Journal of Integrative Agriculture 2016, 15(2): 349–363

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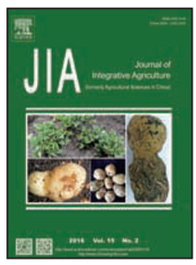

RESEARCH ARTICLE

Occurrence and molecular characterization of *Potato spindle tuber viroid* (PSTVd) isolates from potato plants in North China

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Abstract

China is the largest potato producing country worldwide, with this crop representing the fourth largest staple food crop in China. However, the steady presence of *Potato spindle tuber viroid* (PSTVd) over the past five decades has a significant economic impact on potato production. To determine why PSTVd control measures have been ineffective in China, more than 1 000 seed potatoes collected between 2009 and 2014 were subjected to PSTVd detection at the Supervision and Testing Center for Virus-free Seed Potatoes Quality, Ministry of Agriculture, China. A high PSTVd infection rate (6.5%) was detected among these commercial seed potatoes. Some breeding lines of potato collected from 2012 to 2015 were also tested for PSTVd infection, revealing a high rate of PSTVd contamination in these potato propagation materials. Furthermore, comparison of the full-length sequences of 71 different Chinese PSTVd isolates revealed a total of 74 predominant PSTVd variants, which represented 42 different sequence variants of PSTVd. Comparative sequence analysis revealed 30 novel PSTVd sequence variants specific to China. Comprehensive phylogenetic analysis uncovered a close relationship between the Chinese PSTVd sequence variants and those isolated from Russia. It is worth noting that three intermediate strains and six mild strains were identified among these variants. These results have important implications for explaining the ineffective control of PSTVd in China and thus could serve as a basic reference for designing more effective measures to eliminate PSTVd from China in the future.

Keywords: certification, germplasm, phylogenetic analysis, quarantine, seed potato, viroid

1. Introduction

Potato (*Solanum tuberosum* L.) is the third most important food crop worldwide (after rice and wheat) in terms of human consumption (Devaux *et al.* 2014). China is the largest potato producer, with almost a quarter of the world's potato production; this crop represents the fourth largest staple food crop in China. Potato is widely grown in China and is distributed in four major agroecological zones, zone I to

Received 20 July, 2014 Accepted 18 September, 2014
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doi: 10.1016/S2095-3119(15)61175-3

zone IV, based on climatic conditions and cropping systems (Fig. 1) (Jansky *et al.* 2009). Zone I, i.e., the north single cropping zone, is the largest area of potato production.

Potato yields are significantly affected by virus infection. More than 40 viruses and two viroids can infect potato naturally, but only nine viruses and one viroid have a significant economic impact on this crop worldwide (Wang *et al.* 2011; Palukaitis 2012). *Potato spindle tuber viroid* (PSTVd) causes potato spindle tuber disease, which poses a serious threat to the production of seed potatoes, the maintenance of potato germplasm collections, and potato breeding programs (Owens *et al.* 1992). Over the past few decades, several disease outbreaks caused by PSTVd have been reported to occur in potato production in North America and Europe. With development of rapid, sensitive PSTVd detection methods, PSTVd has been eradicated or significantly reduced from potato breeding programs and commercial production in these regions (Owens 2007).

Potato spindle tuber disease was first detected in China in the 1960s on 'Irish Cobbler' potato. The disease mainly occurs in potato-growing regions in North China, including zone I and II (Cui *et al.* 1992; He *et al.* 1993; Ma *et al.* 1996). However, recent studies have revealed the presence of PSTVd in potato production zone III and IV (Qiu *et al.* 2011; Song *et al.* 2013). Although sensitive molecular detection methods have been widely used to identify and remove PSTVd-infected potato plants (He and Zhou 1992; He *et al.* 1992; Li *et al.* 2001) and PSTVd was included in the list of quarantine pests in 2004, this pathogen has not been eliminated or reduced in China but has instead spread to wider geographic areas. Therefore, the steady presence of PSTVd in China is an important problem that remains to be addressed.

Using PSTVd-free seeds for potato planting is commonly considered to be crucial, representing the most effective strategy for eliminating PSTVd from potato crops. Although virus (including viroid)-free seed potatoes have been produced and sold by a broad array of entities, including research institutes, universities, seed companies, private companies, and even small farmers (Jansky *et al.* 2009), PSTVd still exists in China and has not exhibited an obvious decline. This problem may be explained by the low quality of seed potatoes. To improve seed potato quality, the Supervision and Testing Center for Virus-free Seed Potatoes Quality was established at the Institute of Virus-free Seedling Research, Heilongjiang Academy of Agricultural Sciences under the support of the Ministry of Agriculture of China. One of the tasks of this Center is to test for PSTVd

Fig. 1 *Potato spindle tuber viroid* (PSTVd) sampling sites in China. Different colors represent the four major potato production zones (I–IV) in China. The sites for collecting PSTVd samples are marked with stars.

infection in samples received from seed potato producers and some potato growers.

Here we outline the results of the research conducted in the Center from 2009 to 2014, indicating a high rate of PSTVd infection in both commercial seed potatoes and propagation materials used for breeding programs. In addition, surveys of potato spindle tuber disease were also carried out in the field. The observation of some typical symptoms reveals the increasing need to control this disease. Finally, PSTVd-positive potato samples were subjected to molecular analysis to further characterize PSTVd infection of this crop in China.

2. Results

2.1. Symptoms of potato spindle tuber disease under field conditions

In every year of the survey, typical symptoms of some susceptible potato plants to PSTVd infection were observed under field conditions in the six provinces examined, especially in three northeastern provinces. Diseased potato plants commonly grow upright, with small leaflets, and their growth becomes stunted (Pfannenstiel and Slack 1980) (Fig. 2-A). Tubers of these diseased plants may be distorted, with some growth cracks (Figs. 2-B and C). Notably, spindly tubers (Fig. 2-D) were occasionally found in fields in Heilongjiang Province, although this typical symptom was not expressed previously when several locally bred potato cultivars were inoculated with PSTVd (Singh *et al.* 1993). The common occurrence of some typical symptoms of potato spindle tuber disease under field conditions implies that this disease is persistent in China and, consequently, some potato cultivars used for planting are susceptible to PSTVd infection.

2.2. Incidence of PSTVd infection

The results of analysis of PSTVd infection in seed potato samples are summarized in Table 1. Of the more than 1 000 potato samples collected, 66 were positive for PSTVd, yielding an incidence of 6.5%. Notably, PSTVd-infected seed potatoes were found in all six provinces, implying widespread PSTVd infection in China. Strikingly, a very high infection rate of PSTVd, 50%, was observed for the potato breeding line samples from 2012 to 2015, with the highest incidence, up to 87.3% (48/55), occurring in 2014 (Fig. 3). The high infection rate of PSTVd in breeding lines occurred because these breeding lines were likely derived from the same origin, i.e., contaminated mother plants that were propagated vegetatively. Importantly, the PSTVd incidence from 2012 to 2014 in breeding lines of potato was similar to that observed in the 1980s. These results

Fig. 2 Typical symptoms of potato spindle tuber disease observed in the field. A, infected potato plant of unknown cultivar. B, distorted tuber with crinkled growth. C, tubers of potato cultivar Kexin 18 with obvious crinkled growth. D, spindled potato tuber of unknown cultivar.

Table 1 Incidence (%) of *Potato spindle tuber viroid* (PSTVd) in various provinces from 2009 to 2014[1)]

Locations	2009	2010	2011	2012	2013	2014	Total
Heilongjiang	7.7 (3/39)	1.3 (1/76)	0 (0/12)	0 (0/29)	0 (0/38)	3.8 (2/53)	2.4 (6/247)
Jilin	NT	18.8 (3/16)	0 (0/32)	11.1 (8/72)	5.4 (2/37)	7.5 (3/40)	8.1 (16/197)
Liaoning	NT	NT	4.0 (1/25)	0 (0/9)	NT	NT	2.9 (1/34)
Inner Mongolia	31.3 (5/16)	23.3 (10/43)	3.7 (3/81)	0 (0/33)	4.2 (2/48)	0 (0/41)	7.6 (20/262)
Shandong	NT	100 (11/11)	2.1 (3/142)	100 (2/2)	NT	NT	10.3 (16/155)
Shaanxi	NT	33.3 (2/6)	0 (0/10)	0 (0/6)	NT	5.2 (5/97)	5.9 (7/119)
Total	14.6 (8/55)	17.8 (27/152)	2.3 (7/302)	6.6 (10/151)	3.3 (4/123)	4.3 (10/231)	6.5 (66/1 014)

[1)] The numbers in a bracket mean the number of positive samples/number of tested samples.
NT, not tested.

indicate that over the past 30 years, the presence of PSTVd in China has not been significantly affected, even though some control measures have been carried out. Indeed, eliminating PSTVd from China has become an important issue. Besides, nearly all symptomatic field potato samples were found to be infected by PSTVd (data not shown). Approximately 200 PSTVd-infected potato samples were obtained, and 71 different PSTVd isolates (Table 2) were subjected to sequencing.

2.3. Identification of PSTVd sequence variants

To determine the sequences of different PSTVd isolates, reverse transcription-polymerase chain reaction (RT-PCR) was performed, followed by two different sequencing strategies. For RT-PCR amplification, primers PSTVd-54R and PSTVd-55F were designed based on sequence alignments of all naturally occurring PSTVd variants in GenBank (Appendix A). Although nucleotides covered by the primers are highly conserved in most PSTVd variants, several variants, primarily those isolated from tomato crops and some ornamental plants, have a few mutations in this region. To further verify the sequences of the PCR products amplified using the first pair of primers, a second pair of primers (PSTVd-240F and 239R, their sequences was listed in the section of materials and methods) was also designed. Sequence analysis of 20 out of 71 different PSTVd isolates (Table 2) amplified using the second pair of primers confirmed sequence conservation in the region covered by the first pair of primers among Chinese PSTVd isolates.

2.4. Direct sequencing of RT-PCR products

PCR products from 50 out of 71 different PSTVd isolates (Table 2) were sequenced directly without cloning. In most cases, only one sequence was obtained per isolate, indicating that most of the PSTVd isolates (45/50) contained only one predominate sequence variant of PSTVd. However, in several PSTVd isolates, more than one sequence were detected, suggesting that at least two predominate variants simultaneously exist in these isolates. A detailed inspection of the sequence peaks produced from these isolates revealed that peaks at positions 258, 259, and 306 (refer to GenBank accession no. V01465) were not single but instead overlapped (Appendix B). PSTVd isolates 13J-18, DN27, and 13J-64 produced only one overlapping peak, which was composed of two different peaks. These three isolates were considered to contain two equally predominant variants in each isolate. However, for isolates DN-18 and DN-20, predominant variants were difficult to detect, as there were two different peaks at position 258 and three at position 255. In brief, the sequences of 51 predominant PSTVd variants were obtained from 48 out of 50 different PSTVd isolates (except isolates DN18 and DN20). These results confirm that analyzing uncloned PSTVd products can successfully be used to uncover master PSTVd variants in each isolate (Owens *et al.* 2003, 2009).

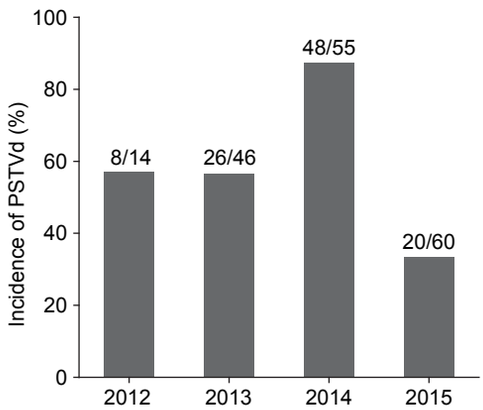

Fig. 3 Infection rate of *Potato spindle tuber viroid* (PSTVd) in potato breeding lines collected from 2012 to 2015. The number over the bar is the infection rate of PSTVd, positive samples/total samples.

2.5. Sequencing cloned PSTVd cDNAs

A total of 21 out of 71 different PSTVd isolates (Table 2) were cloned, followed by sequencing. Four to six clones were randomly selected and sequenced for each isolate, resulting in the detection of 105 sequences (Appendix C). Population

Table 2 Geographical distribution of Chinese PSTVd isolates used for PSTVd sequencing in this study

Isolate name	Potato variety	Region of origin	Collecting time	Tissue[1)]	Sequencing[2)]
11J-16	Kexin 13	Harbin	2011	Tuber	cDNA
C884	Zhongshu 3	Harbin	2011	Tuber	Clone
351-15, 17, 18	Black	Harbin	2011	Tuber	Clone
355-11, 13	Unknown	Harbin	2011	Tuber	Clone
DN-12-2	Breeding materials	Harbin	2012	Plantlets	Clone
DN-12-5, 7	Breeding materials	Harbin	2012	Plantlets	cDNA
DN-1, 3, 8, 9, 10, 12, 13, 15, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 30, 38, 40, 43	Breeding materials	Harbin	2013	Plantlets	cDNA
14W-13, 15	Unknown	Harbin	2014	Plantlets	Clone
37-38	Neishu 7	Keshan	2009	Plantlets	Clone
39-40	Bashu 10	Keshan	2009	Plantlets	Clone
BDH-45	Unknown	Keshan	2012	Plantlets	Clone
HL(1)-ZDX	Favorite	Keshan	2012	Tuber	cDNA
K18(4)-ZDX	Kexin 18	Keshan	2012	Tuber	Clone
K18(8)-ZDX	Kexin 18	Keshan	2012	Tuber	cDNA
K18(9)-ZDX	Kexin 18	Keshan	2012	Tuber	Clone
40N	Favorite	Nenjiang	2012	Tuber	cDNA
12W-70	Unknown	Jilin	2012	Plantlets	Clone
12W-48, 83, 278, 279	Unknown	Jilin	2012	Plantlets	cDNA
13W-81	Unknown	Jilin	2013	Plantlets	Clone
J-9	Zaodabai	Liaoning	2011	Plantlets	cDNA
649-1	Unknown	Inner Mongolia	2011	Tuber	cDNA
13-J-15, 16, 18, 20, 22, 23, 24, 27, 31, 61, 64	Unknown	Inner Mongolia	2013	Plantlets	cDNA
13W-38	Unknown	Inner Mongolia	2013	Plantlets	cDNA
10W-170, 171, 179	Favorite	Shandong	2010	Tuber	Clone
12W-138	Unknown	Shandong	2012	Plantlets	Clone
12W-13	Favorite	Shaanxi	2012	Plantlets	Clone
10W-35	Unknown	Inner Mongolia	2010	Plantlets	cDNA
0076-1	Unknown	Keshan	2009	Tuber	cDNA
648-2	Unknown	Inner Mongolia	2011	Tuber	cDNA
ODN	Breeding material	Harbin	2012	Plantlets	cDNA

[1)] Plantlets indicate the collected isolates were maintained as plantlets *in vitro*; tuber means the collected isolates from field were preserved by the extracted total RNAs.
[2)] cDNA indicates that the full-length sequence of PSTVd was obtained by direct sequencing of uncloned cDNAs generated from RT-PCR; clone indicates that the complete genome of PSTVd was obtained by sequencing of cloned cDNAs produced by RT-PCR.

structure analysis revealed that each isolate contained at least one predominant PSTVd variant. On the whole, three different types of population structures for these 21 different PSTVd isolates were revealed: i) a population containing only one type of sequence variant, such as isolates 10W-171, C884, and 12W-70; ii) a population structure following the typical quasispecies model (Gora-Sochacka *et al.* 1997), as four isolates (DN12-1, 12W-13, 13W-81, and 37-38) comprised different sequence variants but contained a dominant variant; and iii) a population comprising isolates 351-15 and BDH-45, which also included several different sequence variants, with almost identical numbers of these variants, implying the existence of more than one predominant variant. This population structure could explain the presence of overlapping peaks when uncloned PSTVd cDNAs were directly sequenced. In short, the sequences of 23 predominant PSTVd variants were obtained from 21 out of 71 different isolates (Table 2) by sequencing cloned PSTVd cDNAs.

2.6. Novel variants and polymorphisms of PSTVd

Comparative sequence analysis of the 74 predominant PSTVd variants identified from 71 different PSTVd isolates by sequencing of uncloned PCR products and cloned cDNAs revealed 42 different sequence variants of PSTVd (Table 3). Of the 42 different PSTVd variants, 12 completely matched sequences in GenBank (Table 3). It is important to note that six (DN1, DN13, DN15, DN24, DN27, and J9) and three (10W35, 12W83, and DN12-2) of Chinese PSTVd variants were identified as mild and intermediate strains because their sequences were identical to those variants with known pathogenicity. In addition, 30 novel PSTVd variants were detected that were found only in China. These novel PSTVd variants were deposited into the public database GenBank to supplement available sequence information about Chinese PSTVd variants.

The sequences of the 42 different PSTVd variants were

Table 3 Sequence analysis of the Chinese PSTVd variants identified in this study

Variant	GenBank accession no.	Isolates	Size (nt)	Mutations[1)]	Closest sequence[2)]
DN27	KR611340	HL(1)-ZDX K18(8)-ZDX BDH45, DN27	359	–	M14814 (0)
DN15	KR611338	DN-15, 21, 27, 11J-16, BDH45, K18(4)-ZDX K18(9)-ZDX	359	C256U	EU879917 (0)
13W81-2	KR611357	13W81	359	U161C	M14814 (1)
DN3	KR611358	DN3	359	G49A	M14814 (1)
DN1	KR611369	DN-1, 25, 43	359	U310A	X52036 (0)
DN24	KR611360	DN24	359	U310C	X76844 (0)
ODN	KR611362	ODN	358	U310-	JQ889848 (1)
DN8	KR611370	DN-8, 28	359	U257A	M14814 (1)
DN13	KR611373	DN-13, 22, 13J64	359	G254A	M88677 (0)
13J64-1	KR611374	13J64	359	G254A, U306A	M88677 (1)
DN18	KR611355	DN-18, 19, 20	359	C256A, C259U	M88681 (1)
J9	KR611339	J9, 13W38, 12W48, 13J-15, 27, 61	359	U121C, C256U	EF044305 (0)
40N	KR611334	351-15, 18, 355-11, 13, 12W-13, 138, 10W-171, 179, C884, 40N	359	U245A, C256U	EU879917 (1)
DN26	KR611347	DN26	359	C256A, U310C	X76844 (1)
13J22	KR611359	13J-22, 23, 24	359	U121C, U310C	X76844 (1)
DN12-7	KR611361	DN12-7	358	C196G, U310-	JQ889848 (2)
12W279	KR611363	12W279	358	U306A, U310-	JQ889841(0)
10W35	KR611367	10W35, 14W15	358	U310A, U312-	EF044304 (0)
649-1	KR611375	649-1	359	G49A, G254A	M88677(1)
DN38	KR611341	DN38	359	G49A, G54A, C256U	EU879917 (2)
10W170	KR611335	10W170	359	U161C, U245A, C256U	EU879917 (2)
351-15-2	KR611336	351-15	359	U245A, C256U, U310C	X76844 (2)
351-17-1	KR611337	351-17	358	U245A, C256U, U310-	EU879917 (2)
DN10	KR611346	DN-10, 13J-20	359	U121C, C256A, U310C	EU879921 (1)
DN40	KR611349	DN40	358	C256A, U306A, U310-	JQ889841 (1)
DN30	KR611354	DN30	359	G49A, G54A, C256A	JQ889840 (2)
DN9	KR611364	DN9	358	U121C, U306A, U310-	JQ889850 (0)
12W278	KR611372	12W278	358	U257A, U306A, U310-	JQ889841 (1)
12W83	KR611366	12W83	358	U121C, U310A, U312-	EF044303 (0)
DN12-2	KR611368	DN12-2	359	141+U, U310A, U312-	EF044302 (0)
12W70-2	KR611365	12W70	359	C259U, U306A, U310-	JQ889841(1)
13J31	KR611345	13J31	358	U121C, C256U, U306A, U310-	EU879923 (0)
13J16	KR611348	13J16	359	U121C, A225C, C256A, U310C	EU879921 (2)
DN12-5	KR611352	DN12-5	358	G49A, G54A, C256A, U310-	JQ889840 (3)
DN12	KR611353	DN12	359	G49A, G54A, U121C, C193U, C256A, U310A	JQ889846 (3)
13J18	KR611344	13J18	361	49+A, 119+CC, U121C, C256U, U310-	JQ889849 (3)
13J18-1	KR611351	13J18	361	49+A, 119+CC, U121C, C256A, U310-	JQ889851 (4)
14W13-3	KR611350	14W13	359	49+A, U121C, U161C, C167U, C256A, U310-	JQ889851 (4)
0076-1	KR611343	0076-1	360	49+A, G54A, G61A, 146+C, C256U, U310-	JQ889851 (4)
39-40-1	KR611371	39-40	359	119+A, U121C, U161C, U257A, U310A, U312-	JQ889844 (2)
648-2	KR611376	648-2	359	U15G, G49A, G54A, G61A, C193U, C196U, G254A	M88677 (6)
37-38-3	KR611342	37-38	359	G49A, U121C, 146+C, C256U, U310A, C311-, U312-, U316A, 319+C	JQ889843 (5)

[1)] nucleotide changes compared with PSTVd sequence variant DN27. The position of mutations referred to Appendix D. – means no data. - after mutations means deletion.
[2)] Numbers in brackets indicate mutations of Chinese sequence variant compared with the corresponding closest sequence variant.

first compared with the sequence of the type strain (GenBank V01465) to identify new polymorphisms in PSTVd. The results reveal that mutations at 25 positions formed limited clusters, including the following: deletion of A at 119; A/U

or A/C substitution at 121; C/U or C/A substitution at 256; A/U or A/C substitution at 310; and insertion of U between 311 and 312 (Appendix D). These clusters of sequence changes were also observed in Russian PSTVd variants (Owens *et al.* 2009). Thus, a more comprehensive comparison of these 42 PSTVd variants with all PSTVd sequence variants from GenBank (data not shown) was performed. Several new polymorphisms of PSTVd were identified, including insertions of C between 318 and 319 and between 119 and 120, as well as substitutions of G/A at 54, A/C at 119, U/A at 254, and U/A at 316. These polymorphisms were found only in variants from China, indicating that they were specific to Chinese PSTVd variants. In addition, the identification of two mutations in PSTVd isolates from potato plants, at 161 (U/C) and 196 (C/G), supports the notion that PSTVd can be transmitted between different plant species (Verhoeven *et al.* 2010a, b), because these two mutations were also observed in the ornamental plants *Brugmansia* spp. and *Cestrum aurantiacum* (Verhoeven *et al.* 2008a; Luigi *et al.* 2011).

2.7. Effects of mutations on loops or motifs in the secondary structure of PSTVd

The effects of the identified mutations, especially novel mutations affecting the conformation of the predicted secondary structure of PSTVd, were examined (Fig. 4). Some loops that are associated with important biological functions of viroids (Zhong *et al.* 2008) were found to be conserved in these PSTVd variants. Loops 23, 26, and related RY motifs, which are essential for proliferating or establishing a viroid infection (Gozmanova *et al.* 2003; Kalantidis *et al.* 2007), were strictly conserved. Although mutations at 256 (C/W), 257 (U/A), and 259 (C/U) were observed in loop 15 (also known as loop E), these mutations have no effect on the integrity of the loop E motif according to the model proposed by Zhong *et al.* (2006). This observation is in line with the results of analysis for some natural PSTVd variants isolated from Russia (Owens *et al.* 2009; Kastalyeva *et al.* 2013).

Several mutations may alter the conformation of other functional loops. Substitution of U by G at position 15 was identified in the TCR (terminal conserved region) motif. This motif may be involved in some unknown, critical functions in the genera *Pospiviroid*, *Apscaviroid*, and *Coleviroid* (Di Serio *et al.* 2014). Importantly, this mutation enlarges loop 3 and disrupts one base pair in the GC box, which may constitute the transcription domain of PSTVd (Kolonko *et al.* 2006). In addition, several mutations observed in loops 7, 17, 19, 20, 24, and 25 which are associated with PSTVd trafficking (Fig. 4) (Zhong *et al.* 2008). The insertion of C at position 318 and substitution U316A for variant 37-38-3 not altered loop 7 but resulted in a new bulge. In case of loop 17, the substitutions of A118C and U121C and the insertion of C at position 119 in variant 13J18 and 13J18-1 partially closed

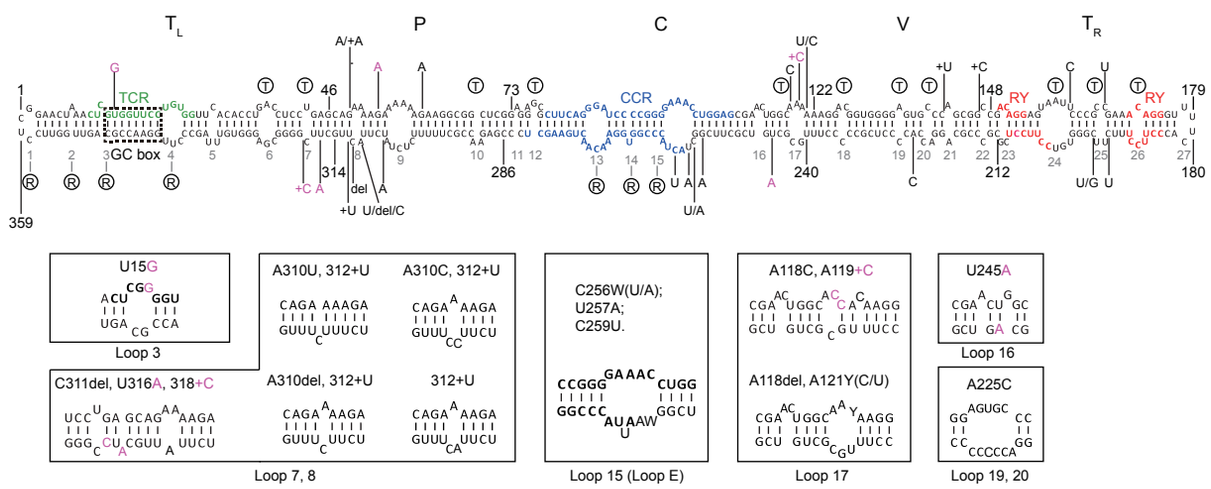

Fig. 4 Potential effects of the observed nucleotide changes on the structures of conserved motifs and functional loops in the PSTVd genome. The minimum free energy structure of PSTVd was predicted using the reference sequence (accession no. V01465). From left to right, the predicted secondary structure of PSTVd contains 25 interior or bulge loops and two hairpin loops. Loops essential for PSTVd replication (R) or trafficking (T) are marked with numbers (Zhong *et al.* 2008) at the boundaries of the five *Pospiviroid* structure domains: the terminal left (T_L), terminal right (T_R), pathogenicity (P), central (C), and variable (V) domains. Three conserved motifs, including the terminal conserved region (TCR), central conserved region (CCR), and RY, are indicated in green, blue, and red, respectively. Nucleotides forming a GC box are indicated in the rectangle with a dotted line. Nucleotides in pink represent mutations found only in Chinese PSTVd variants, whereas nucleotides in black outside the secondary structure of PSTVd indicate mutations that were reported in PSTVd variants from other countries. The detailed changes in the structures of loops 3, 7, 8, 15, 16, 17, 19, and 20 are shown in the enlarged panels at the bottom.

this loop and produced a new loop 7. By contrast, loops 19 and 20 were merged into a larger loop by the substitution A225C in variant 13J16 (Appendix D).

Although functions of loops 8, 9, 16, 21, and 22 have yet to be elucidated (Zhong *et al.* 2008), their structures were also modified due to mutations. Mutations occurred at positions 310 to 312 were complex and resulted in five different structures for loop 8 (Fig. 4). The nucleotide change (U/A) at position 306 found in six variants (Appendix D) partially enlarged loop 9 by disruption of a neighboring A:U base pair. A similar situation was found with loop 16. Mutation (U245A) in the short helix close to loop 16 decreased this loop to a bugle and generated a new bugle. Strikingly, although functions of these loops are poorly understood, they were not found to be closed by naturally occurring sequence changes. These results imply that these loops may be helpful, if not critical, for the accomplishment of viroid biological functions.

2.8. Common PSTVd variants in China

Determining sequence variants from 71 different isolates allowed us to identify common PSTVd variants in China. Blast and comparative sequence analyses revealed several widespread PSTVd sequence variants in different potato cultivars collected from different provinces. Sequence variant 40N was detected in ten different isolates of five different cultivars, which were collected from three different provinces (Heilongjiang, Shandong, and Shaanxi) (Tables 2 and 3). Similarly, PSTVd variant J9 was detected in six different isolates, representing four different cultivars from three different provinces (Jilin, Liaoning, and Inner Mongolia). These data suggest that PSTVd variants 40N and J9 are common in potato production regions in North China. In addition, variant DN15 was found in seven different isolates. Although these isolates were collected from a single province, they were collected from two different regions in the province and, importantly, many of the isolates were found on breeding materials. Therefore, DN15 appears to be a common variant in China. Similarly, DN15, DN1 and DN18 (Tables 2 and 3) also appear to be common variants due to their presence in some potato breeding materials. It should be noted that the sequences of the major PSTVd variants J9, DN15, and DN1 are identical to those of mild strains isolated from other countries (right column of Table 3). Only one nucleotide change was detected between the common variant 40N and EU879917, known as mild strain of PSTVd.

2.9. Phylogenetic analysis of Chinese PSTVd sequence variants

To elucidate the evolutionary relationship between Chinese PSTVd variants and those from other countries, a comprehensive phylogenetic analysis was performed using a Clustal X version 2.0 (Larkin *et al.* 2007) alignment of the sequences from the 42 PSTVd variants and 165 naturally occurring PSTVd variant sequences deposited in GenBank (Appendix A). The results obtained using the neighbor-joining method, maximum likelihood method, and minimum evolution analysis in MEGA 6 are essentially identical. PSTVd variants were clearly clustered into a small group (I) and a large group (II) with a high bootstrap value. The large group (II) contained more than 180 variants, which did not form distinct clusters due to low bootstrap values. The low sequence diversity of PSTVd, which was also observed in Russian variants (Owens *et al.* 2009), may explain the absence of distinct clusters in group II in the phylogenetic trees constructed by MEGA 6.

When evolution adopts in a tree-like manner, analysis of the data may not be best served by a tree or an assumed tree-like model. A number of different types of phylogenetic networks could resolve this issue (Huson and Bryant 2006). Thus, a phylogenetic network of PSTVd variants was constructed using SplitsTree 4 (Huson and Bryant 2006) to detect possible relationships among the Chinese PSTVd variants. In line with the phylogenetic tree obtained by MEGA 6, PSTVd was classified into group I and group II (Fig. 5). However, group II was divided into three subgroups (group IIA–C). Based on the phylogenetic network, it is interesting to note the following: i) PSTVd variants, to some extent, are host specific. All variants from potato, except for variant DQ308556 from Iran, were classified into group II (Appendix A), whereas variants from pepino (*S. muricatum*), pepper (*Capsicum. annum*), *S. nigrum*, and cape gooseberry (*Physalis peruviana*) were classified into group I. Moreover, a variant from dahlia that was recently detected in Japan (Tsushima *et al.* 2011) was not included in any cluster. ii) PSTVd variants from tomato are dispersed among groups and subgroups except subgroup IIC. This result is in accordance with the previous observation that PSTVd variants from tomato do not form a separate cluster but instead cluster with PSTVd variants from vegetatively propagated solanaceous plant species (Verhoeven *et al.* 2010b), which confirms that solanaceous plant species infected with PSTVd may be the source of PSTVd for diseased tomato crops (Verhoeven *et al.* 2010b). It should be noted that PSTVd variants from potato are dispersed among all groups and subgroups. Thus, they may originally derived from PSTVd infected solanaceous plant sepecies as the origin of PSTVd variants from tomato (Verhoeven *et al.* 2010b). iii) All PSTVd variants from Asian countries and Russia belong to group II, and importantly, most of these variants clustered together. iv) Chinese PSTVd variants disperse among three subgroups.

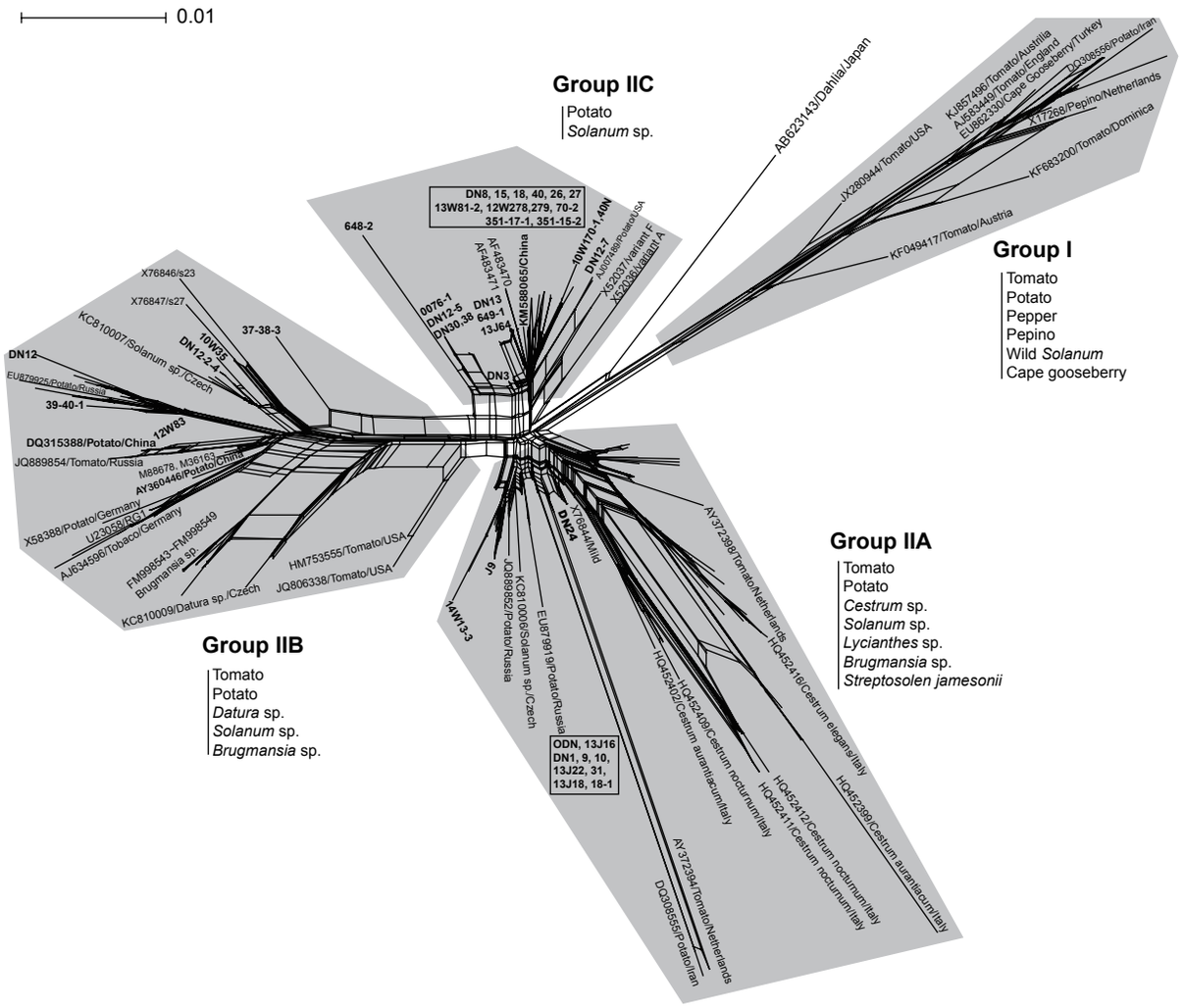

Fig. 5 Phylogenetic relationships among most known naturally occurring PSTVd variants. The network was constructed by SplitsTree4 using the neighbor-joining method with 1 000 replicates. Bold labels indicate Chinese PSTVd variants identified in this work and reported previously. Labels for many variants have been omitted.

To elucidate the possible evolution process among Chinese PSTVd variants, a two-dimensional network was constructed as described by Owens *et al.* (2009). This analysis was based on the principle that the number of mutations between two variants must be the least and no more than three. To help detect possible links among these PSTVd variants, the variant DN27 in China was used as a reference. Thus, 42 different PSTVd variants were listed in order of number of mutations compared with the reference (Table 3). The result shows that all PSTVd variants were included in a two-dimensional network with the exception of four variants (0076-1, 648-2, 39-40-1, and 37-38-3; Fig. 6). Strikingly, 35 out of 42 variants were linked to each other with only one mutation. Given that 12 variants in the network (indicated with a gray background in Fig. 6) were identical to those from other countries, it is probable that most of the variants found only in China originated from those introduced variants through alteration of a single nucleotide. By contrast, several variants could not have been generated from those introduced variants through a single nucleotide change, instead arising from the original variant though a relatively complex pathway. For example, generation of 14W13-3 from the closest introduced variant DN27 proceeded in seven steps involving 11 mutations.

3. Discussion

Potato spindle tuber disease was first observed in China in the 1960s. The causal agent of this disease (PSTVd) was detected subsequently by bioassays using indicator host of tomato (*cv.* Rutgers) (Li *et al.* 1979). Until now, PSTVd is still persistent in China. In principle, the purpose to prevent the introduction and/or spread of PSTVd in a country could be achieved by phytosanitary measures and elimination of

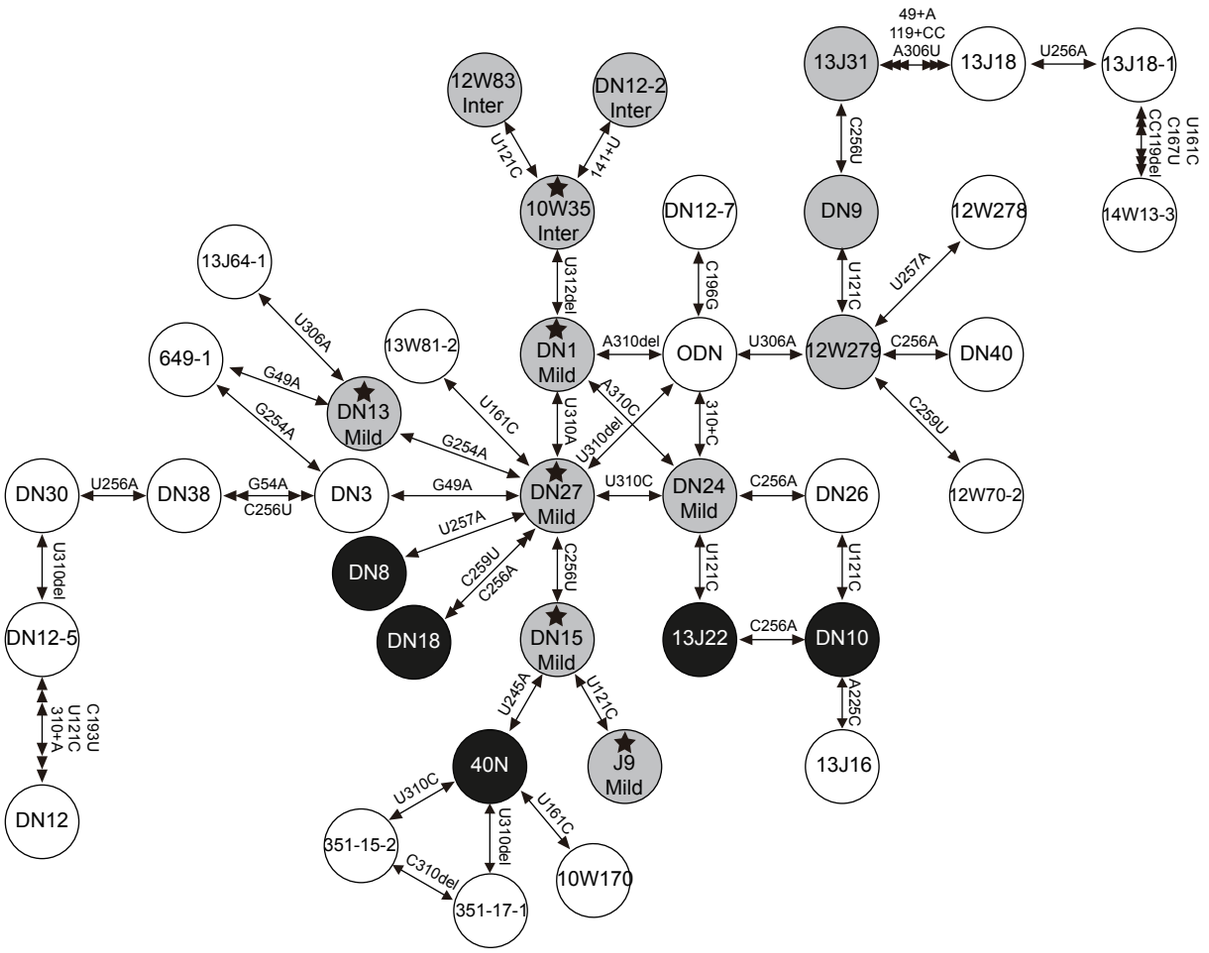

Fig. 6 Sequence relationships among most of the identified Chinese PSTVd variants listed in Table 3. The network was created based on the principle that the number of mutations between each of two variants must be the lowest. Variants marked with a gray background are the same as those that were reported. Popular PSTVd variants in China are denoted with a black background or star.

this pathogen from a geographic region could be realized through implementation of some effective strategies, such as strict certification of seed potatoes and eradication of PSTVd infected propagation materials. This has been confirmed by the successful elimination of PSTVd from North America and some European countries (Owens 2007). In order to exclude PSTVd from China, PSTVd was covered by Chinese phytosanitary rules in 2004. Furthermore, sensitive molecular methods have been applied to detected PSTVd in infected plant samples (He and Zhou 1992; He *et al.* 1992; Li *et al.* 2001) and virus/viroid-free seed potatoes have been produced and used for potato planting (Jansky *et al.* 2009). However, these control strategies are clearly ineffective for exclusion of PSTVd from China. The data obtained in this study could partially explain why PSTVd continues to persist in China based on the possible origin of Chinese PSTVd isolates, the quality of seed potatoes produced and sold for potato planting, and pathogenicity of Chinese strains of this pathogen.

Although PSTVd was likely introduced into China through the import of potato cultivar Irish Cobbler from North America (Singh *et al.* 1993), this does not explain the presence of PSTVd in China in recent years. The elimination of PSTVd from North America (Singh and Crowley 1985; Owens 2007) excludes the possibility that PSTVd-infected potatoes are still imported into China from North America. In addition, PSTVd was listed as a quarantine pest in 2004 in China, which, to some extent, should also prevent re-introduction of propagation materials infected by this viroid. While North America is not the main source of imported potato propagation materials in China, the possibility that PSTVd is imported into China from other countries through potato materials, especially those used for breeding, cannot be completely excluded. The results of phylogenetic analysis show that Chinese PSTVd isolates share the closest relationships with those from Russia (Fig. 5), indicating that cross-transmission of PSTVd may occur between China and Russia. This notion is supported by the fact that frequent

exchange of potato germplasm between the former Soviet Union (Russia) and China has occurred from the 1940s to the present. Therefore, the introduction of PSTVd through exchanging potato germplasm from other countries may be still associated with the steady presence of PSTVd in China.

With respect to the origin of PSTVd in potato crops, cross-host transmission may occur, as vegetatively propagated ornamental plant species act as a source of PSTVd infection in tomato (Verhoeven *et al.* 2010b). Like PSTVd isolates from tomato, PSTVd isolates from potato plants could be classified into all groups and subgroups (Fig. 5). Thus, it appears that some ornamental plants infected by PSTVd may be sources of PSTVd infection in potato (Matousek *et al.* 2014). Although PSTVd has been isolated from ornamental plants in many countries (Di Serio 2007; Verhoeven *et al.* 2008a, b; Tsushima *et al.* 2011; Matousek *et al.* 2014; Milanović *et al.* 2014), PSTVd from ornamental plants has not been reported in China. However, recognition of possible cross-host transmission still has important implications for the control of PSTVd in China. The lack of reported PSTVd from ornamental plants in China does not prove that PSTVd is not present in these plants, as few studies have been performed to identify PSTVd in ornamentals. Given that the exchange of ornamental plant germplasm between China and other countries has significantly increased in recent years, it will be important to perform PSTVd quarantine measures for imported ornamental plants.

Using PSTVd-free seeds in potato planting is commonly considered to be crucial, representing the most effective strategy for eliminating PSTVd from potato crops. Although virus (including viroid)-free seed potatoes are produced and sold by a variety of entities in China, the presence of PSTVd has not been reduced for two possible reasons: (1) So-called virus-free seed potatoes are of low quality, and (2) not all farmers use virus-free seed potatoes. The results of the current study verify that the first possibility is correct. The high PSTVd infection rate (Table 2) in commercial seed potatoes (6.5%) can, to a major extent, explain why PSTVd has remained persistent in China. The second reason may also help explain this problem, as only approximately 20% of the total potato production area in China was planted using virus-free seed potatoes (Jansky *et al.* 2009). The ratio of utilization of certified seed potato tubers in China has been steadily increasing (Wang *et al.* 2011; Devaux *et al.* 2014). However, the expected decrease in PSTVd infection has not occurred, implying that the second reason does not explain the steady presence of PSTVd in China. The eradication of PSTVd in the seed potato crop in North America was attributed to higher standards and stricter regulation in seed certification programs and the rigorous use of clean stocks (Singh and Crowley 1985; Owens 2007), suggesting that effective actions must be taken to improve the quality of seed potatoes in China.

In line with several previous studies on the identification of strains of Chinese PSTVd isolates (He *et al.* 1993; Singh *et al.* 1993; Ma *et al.* 1996), comparative analysis of PSTVd sequence variants identified in the present study confirmed that some PSTVd variants from China are mild strains, as they are identical to mild strains from Russia (Table 3, Fig. 6). These mild strains can infect potato plants but generally do not cause obvious symptoms, although the typical symptoms of potato spindle disease were occasionally observed in a few susceptible potato cultivars (Fig. 2). Therefore, farmers incorrectly assume that PSTVd has no effect on potato production, which may explain why many potato famers use their own harvested potatoes as seed for the next season rather than buying certified seed (Jansky *et al.* 2009). Even though the use of self-harvested seed potatoes is not the main reason for the ineffective control of PSTVd in China, the notion that PSTVd is not harmful to potato production has a serious impact on the attempt to control PSTVd. The current finding that some Chinese PSTVd variants are mild strains may also be associated with the steady presence of PSTVd in China.

Indeed, PSTVd directly damages potato, as a recent study revealed that four different potato cultivars showed obvious symptoms when inoculated by PSTVd under experimental conditions, and importantly, the yields of one of these cultivars decreased significantly (Qiu *et al.* 2014). Moreover, several intermediate strains were also identified among the Chinese PSTVd variants identified in the current study (Table 3, Fig. 6). Notably, some novel PSTVd variants specific to China, such as 648-2 and 37-38-3, are significantly divergent from known PSTVd variants, with six and five mutations compared with the closest variants, respectively (Table 3, Fig. 6). These results indicate that PSTVd variants from China likely have different levels of pathogenicity rather than only the mild type. We cannot obtain a more comprehensive understanding of Chinese PSTVd variant strains, as most samples used in this study were collected from North China and the pathogenicity of some divergent PSTVd strains was not tested using bioassays. It will be important to identify mutations generated during the adaptation of PSTVd to different local potato cultivars and to determine the pathogenicity of the PSTVd variants identified in this study on different major commercial cultivars used in China.

In addition to explaining persistence of PSTVd in China, the identified naturally occurring mutations provided some compelling supports to the functional motif model for PSTVd (Zhong *et al.* 2008). This model shows that most of loops distributed in the secondary structure of the viroid genome

are critical for viroid replication and trafficking. Despite many naturally occurring sequence changes were found in loops, these loops were not able to be completely disrupted (Fig. 4). These data confirmed the essential roles of these loops for replication and systematic trafficking. Loop E (Loop 15) in PSTVd genome is most used in mutational analysis and can be best understood among these loops. It is involved in host adaptation (Wassenegger *et al.* 1996), replication (Zhong *et al.* 2006), and pathogenicity (Qi and Ding 2003). Maintenance of the integrity of the structure of Loop E in natural PSTVd variants (Fig. 4) further support the notion that an intact loop E is essential for PSTVd viability (Zhong *et al.* 2006). The C259U substitution within Loop E found in variants of DN18 and 12W70-2 indicated that this mutation not only occurred in tobacco (Wassenegger *et al.* 1996) but also exists in potato in nature. Most strikingly, the U257A substitution was found in three different variants. This mutation can convert the intermediate PSTVd strain to a lethal strain that causes severe growth stunting with "flat-top" symptom in infected tomato plants (Qi and Ding 2003). The pathogenicity of the PSTVd variants containing this nucleotide change in potato plants was unknown, because these variants were isolated from plantlets *in vitro* which were too small to express symptoms. Thus, a hypothesis that the U257A substitute may also confer this PSTVd variant stronger pathogenicity in potato plants is worthwhile to be addressed by mutational analysis.

Finally, the results of this study reveal several reasons for the ineffective control of PSTVd in China over the past five decades. There have been many difficulties during each step of the strategy used to control PSTVd, including the import of PSTVd-infected potato germplasm through exchange propagation material, the low quality of virus-free seed potatoes, and the lack of awareness about the damage caused by PSTVd infection in potato crops. Therefore, the strategy used to control PSTVd in China should be modified, and more effective actions must be taken to eliminate PSTVd from China in the future.

4. Conclusion

We summarized the results of PSTVd detection in seed potatoes collected from 2009 to 2014, which were analyzed at the Supervision and Testing Center for Virus-free Seed Potatoes Quality, Ministry of Agriculture of China, at the Institute of Virus-free Seedling Research, Heilongjiang Academy of Agricultural Sciences, China, revealing a high infection rate of 6.5%. Strikingly, breeding lines of potato collected from 2012 to 2015 were also infected by PSTVd, with a high incidence of up to 87.3% in 2014. These data indicate that the quality of commercial seed potatoes is low, which has strongly contributed to the ineffective control of PSTVd in China. In addition, 71 different PSTVd isolates from China were sequenced, revealing 74 predominant PSTVd sequence variants. Comparative analysis of these sequence variants revealed 42 different sequence variants and 30 novel variants specific to China. Phylogenetic analysis showed that the PSTVd sequence variants from China share close relationships with those from Russia. Like the Russian PSTVd variants, some of the Chinese PSTVd variants are mild strains, and several intermediate strains were also identified among the Chinese PSTVd variants. Based on the results of this study, we provide several possible reasons for the steady presence of PSTVd in China. The results provide a basic reference for designing more effective measures for controlling PSTVd in China in the future.

5. Materials and methods

5.1. Sample collection

Three different types of potato samples, including seed potatoes, potato breeding lines, and field samples, were collected from six provinces in North China (Fig. 1, Table 1). Seed potatoes were collected by the Supervision and Testing Center for Virus-free Seed Potatoes Quality, Ministry of Agriculture of China, which is affiliated to the Institute of Virus-free Seedling Research, Heilongjiang Academy of Agricultural Sciences. Every year, many different seed potatoes were sent to the Center mainly from research institutes, universities and seed companies, and few from private companies and small farms for viral infection testing. The north cropping region (zone I) is the most important seed potato production region in China (Jansky *et al.* 2009). The collected seed potatoes were mainly obtained from this region. In addition, potato breeding lines were collected from universities and research centers from 2012 to 2015 and tested for PSTVd infection. Lastly, some diseased potato plants in the field were collected during the growing seasons. The disease was diagnosed under field conditions according to the typical symptoms including upgrowth or stunted plants, short petioles, and small leaflets (Cui and Li 1990; Singh *et al.* 1991). Some tubers of diseased plants were also checked upon harvest.

5.2. PSTVd detection and maintenance of PSTVd isolates

The collected samples were tested by nucleic acid spot hybridization (NASH) using digoxin-labeled PSTVd cDNA probes and RT-PCR as previously described (Li *et al.* 2001; Lv *et al.* 2005). Most of PSTVd-positive leaves or tubers verified by these two methods were cultured into plantlets

in vitro and maintained by yearly propagation in a culture room at (25±2)°C with a 16-h photoperiod. Potato tubers were not chosen for preservation because some PSTVd-infected tubers often become too small to be replanted after several passages, resulting in the loss of materials. This phenomenon was also observed by Owens *et al.* (2009). In addition, total RNAs of some diseased potato tubers collected from field were extracted and preserved in –80°C (Table 2).

5.3. RNA extraction, RT-PCR, cloning, and sequencing

Total RNAs of the potato tubers and leaflets *in vitro* (Table 2) were extracted using TRNzol-A+ reagent according to the manufacture's instructions (Tiangen Biotech, Beijing). Reverse transcription (RT) of the RNAs were performed using M-MLV reverse transcriptase (Promega, Madison, USA) with PSTVd-specific primer PSTVd-54R (5′-GGATCCCTGAAG CGCTCCTCCGAGCCG-3′, complementary to positions 92–66). The cDNAs were directly used as templates to amplify the PSTVd genome. Full-length double-stranded PSTVd cDNAs were synthesized using primer pair PSTVd-54R and PSTVd-55F (5′-CCGGGGAAACCTGGAGC GAACTGG-3′, positions 93–116). The overlapping PSTVd cDNAs were also amplified using primers PSTVd-239R (5′-AAAGGGGGCGAGGGGTGRTC-3′, complementary to positions 239–220) and PSTVd-240F (5′-GCGCTGTC GCTTCGGMTACTAC-3′, positions 240–261) to confirm the sequence amplified by the first pair of primers.

Two different sequencing strategies (direct sequencing of PCR products and sequencing of cloned cDNAs) were used independently. PSTVd cDNAs used for cloning followed by sequencing were produced by high-fidelity *pfu* DNA polymerase (Tiangen Biotech, Beijing), whereas uncloned PSTVd cDNAs used for direct sequencing were synthesized using Blend *Taq* (Toyobo Life Science, Shanghai, China) DNA polymerase. Although low fidelity DNA polymerase was used for direct sequencing of PCR products, analysis of uncloned PCR products enables sequences of the predominant variant(s) in each isolate to be obtained due to the insensitivity of uncloned cDNA sequencing to the mutations (Owens *et al.* 2003; Owens *et al.* 2009).

For cloned cDNA sequencing, the PCR products of PSTVd were cloned into the pMD18-T vector (TaKaRa, Dalian, China). Recombinant plasmids containing PSTVd cDNAs were transformed into *E. coli* DH5α competent cells. Four to six positive clones were selected randomly. The selected PSTVd-positive clones and the uncloned PSTVd cDNAs were subjected to direct sequencing at Sangon Biotech (Shanghai) Co., Ltd., China, using an ABI 3730×L DNA analyzer (Sangon).

5.4. Prediction of secondary structure of PSTVd

The secondary structures of different PSTVd isolates with the lowest free energy were predicted by Mfold (Zuker 2003) at a folding temperature of 37°C. The predicted secondary structure of the reference sequence of PSTVd (GenBank accession no. NC_002030) (Gross *et al.* 1978) was further drawn using Rnaviz2 (De Rijk *et al.* 2003).

5.5. Sequence alignment and phylogenetic analysis

The sequences of the predominant variant(s) in each collected isolate were used for phylogenetic analysis, along with the sequences of naturally occurring PSTVd isolates deposited in GenBank. The predominant variants(s) of isolates were directly identified by uncloned PSTVd cDNA sequencing. For the isolates sequenced from cDNA clones, the predominant variant(s) was determined by alignment of the sequences of all obtained clones from each isolate using Clustal X version 2.0 (Larkin *et al.* 2007). Sequences of 165 naturally occurring PSTVd isolates were selected from the 253 PSTVd sequences deposited in GenBank. Information about these isolates, including their GenBank accession numbers, hosts, and country of origin is summarized in Appendix A. All PSTVd sequences were aligned using MUSCLE (Edgar 2004) with manual adjustment. Phylogenetic and molecular evolutionary analysis were performed using the MEGA version 6 (Tamura *et al.* 2013) and SplitsTree4 software packages (Huson and Bryant 2006).

Acknowledgements

The work was supported by the China Agriculture Research System (CARS-10-P14), the National Science Foundation for Post-doctoral Scientists of China (20110491125), the Heilongjiang Funds for Distinguished Young Scientist, China (JC 201018), the Harbin Application Technology Research and Development Projects, China (2013AE6AW059) and the Young Scientists Science Foundation of Heilongjiang Province, China (QC2015026).

Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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(Managing editor ZHANG Juan)