742
China-P-X96538
Philippines-P-AF374863
Thailand-W-AY010722
Indonesia-P-AF374865
Thailand-P-U14743
China-W-DQ868880
Taiwan-P-X78557
Japan-P-AB044339
SouthKorea-W-AB369277
Taiwan1-W-AY027811
Taiwan-W-AY027810
0.02
Subgroup-I Blaine County
Subgroup-I Atoka County
Subgroup-II Jefferson County
Subgroup-I Jefferson County
Subgroup-II Atoka County
Subgroup-II Blain County
Subgroup-II Tulsa County
Subgroup-I Tulsa County
Australian isolates
North and South American isolates
Asian isolates

◀ **Fig. 1** Maximum-likelihood phylogenetic tree based on the complete coat protein (CP) nucleotide sequences of 64 Oklahoman PRSV-W isolates (2008-2009) and 35 other PRSV-W and P strains sequenced previously (available from GenBank). Trees were generated using MEGA5 with default parameters. Bootstrap values (with 1,000 replications) greater than 50% are indicated at the tree nodes. Detailed information for Oklahoman isolates is listed in Table 1, while isolates from other locations are listed in Table 2

is that all of these counties are located at least 160 kilometers from each other. Since PRSV-W isolates are transmitted in a non-persistent manner, it is difficult for the aphids to cover that much distance naturally in a few minutes while still retaining the active virus in their stylet. Thus, it is evident that these isolates are circulating locally among the cucurbit crops, and genetic variability is most probably generated by substitutions and deletions rather than insertions, frameshifts or recombination. We confirmed previous results that genetic diversity among PRSV isolates is related to the geographical origin of the isolates [7].

It has been reported for PRSV isolates [33] that both nt and aa sequences show high variability in the N-terminus of the CP gene, while the core region and C-terminus are more conserved. Our results showed that both the N and core regions of the CP genes of PRSV-W isolates had high nt and aa variability, while only the C-terminus was conserved (Supplementary Table 2).

In three of the four counties, at least two sequences (A1a, A1b, J15a, J15b, T11a, T11b, T14a and T14b) were isolated from the same sample, indicating that there is mixed infection of PRSV-W isolates in these samples. It would be interesting to study the biological properties of the Oklahoman PRSV-W isolates that belongs to different subgroups in different counties to determine the effect of these aa changes on their biological properties.

Genetic diversity was not distributed evenly among the PRSV-W isolates collected from four different counties in Oklahoma. For example, PRSV-W isolates from Atoka County showed a maximum divergence of 4.5%, followed by isolates from Blaine County (4.1%), Tulsa County (2.8%) and Jefferson County (0.7%). This variation may reflect the time of the dispersal event of PRSV in their respective locations.

The EK (glutamic acid and lysine) repeat boxes [34] associated with host specificity and the DAG motif [2, 11] responsible for aphid transmission, reported previously in the N-terminal regions of the CP gene of PRSV, were present in all Oklahoman isolates. Seven aa substitutions were observed in the KE regions of Oklahoman PRSV-W isolates, while none were found in the DAG motif.

Previous PRSV-W or P isolates from other geographical locations grouped together, as is evident from the phylogenetic tree (Fig. 1). However, Oklahoman PRSV-W isolates formed separate clusters from the rest of the PRSV-P or W isolates, indicating that they are unique.

Genetic variation of Oklahoman PRSV-W isolates was as high as 0.7-4.5% in the 3′-terminal region, which indicates that all of them belong to one strain, as the threshold for classifying distinct strains is 10% variation [10]. Variation in the aa sequences of the Oklahoman PRSV-W isolates was less than in their nt sequences, which can be explained by the fact that most of the nt changes in PRSV occurred at wobble bases (third base of the genetic code). This degeneracy of the third base preserves the primary structure of the protein [14, 38]. The variability of PRSV CP genes in Oklahoma is similar to the variability reported from other countries, including Australia (1.5%), Brazil (3.9%), Vietnam (4.1% among W isolates), Mexico (5.7%), and Venezuela (5.8%), while it is lower than the variability reported in India (10.1%), China (6.1%), and Thailand (7.6% among W isolates) [3, 9, 20, 22, 34–40].

We suggest that the difference in the degree of variation in PRSV reported in several studies does not necessarily reflect an actual difference in variation in PRSV between these geographic regions but instead may be due to a difference in the number of isolates, the distance between locations where the isolates were collected, and whether the isolates were collected in one or several seasons. The best example was provided by Chin et al. [7], who found that the CP gene among isolates collected from Jamaica during 1999 shared 98-100% sequence similarity compared to the isolates collected in 1990, which showed 92.2% sequence similarity.

Sequence variation between CP genes of Oklahoman PRSV-W isolates and Asian isolates was as high as 13%. These results are in accordance with other studies that reported variation in the nt sequence of the CP gene of PRSV isolates up to 12% [3], 14% [20], 15% [22], and 17% [23]. This level of variation is still lower than the nt variation of 28% and 22.4% reported for the RNA viruses yam mosaic virus (YMV) and rice yellow mottle virus, respectively [6, 28].

In conclusion, our data show that genetic diversity in the CP gene exists among the Oklahoman PRSV-W isolates originating from a single host in four different geographical locations. In future studies, we emphasize the importance of determining the biological properties of the representative isolates from each subgroup within each county before formulating any control strategy, especially transgenic resistance in watermelon. The success of both conventional and genetically engineered resistance strategies depends on the genetic diversity of PRSV populations [13, 37].

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