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Aphid vectors impose a major bottleneck on *Soybean dwarf virus* populations for horizontal transmission in soybean

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Abstract

Many RNA viruses have genetically diverse populations in a single host. Important biological characteristics may be related to the levels of diversity, including adaptability, host specificity, and host range. Shifting the virus between hosts might result in a change in the levels of diversity associated with the new host. The level of genetic diversity for these viruses is related to host, vector and virus interactions, and understanding these interactions may facilitate the prediction and prevention of emerging viral diseases. It is known that luteoviruses have a very specific interaction with aphid vectors. Previous studies suggested that there may be a tradeoff effect between the viral adaptation and aphid transmission when *Soybean dwarf virus* (SbDV) was transmitted into new plant hosts by aphid vectors. In this study, virus titers in different aphid vectors and the levels of population diversity of SbDV in different plant hosts were examined during multiple sequential aphid transmission assays. The diversity of SbDV populations revealed biases for particular types of substitutions and for regions of the genome that may incur mutations among different hosts. Our results suggest that the selection on SbDV in soybean was probably leading to reduced efficiency of virus recognition in the aphid which would inhibit movement of SbDV through vector tissues known to regulate the specificity relationship between aphid and virus in many systems.

Keywords: *Soybean dwarf virus*, Luteovirus, Soybean aphid, Genetic diversity

Background

Soybean dwarf virus (SbDV) is a member of the *Luteoviridae* family, found in multiple locations around the world, including Japan (Tamada 1973) and the United States (Damsteegt et al. 1990). Like other luteoviruses, SbDV is restricted to vascular phloem tissues in plant, and is transmitted by aphid vectors in a persistent, non-propagative manner (Gray and Gildow 2003), which appear to have played a key role in the evolution and diversification of the virus (Terauchi et al. 2003). SbDV is a single-stranded, positive-sense RNA virus organized into five open reading frames (ORFs). ORF-1 and -2 encode essential elements for replication, and ORF-3, -4 and -5 encode proteins involved in structure, movement, and vector transmission (Mayo and Miller 1999). SbDV isolates have been found in many different plant hosts such as white clover (*Trifolium pratense* L.), subterranean clover (*T. subterraneum* L.), broad bean (*Vicia faba*), pea (*Pisum sativum*), and lentils (*Lens culinaris* Medik.), however, it usually causes an economically important disease only in soybean crops. There are several distinct isolates based on symptomatology in soybeans, aphid vector specificity, and molecular makeup (Yamagishi et al. 2006). Indigenous SbDV was found commonly in clover in the United States, but rarely caused disease on soybean (Damsteegt et al. 1999). It was suggested that SbDV outbreaks were limited because of the lack of aphids colonizing soybean before 2000 (Damsteegt et al. 2011). Since 2000, a soybean aphid (*Aphis glycines*) capable of colonizing soybeans was introduced into the United States, and researchers identified SbDV in soybean crops in Wisconsin and Illinois (Phibbs et al. 2004; Harrison

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et al. 2005). Soybean aphids and SbDV isolates transmitted by soybean aphids were also discovered in the eastern US (Schneider et al. 2011). However, SbDV infections in fields were limited, and only occurred on the very edges of the fields.

Population genetic diversity is the essential component that allows a species to adapt to an ever-changing environment. The error-prone replication, large population, and rapid replication associated with RNA viruses lead to genetically diverse populations even within a single host (Roossinck 1997). It has been thought that unlike most animal viruses, most plant viruses need to be generalists for survival (Garcia-Arenal et al. 2003). Viral populations are complex and dynamic when the level of genetic diversity in a population reacts to changes in selection pressures (Lech et al. 1996). Maintaining a high level of genetic diversity would be an advantage for a virus to access a new environment, such as a different, distantly related host species. Most comparative studies of viral evolution have utilized consensus sequences that represent something of an average of the entire viral population in single host, but more detailed studies of evolutionary dynamics relying on genetic diversity would require more detailed gene resolution (Holmes 2009). Studies indicated that the diversity level of plant viruses correlated to the size of their reported host range (Schneider and Roossinck 2000). Meanwhile, in different hosts, plant viruses also have different ability to maintain diverse population (Schneider and Roossinck 2001). For instance, the diversity level of *Wheat streak mosaic virus* did not change significantly when passaged in different cereal species (French and Stenger 2003), while members of the family *Flexiviridae* have been reported to maintain high diversity levels and a strong constrained pressure on coding sequences (Chare and Holmes 2006; Shi et al. 2004; Alabi et al. 2010).

Genetic bottlenecks play an important role in the virus evolution that leads to the reduction of genetic variation in virus population and intensification of genetic drift. Transmission bottlenecks have been documented during a process of inter-host transmission, particularly as mediated by aphid vectors (Ali et al. 2006; Ali and Roossinck 2010). Phylogenetic analysis of coat proteins of different plant viruses indicated that vector-borne viruses are subject to significantly stronger purifying selection than non-vector-borne viruses (Chare and Holmes 2006). Similar population bottlenecks have also been observed during the mosquito stage in the vector-borne RNA viruses that infect animals (Smith et al. 2008). However, there are some indirect studies of viral population which suggest that transmission bottlenecks in nature may not be as extensive as often thought (Holmes 2009). Detail studies of *Dengue virus* intra-host genetic diversity suggested that transmission bottlenecks are not especially severe (Aaskov et al. 2006). Similarly, multicomponent viruses requiring mixed infections to form a fully functional unit could be considered to select against a transmission bottleneck in the case of some plant RNA viruses (Manrubia et al. 2005).

Luteoviruses have a very specific interaction with aphid vectors. The selective specificity of the luteovirus transmission can occur at a minimum of three cellular sites, including the gut cell membrane, and the basal plasmalemma (cell membrane) and basal lamina of the accessory salivary gland (ASG) (Gildow and Rochow 1980; Garret et al. 1993; Gildow 1993; Gildow and Gray 1993; Gildow et al. 2000). These specific recognition sites are likely determined by multiple protein domains on the virus capsid and multiple cell surface receptors of aphid vectors (Gray and Banerjee 1999; Gray and Gildow 2003). Little is known about the evolutionary process and genetic diversity of populations in luteoviruses, which are highly vector specific and host tissue specific (Gildow 1999). Transmission bottlenecks have been considered as a key factor to reduce effective population size that limits genetic diversity of luteovirus populations.

Our previous studies indicated that there was a trade-off effect between the SbDV fitness in soybean and aphid vector transmissions when SbDV was transmitted from clover to soybean sequentially in the greenhouse (Tian et al. 2017). The objective of this study was to investigate SbDV transmission and bottleneck effects in different aphid vectors, and to examine intra-host genetic diversity of viral population in various plant host species when a virus adapts to changing environments. Our results provide an insight into vector effects on the luteovirus genome, particularly replicase-related regions when adapting in new environment. Identifying vector effects affecting the diversity level of viral populations is an important step to the understanding and management of vectored pathogens of new/emerging plant diseases.

Results

SbDV transmission efficiency by different aphid vectors

To examine SbDV transmission efficiency by different aphid vectors, SbDV-MD6 was initially transmitted from clover to pea or soybean based on their plant feeding preference by *Nearctaphis bakeri* (Cowen 1895) and *Acyrthosiphon pisum* (Harris), respectively, and then repeatedly transmitted to the same host plant by the same aphid species for four sequential transmissions. The transmission efficiency of each aphid species was then determined following each transmission or passage (Fig. 1a). Transmission efficiency for *A. pisum* fed sequentially on peas for passages 1–4, was 30%, 40%, 60%, and 50%, respectively. Results indicated that ability of *A. pisum* to transmit SbDV to peas increased in efficiency in passages following the first adaptive transmission from clover to peas. By comparison, transmission efficiency of *N. bakeri* fed

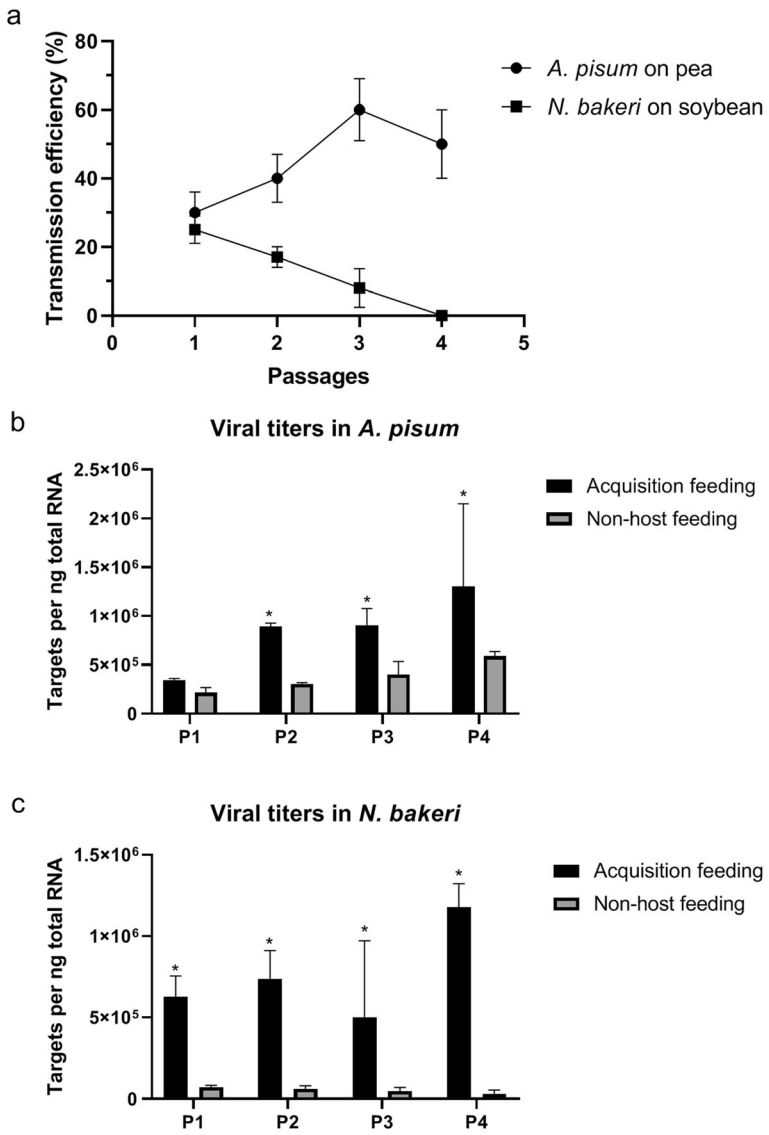

Fig. 1 Transmission efficiency and virus titers of SbDV-MD6 transmitted by aphids *A. pisum* and *N. bakeri* on pea and soybean, respectively. **a** The transmission efficiency on pea and soybean hosts. The percentages were determined by infected plants of total plants in each experiment. Two independent experiments were performed. **b** Virus titers of SbDV-MD6 in *A. pisum* at the acquisition and retention stage through serial passages. **c** Virus titers of SbDV-MD6 in *N. bakeri* at the acquisition and retention stage through serial passages. Error bars represent the standard deviation of the mean. Statistical significance levels (determined by ANOVA test, $P < 0.5$) were denoted by asterisks

sequentially on soybeans transmitted SbDV in passages 1–4 at 25%, 16%, 8%, and 0%, respectively. In soybean, SbDV transmission by *N. bakeri* was reduced with each subsequent passage until transmission ability was lost after the third passage.

SbDV presents in aphid during the serial transmission

To examine the ability of *A. pisum* and *N. bakeri* to acquire and retain SbDV through sequentially serial passages, virus titers in aphids acquisition fed on plants from each of four virus passages were analyzed by RT-qPCR. To acquire virus, aphids were allowed a 24 h acquisition feeding on infected plants from each passage, and then 25 aphids were analyzed by RT-qPCR. A cohort of 25 aphids from each passage was allowed to feed 2 days on red clover (non-host) to clear viruses in the alimentary canal and then tested similarly. Results from two independent experiments indicated that both *A. pisum* and *N. bakeri* effectively acquired SbDV into the gut during a 24 h feeding on pea or soybean and retained the virus in the hemocoel following a 48 h feeding on red clover to clear the gut lumen content (Fig. 1b, c). In *A. pisum* on pea, SbDV concentrations acquired into the hemocoel were consistently high for viruses acquired following serial passages. In *N.*

bakeri on soybeans, SbDV accumulation were high during the serial transmission. This was to be expected because SbDV was shown to increase in concentration in soybeans through sequential passages over time (Tian et al. 2017). However, viral titers were significantly decreased after non-host feeding compared to peas. After passage 3, *N. bakeri* were unable to transmit SbDV even though SbDV were still detected in the aphid hemocoel.

To compare acquisition and retention of SbDV in soybean aphid *A. glycines*, SbDV-MD6 were established in soybeans through 3 serial passages using *N. bakeri*. Then two different aphid vectors, *A. pisum* and *A. glycines*, were acquisition fed for 48 h on infected soybean, following with a non-host 48 h feeding. Both *A. glycines* and *A. pisum* acquired and retained SbDV-MD6 in the hemocoel (Table 1). The low virus concentrations in *A. glycines*, following a 48 h feeding on non-hosts suggested that SbDV uptake from the gut lumen and transport into the hemocoel were inefficient. However, both species acquired SbDV-MD6 into the hemocoel and were genetically competent to transmit the virus as indicated in Table 1.

SbDV genetic diversity and mutation frequency in plant hosts

To identify the extent and structure of intra-host viral genetic diversity of SbDV in different plant hosts with serial passaging, we sequenced the clones from 9 samples. SbDV-MD6 from infected clover was used as the inoculation source. Young pea and soybean plants were inoculated by aphid vector *A. pisum* and *N. bakeri*, respectively. Samples were from two individually infected plants for the first and last passages used in our previous study (Tian et al. 2017) (Genebank ID of the consensus sequences of SbDV-MD6 isolates: JN674402, JN674403, JN674406, JN674407, and JN674409 as references), and the viral populations were cloned separately. It was indicated that most of non-synonymous and synonymous mutations occurred on the replication-related genes during the serial transmission assays (Tian et al. 2017). Therefore, in this study, the examined targeting sequences included 3 fragments covering approximately 2.5 kb in length, which included the replication-related genes (ORF1 and ORF2) and partial flanking sequences. They were cloned separately, and 11 to 20 clones were analyzed for each fragment from each virus population. The mutation frequencies were very similar between passage lines for both hosts, so mutation frequencies within the passages were pooled. In theory, each clone represented a unique viral RNA, and comparing the sequences of the viral clones to the consensus sequence provided a snapshot of the genetic diversity generated within a given viral population. Using these clones as a representative sample of viral populations, mutation frequency and the percentage of mutated viral clones were used as indicators of population diversity (Schneider et al. 2011). Control reactions were done using in vitro transcripts as the template RNA to estimate the level of variability introduced by transcription, RT, and thermal cycling. There were 4 of 22 control clones (18%) containing a single mutation, giving the background level of experimental mutation frequency at 0.02%, significantly lower than levels observed in the viral populations.

Mutation frequency is the number of bases that differ from the consensus sequence divided by the total number of bases sequenced. Two plants were sampled for each plant treatment. Cloning and sequencing two RT-PCR sets for each treatment would detect potential differences in levels of error introduced in independent RT-PCRs. There were no significant differences between the sampled plants of any given passage, so the data from clones for each passage were pooled. The replication-related genes (ORF1 and ORF2) were used to exam the genetic diversity in a single infected plant. The level of intra-host genetic diversity increased from 0.08% on average in clover to 0.11% in peas and 0.12% in soybeans after reaching equilibrium (Table 2). After SbDV was passaged in the new host for several times, the level of diversity were decreased slightly to 0.10% in both pea and soybean passages. There were no significant changes over the courses of passaging in the same host species. The only significantly different mutation frequency observed in the plant occurred in passage 1 soybeans where the mutation frequency reached the 0.12%.

Table 1 The concentration of *Soybean dwarf virus* (SbDV) in the aphid vectors, *A. glycines* and *A. pisum*, following a 48 h acquisition feeding on soybeans infected with the North American SbDV-MD6 and a 48 h non-host feeding, as indicated by viral copy numbers

SbDV isolate	Aphid Vector			Average viral copy numbers in aphids [b]	
				48 h acquisition feeding	48 h non-host feeding
MD6	*A. glycines*	1/4	1/4 [a]	1.4×10^5+/− 0.0	2.3×10^3+/− 0.0
	A. pisum	3/3	2/3	4.6×10^6+/− 1.1×10^5	5.8×10^5+/− 1.7×10^4

[a] Acquisition and retention values represented by two fractions. The first fraction represents the number of times the aphid acquired the virus from feeding on infected host tissue/total number of experimental repetitions. The second fraction represents the number of times the aphid retained the virus after feeding on non-infected non-host tissue/total number of experimental repetitions

[b] Mean copy number values for all positive results with standard deviations determined by RT-qPCR. In cases where only a single sample tested positive, the standard deviation is listed as 0.0

Table 2 Genetic variation in SbDV-MD6 populations in different host species and passages

Host and passage	Mutated clones	Total mutations/ bases sequenced	Mutation frequency (%)
Clover	60% (6/10)	25/29, 590	0.08[a]
Pea and passage 1	55% (11/20)	66/59, 180	0.11[a]
Pea and passage 8	40% (8/20)	64/59, 180	0.10[a]
Soybean and passage 1	56% (10/18)	64/53, 262	0.12[b]
Soybean and passage 6	44% (8/18)	53/53, 262	0.10[a]

Substitutions are counted for determining mutation frequency. Mutation frequencies with the same letter were not statistically different. Least significant differences were determined by the ANOVA test ($P < 0.05$)

Mutation distribution

An examination of the locations of the mutations suggests that mutations were randomly distributed but not evenly distributed (Fig. 2). The observed mutations in the SbDV populations were distributed throughout the sequenced region, with a bias for ORF1 (from nucleotide position 145 to 1230) region over ORF2 region (from nucleotide position 1221 to 2828). There were some areas where many mutations were observed, in particular the area between nucleotides 257 and 1046 of ORF1 gene, but no mutation hot spots were observed. In addition, there was a region from 1942 to 2238 where mutations occurred on ORF2 region, and there were two mutation free regions on ORF2.

Type of mutations

There were more synonymous mutations observed than non-synonymous in pea passages but no bias observed in the clover and soybean population (Table 3). Occasionally, a couple bases were mutated close to one another. In these cases, each individual mutated base was counted as a mutation, even though the mutations may have arisen from a single mutational event. However, if these mutations are grouped and counted as a single mutation, it does not affect the statistical comparisons significantly.

All of the observed mutations in SbDV populations were substitutions except one insertion in the pea passage 1. Close examination of the specific changes indicates a bias for transitions (Table 4), in particular A-to-G and U-to-C transitions. There was a strong bias for A-to-G transitions in both pea and soybean passages (61 of 116 substitutions in pea and 58 out of 130 substitution in soybean, Table 4). The SbDV populations in clover demonstrated a slight preference for U-to-C (13 substitutions) over A-to-G (6 substitutions) transitions.

Discussion

From previous studies, we found that the clover strain of SbDV-MD6 was able to be transmitted by *A. pisum* on pea passages with increasing aphid transmission efficiency and viral fitness. For serial passages on soybean, SbDV-MD6 also adapted readily to soybean by improved replication and/or movement, however, the selection for host adaptation created tradeoff effects decreasing host-to-host transmissibility by aphid vectors (Tian et al. 2017). Here, we investigated the factors that affect the interaction between viruses and aphids, and the intra-host genetic diversity of viral population that influence SbDV evolution during the environmental adaptation.

There are multiple barriers to luteovirus transmission by aphids. Transmission requires multiple steps, any of which could be blocked. First, aphids may not acquire virions from plant host. Second, if the aphid does acquire virions, there are still barriers to transmission in the aphid gut, at the basal lamina and at basal plasmalemma of the ASG (Gildow 1993; Gildow et al. 2000; Gray and Gildow 2003). When SbDV-MD6 passaged on soybeans, mutations were observed on ORF1, ORF2, and ORF 5 (Tian et al. 2017). ORF1 and 2 are replication related genes, and ORF5 is a readthrough protein (RTP) extension of coat protein (CP) that is proved to be related to aphid transmission (Brault et al. 2007; Thekke-Veetil et al. 2017). Numerous factors that affect aphid transmission were the mutations accumulated including ORF5 and CP-RTP of luteoviruses (Van den Heuvel et al. 1997; Terauchi et al. 2003; Peter et al. 2009). The mutations in replication-related proteins (ORF1 and ORF2) could lead to the improvement of virus titers in plant hosts. It is also possible that these mutations affected virus distribution or movement in host plants in such a way that acquisition of

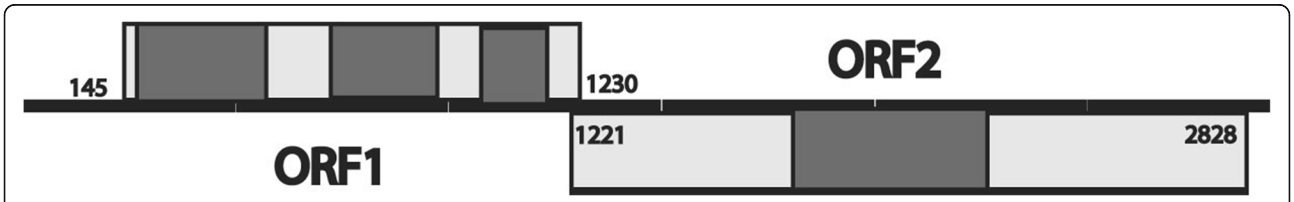

Fig. 2 Distribution of accumulated mutations observed in clones derived from SbDV-MD6 populations. The region sequenced extends from base 1 to base 2959 covering both ORF1 and ORF2 regions. Mutations are indicated by dark, and mutation free zones are white regions on the map

Table 3 Types of mutations observed in SbDV-MD6 populations in different host species and passages

Host and passage	Translated		Non translated	Substitution
	syn	Non-syn		
Clover	15	7	3	25
Pea and passage 1	43	18	5	66
Pea and passage 8	48	13	3	64
Soybean and passage 1	34	26	3	63
Soybean and passage 6	20	32	1	53

Syn synonymous mutation

the virions of infected plant became more and more difficult following virus serial passages on soybeans.

The soybean aphid, *A. glycines*, was first introduced into the US in 2000. It is now widely distributed in the US and there is a concern for the potential ability of *A. glycines* to function as a major vector transmitting devastating plant pathogen, like SbDV (Wang et al. 2006). To determine if the lack of the transmission was related to virus acquisition and/or retention, soybean aphid *A. glycines* was tested separately, and *A. pisum* was also tested for comparison. The SbDV-MD6 isolate was transmissible by both aphid species, although the titers in *A. glycines* were lower compared with *A. pisum* after the acquisition feeding (Table 1). It is consistent with the observation of other SbDV isolates in different aphid vectors that regardless of whether or not the isolates were transmissible by that particular aphid species, SbDV were always acquired through feeding (Damsteegt et al. 2011). Following the non-host feeding for 48 h, the SbDV-MD6 was retained in both aphid species, although the titer was significantly low in soybean aphids. MD6 isolate was also efficiently transported into hemocoel by *A. pisum* but not efficiently by *A. glycines*, despite the fact that many SbDV isolates are efficiently transmitted by *A. pisum* and poorly transmitted by *A. glycines* (Wang et al. 2006; Damsteegt et al. 2011). Based on these results, it indicated the acquisition of Maryland isolates of SbDV by aphids was not a major problem for aphid transmission, and other selective sites must exist to limit the aphid transmission. The most probable site is at the ASG which is known for specific interaction between aphid and virus in many systems (Gildow and Rochow 1980; Gildow and Gray 1993). As with other luteovirus/aphid vector relationships, SbDV appears to have multiple levels of vector specificity to overcome.

Table 4 Substitution types of SbDV-MD6 populations in different host species

Host	Original bases	No. of substitutions at mutated base			
		G	A	U	C
Clover	G	–	4	0	0
	A	6	–	1	0
	U	0	0	–	13
	C	0	0	1	–
Soybean	G	–	6	0	2
	A	58	–	0	0
	U	1	2	–	43
	C	0	6	6	–
Pea	G	–	16	0	2
	A	61	–	0	0
	U	1	3	–	36
	C	0	0	11	–

The levels of diversity observed in soybean increased significantly at passage 1, however, different passages have no significant difference. This suggests that the SbDV is capable of generating variations when shifting to a new host, and other factors limit the eventual accumulation of variation in whole plants. It supported results that SbDV-MD6 was capable of adapting to new hosts rapidly. It is interesting to note that the greatest increase of SbDV-MD6 diversity occurred under the strong selection pressure during the shift to the soybean host. The diversity of SbDV-MD6 maintained statistically similar levels for all other populations except for passage 1 in soybeans (Table 2). This is different from other viruses, where different levels of diversity are observed on different hosts (Schneider and Roossinck 2000). However, it is the first observation of population diversity on luteoviruses. The fact that luteovirus are limited to phloem may contribute to similarities in mutation frequencies between hosts, as phloem environment may be similar for virus replication from host to host. In addition, three hosts were used in this study were all related legumes.

The level of intra-host diversity we report for SbDV (mean = 0.10%) was in the normal range as that previously observed in intra-host studies of other plant and animal viruses (Schneider and Roossinck 2000; Murcia et al. 2010). Therefore, SbDV appears to exhibit mutational dynamics broadly similar to those observed in some rapidly evolving RNA viruses, and as expected given the intrinsically error-prone nature of replication with RNA-dependent RNA polymerase. Our results are compatible with the notion that the majority of intra-host mutations in SbDV are deleterious and removed by purifying selection during maintenance in the same host species. In addition, a few mutations fixed in the final passages especially in soybeans suggested that positive selections canoccur when SbDV moved to new hosts by aphid vectors. This suggested that the bottleneck imposed by the aphid is not substantial in this persistent

transmission manner, which may play an important role in selection pressure on viruses.

Transitions are the most common mutations. In this study, the transitions from A-G and U-C are the most common substitutions. In both pea and soybean, A-G was more common than U-C, but in clover, U-C was the most common mutations observed. This may indicate that the environments in different hosts are not exactly the same. The A-G and U-C transition bias could be caused by base pairing between the guanidine and uridine in viruses (Schneider and Roossinck 2000), and it has been noted in other RNA viruses (Schneider and Roossinck 2001; Ali and Roossinck 2017). The occurrence of A-G or U-C transitions is related to whether U-G base pairing occurs during positive or negative strand synthesis. The preference for U-C transition in clover may suggest that more mutations occurred during the negative strand synthesis in that host.

There were three regions on SbDV-MD6 replicase–related genes where mutations were not observed (Fig. 2). One region (nucleotide 658 to 846) is in ORF1, and two large regions are on ORF2. Overall, ORF1 had more mutations than ORF2. This may suggest that the mutation free regions on ORF2 are conserved and important for virus replication. Mutations likely do occur in these mutation free regions, but viral RNAs with mutations in these regions are selected out of population. It is also important to note that the cloned viral populations are being selected for at the RNA level. There are more non-mutated copies of genes to produce functional replicases. Therefore, the presence of regions where mutations are not recovered indicates the presence of selection for RNA sequence. SbDV has regions with higher mutation rates, which suggest that the mutation free regions are not occurring by chance. The observed mutations are not restricting the infection of SbDV. If so, deleterious mutations will be rapidly selected out of the populations.

It is clear that selection plays an important role, because mutations are biased for synonymous changes in pea host (Table 3), and they are not evenly distributed (Table 2). We also assume that bottlenecks associated with persistent aphid transmission and long-distant movements in plant host are limiting the diversity, because the observed mutation frequency in whole plants is low, and even in the most densely mutated regions it does not approach the theoretical mutation frequency.

Theoretically, the ability to maintain genetic diversity in viral populations should enhance chances for adaptation to new selective regimes. Alternatively, if high diversity in the viral population resulted in fitness losses with Muller's Rachet theory (Escarmis et al. 2009), the forces of selection would rapidly eliminate viruses that surpass viable limits of population diversity. This study has important evolutionary implications for the intra-host genetic structure of viral populations since it is the first study of a phloem-limited luteovirus. It has been suggested that SbDV has a chance of expanding into a new niche and thus pose a threat of emerging as new crop diseases. However, the bottleneck imposed by persistent aphid transmission may be the major factor to limit the spread of SbDV in the nature.

Conclusions

Previous study (Tian et al. 2017) has indicated that SbDV titer increases in peas and soybeans with each succeeding transmission during serial passages. Therefore, one might expect transmission efficiency to increase on both host plant species in later passages, as was demonstrated for the pea treatment. However, the eventual loss of transmission ability via *N. bakeri* on soybean suggested that mutations accumulated in soybean host somehow interrupt the specific interaction between aphid and viruses. Although current work does not address the direct effects of SbDV mutations acquired during serial passage in soybeans, the fact that the same mutations occurred in the independent passage experiments resulting in the exact same loss of transmission by multiple previously vector competent aphid species strongly suggests that the mutations are somehow both advantageous in soybean but interfering with the transmission of MD6 into the ASG. It is also hard to figure out a mechanism that would select a specific level of diversity for host-virus relationship. One possible scenario could be that the viral replicase may make different error rates due to host components associated with viral replication process. Any of environmental factors, such as concentrations of available nucleotides, pH, and other soluble components, may affect the fidelity of viral replicase complex. Alternatively, these viruses may be capable of generating equivalent levels of diversity in different hosts, but selection pressure specific to a particular host and/or aphid vectors, will act as a bottleneck limiting the accumulation of diversity.

Methods

Plants, viruses, and aphid vectors

The SbDV-MD6 isolate was used in this study, which was obtained from the field in Maryland, US. The isolate maintained in white clover (*Trifolium repens*). Puget pea (*Pisum sativum* cv. Puget), soybeans (*Glycine max* cv. Williams), and white clover seedlings were used for host serial transmissions. Red clovers (*Trifolium pratense* L.) were used as non-host feeding for aphid vectors. In the aphid transmission experiments, *A. pisum* (pea aphid), *N. bakeri* (clover aphid), and *A. glycines* (soybean aphid) were used as the vectors for serial transmission and maintained.

Infections with SbDV were initiated with SbDV population from wild stock clover plants. Plant hosts include soybean and pea. Two independent passage experiments

were done by aphid transmission as previously described by Tian et al. (2017). Briefly, SbDV was transmitted from indigenous infected white clover (*T. repens*) (passage 0) to soybean (*G. max*), and pea (*P. sativum*) by aphid *N. bakeri* and *A. pisum*, respectively. Two individual infected plants randomly selected from the first and last passage in host plants were used for sequencing analyses.

Virus acquisition and retention assays

For the serial passages, aphids acquired viruses by feeding on detached leaves of infected white clover as source for 48 h. Then, the aphids were transferred to healthy pea or soybean seedlings for 5 days. At the same time, three seedlings of each plant species were fed by healthy aphids that feeding on healthy tissue as a negative control. After 25 days post inoculation (dpi), the percentage of infected plants was determined by ELISA according to manufacturer directions (Agdia, Elkhart, IN). Each passage line was continued through four passages, or until transmission failed. SbDV-MD6 was transmitted by both aphids in the white clover, as a positive control for transmission.

To test for virus acquisition by aphids from SbDV-infected plants of each passage, approximately 100 *N. bakeri* and *A. pisum* were put on each of 15 SbDV-MD6 infected soybean and pea seedlings, respectively, for a 48 h acquisition feeding at 20 °C. Infected plants at 30 dpi were used as virus sources in each passage. After the 48 h acquisition feeding, 25 aphids on each infected plant were collected immediately, flash frozen and stored at − 80 °C before RNA extraction. The remaining aphids of each species were then transferred to healthy red clover for 48 h. The SbDV-MD6 does not infect or replicate in red clover and during this feeding time the aphid gut lumen is cleared of ingested virus. Following the 48 h feeding on red clover, 25 aphids of each plant were flash frozen, and stored at − 80 °C before RNA extraction and analysis for SbDV retention in the aphid hemocoel.

Persistence of SbDV-MD6 in soybean aphid *A. glycines* following acquisition

Soybean plants infected with the SbDV-MD6 isolate was used as source plants for aphid acquisition and retention test. The isolate was maintained in soybeans prior to use, and the infected source soybean were confirmed by both ELISA and RT-PCR. Similar to previous transmission assay, about 75 *A. glycines* or *A. pisum* were put on each of 3 or 4 infected soybeans for a 48 h acquisition feeding. Immediately following the acquisition feeding, 15 aphids with two replicates were removed at random and flash-frozen for RNA extraction. The remaining aphids were transferred to healthy red clover plants for 2 days non-host feeding to allow SbDV to clear the alimentary canal lumen. Then 15 aphids with two replicates were sampled as described before. Individual samples consisted of RNA extracted from the combined total of 15 aphids with two disks from a healthy plant. Preliminary studies indicated that multiple aphid samples with plant tissue were required for consistent detection of SbDV in samples. The experiments were conducted 4 replicates for *A. glycines*, and 3 replicates for *A. pisum*.

Total RNA extraction and real time RT-qPCR

Total RNA of plants at 30 dpi were extracted from systemically infected leaves of infected plants. Total RNA extraction was used as a template for reverse transcription with SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer. Thermal cycling reactions were carried out for 20 cycles (94 °C denaturation for 30 s, 52 °C annealing for 1 min, 72 °C extension for 1 min), and included a polymerase with proofreading capability (*Pfu*; Invitrogen). There were three PCR products generated covering ORF1 and ORF2 region of SbDV-MD6. The primers used in the PCR assay were 1UF (5′-GAC TAT GGG TTT GAC ATG CAG-3′) and 1090UR (5′-GTT TGA ATC CCC GTT TTC T-3′), 900UF (5′- GCA ACC ATC AAC CGA TAT GCG-3′) and 2126UR (5′-GTT TGA ATC CCC GTT TTC T-3′), and 1235UF (5′-GTT TGA ATC CCC GTT TTC T-3′) and 2959UR (5′-GAG TGC TTC TAT TTT GAA AGT ATT GG-3′).

In each passage, the same number of aphids was collected in the tube and flash frozen, and stored at − 80 °C before RNA extraction. The aphid total RNA extraction was followed the same protocol as described by Wallis et al. (2007). The final RNA pellet was dried by vacuum and resuspended in 40 μL distilled H_2O. SbDV RNA was amplified using first strand cDNA synthesis by SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The RT-qPCR was followed the same protocol as previous study (Tian et al. 2017) using a SmartCycler thermal cycler (Cepheid, Sunnyvale, CA) with two biological replicates and three technical replicates. Similarly, to determine the effectiveness of quantification, a fragment of SbDV-MD6 was cloned into a plasmid with a T7 RNA polymerase promoter. This plasmid was linearized and used to make in vitro transcripts, which served both as positive controls for qRT-PCR and as a directly quantifiable RNA template to establish standard curves.

Cloning and sequencing of viral populations

The PCR products of viral RNAs from individual plants of each treatment were cloned separately and treated as unique populations. The amplified products generated from the viral RNAs were cloned into the vector pCR2.1-TOPO (Invitrogen). The viral clones were sequenced at the genomic facilities (Pennsylvania State University). Eleven to 20

clones were sequenced from each individually infected plant. The alignment of all the cloning sequences were done by using Bioedit (Ibis Theraputics, Carlsbad, CA) and checked manually. Changes between the sequences of the viral population clones and the consensus sequence of the source population were recorded as mutations. In cases where multiple mutations occurred in close proximity in the same clone, each mutated base was considered as a unique mutation. The mutation frequency was calculated as the total number of mutations observed in all clones for a given viral population divided by the total number of bases sequenced for the population.

Statistical analysis

Comparisons between viral populations in different hosts were tested for statistical significance using the ANOVA (analysis of variance) test from the statistical package Minitab 15 (Minitab Inc., State College, PA) to determine least significant differences. Comparison of mutation free zones was done between species using sequencing alignment.

Abbreviations

ASG: Accessory salivary gland; CP: Coat Protein; ORF: Open reading frame; RTP: Readthrough Protein; RT-qPCR: Quantitative reverse transcription polymerase chain reaction; SbDV: *Soybean dwarf virus*

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Authors' contributions

BT, WLS, FEG and VDD conceived and designed the experiments; BT performed the experiments; BT and WLS analyzed the data; ALS and DJS contributed to real time RT-PCR, SbDV strains collection, maintaining, and experimental materials; BT and WLS wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

Aaskov J, Buzacott K, Thu HM, Lowry K, Holmes EC. Long-term transmission of defective RNA virus in humans and Aedes mosquitoes. Science. 2006; 311(5758):236–8.

Alabi OJ, Martin RR, Naidu RA. Sequence diversity, population genetics and potential recombination events in grapevine rupestris stem pitting-associated virus in Pacific north-west vineyards. J Gen Virol. 2010;91:265–76.

Ali A, Li H, Schneider WL, Sheman DJ, Gray SM, Smith D, et al. Analysis of genetic bottlenecks during horizontal transmission of *Cucumber mosaic virus*. J Virol. 2006;80:8345–50.

Ali A, Roossinck MJ. Genetic bottlenecks during systemic movement of *Cucumber mosaic virus* vary in different host plants. Virology. 2010;404(2):279–83.

Ali A, Roossinck MJ. Analysis of quasispecies variation in single and mixed viral infection. Virus Evol. 2017;3(2):vex037.

Brault V, Herrbach E, Reinbold C. Electron microscopy studies on luteovirid transmission by aphids. Micron. 2007;38:302–12.

Chare ER, Holmes EC. A phylogenetic survey of recombination frequency in plant RNA viruses. Arch Virol. 2006;151:933–46.

Damsteegt VD, Hewings AD, Sindermann AB. *Soybean dwarf virus*: experimental host range, soybean germ plasm reactions, and assessment of potential threat to U.S. soybean production. Plant Dis. 1990;74:992–5.

Damsteegt VD, Stone AL, Kuhlmann M, Gildow FE, Domier LL, Sherman DJ, et al. Acquisition and transmissibility of U.S. *Soybean dwarf virus* isolates by the soybean aphid, *Aphis glycines*. Plant Dis. 2011;95:945–50.

Damsteegt VD, Stone AL, Russo AJ, Luster DG, Gildow FE, Smith OP. Identification, characterization, and relatedness of luteovirus isolates from forage legumes. Phytopathology. 1999;89:374–9.

Escarmis C, Perales C, Domingo E. Biological effect of Muller's ratchet: distant capsid site can affect picornavirus protein processing. J Virol. 2009;83:6748–56.

French R, Stenger DC. Evolution of *Wheat streak mosaic virus*: dynamics of population growth within plants may explain limited variation. Annu Rev Phytopathol. 2003;41:199–214.

Garcia-Arenal F, Fraile A, Malpica JM. Variation and evolution of plant virus populations. Int Microbiol. 2003;6:225–32.

Garret A, Kerlan C, Thomas D. The intestine is a site of passage for potato leafroll virus from the gut lumen into the haemocoel in the aphid vector, *Myzus persicae*. Arch Virol. 1993;131:377–92.

Gildow FE. Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology. 1993;83:270–7.

Gildow FE. Luteovirus transmission and mechanisms regulating vector specificity. In: Smith HG, Barker H, editors. The Luteoviridae. Oxon: CAB International; 1999. p. 88–112.

Gildow FE, Damsteegt VD, Stone AL, Smith OP, Gray SM. Virus-vector cell interactions regulating transmission specificity of *Soybean dwarf luteoviruses*. J Phytopathol. 2000;148:333–42.

Gildow FE, Gray SM. The aphid salivary-gland basal lamina as a selective barrier associated with vector-specific transmission of *Barley yellow dwarf luteoviruses*. Phytopathology. 1993;83:1293–302.

Gildow FE, Rochow WF. Role of accessory salivary glands in aphids transmission of *Barley yellow dwarf virus*. Virology. 1980;104:97–108.

Gray SM, Banerjee N. Mechanisms of arthropod transmission of plant and animal viruses. Microbiol Mol Biol Rev. 1999;63:128–48.

Gray SM, Gildow FE. Luteovirus-aphid interactions. Annu Rev Phytopathol. 2003; 41:539–66.

Harrison B, Steinlage TA, Domier LL, D'Arcy CJ. Incidence of *Soybean dwarf virus* and identification of potential vectors in Illinois. Plant Dis. 2005;89:28–32.

Holmes EC. The evolutionary genetics of emerging viruses. Annu Rev Ecol Evol Syst. 2009;40:353–72.

Lech WJ, Wang G, Yang YL, Chee Y, Dorman K, McCrae D, Lazzeroni L C, Erikson JW, Sinsheimer JS, Kaplan AH. In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. 1996:70:2038–43.

Manrubia SC, Escarmis C, Domingo E, Lazaro E. High mutation rates, bottlennecks, and robustness of RNA viral quasispecies. Gene. 2005;347(2): 273–82.

Mayo MA and Miller WA. The structure and expression of luteovirus genomes. In: Smith HG, Barker H, editors. The Luteoviridae. Oxon, United Kingdom: CAB International; 1999. p. 23–38.
Murcia PR, Baillie GJ, Daly J, Elton D, Jervis C, Mumford JA, et al. Intra- and interhost evolutionary dynamics of equine influenza virus. J Virol. 2010;84:6943–54.
Peter KA, Gildow FE, Palukaitis P, Gray SM. The C terminus of the polerovirus P5 readthrough domain limits virus infection to the phloem. J Virol. 2009;83:5419–29.
Phibbs A, Barta A, Domier LL. First report of *Soybean dwarf virus* on soybean in Wisconsin. Plant Dis. 2004;88:1285.
Roossinck MJ. Mechanisms of plant virus evolution. Annu Rev Phytopathol. 1997;35:191–209.
Schneider WL, Damsteegt VD, Stone AL, Kuhlmann M, Bunyard BA, Sherman DJ, et al. Molecular analysis of *Soybean dwarf virus* isolates in the eastern United States confirms the presence of both D and Y strains and provides evidence of mixed infections and recombination. Virology. 2011;412(1):46–54.
Schneider WL, Roossinck MJ. Evolutionarily related Sindbis-like plant viruses maintain different levels of population diversity in a common host. J Virol. 2000;74(7):3130–4.
Schneider WL, Roossinck MJ. Genetic diversity in RNA virus quasispecies is controlled by host-virus interactions. J Virol. 2001;75(14):6566–71.
Shi B, Habili N, Gafny R, Symons RH. Extensive variation of sequence within isolates of *Grapevine Virus B*+. Virus Genes. 2004;29(2):279–85.
Smith DR, Adams PA, Kenney JL, Wang E, Weaver SC. Venezuelan equine encephalitis virus in the mosquito vector *Aedes taeniorhynchus*: infection initiated by a small number of susceptible epithelial cells and a population bottleneck. Virology. 2008;372(1):176–86.
Tamada T. Strains of *Soybean dwarf virus*. Jpn J Phytopathol. 1973;39:27–34.
Terauchi H, Honda K, Yamagishi N, Kanematsu S, Ishiguro K, Hidaka S. The N-terminal region of the readthrough domain is closely related to aphid vector specificity of *Soybean dwarf virus*. Phytopathology. 2003;93:1560–4.
Thekke-Veetil T, McCoppin KN, Domier LL. Strain-specific association of *Soybean dwarf virus* small subgenomic RNA with virus particles. Virus Res. 2017;242:100–5.
Tian B, Gildow FE, Stone AL, Sherman DJ, Damsteegt VD, Schneider WL. Host adaptation of *Soybean dwarf virus* following serial passages on pea (*Pisum sativum*) and soybean (*Glycine max*). Viruses. 2017;9:155.
Van den Heuvel JF, Bruyere A, Hogenhout SA, Ziegler-Graff V, Brault V, Verbeek M, et al. The N-terminal region of the luteovirus readthrough domain determines virus binding to Buchnera GroEL and is essential for virus persistence in the aphid. J Virol. 1997;71:7258–65.
Wallis CM, Stone AL, Sherman DJ, Damsteegt VD, Gildow FE, Schneider WL. Adaptation of plum pox virus to a herbaceous host (*Pisum sativum*) following serial passages. J Gen Virol. 2007;88:2839–45.
Wang RY, Kritzman A, Hershman DE, Ghabrial SA. *Aphis glycines* as a vector of persistently and nonpersistently transmitted viruses and potential risks for soybean and other crops. Plant Dis. 2006;90:920–6.
Yamagishi N, Terauchi H, Honda K, Kanematsu S, Hidaka S. Discrimination of four *Soybean dwarf virus* strains by dot-blot hybridization with specific probes. J Virol Methods. 2006;133:219–22.