Assessment of Common Soybean-Infecting Viruses in Ohio, USA, Through Multi-site Sampling and High-Throughput Sequencing

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ABSTRACT

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Multi-site sampling was conducted during 2011 and 2012 to assess the scope of virus disease problems of soybean in Ohio, USA. A total of 259 samples were collected from 80 soybean fields distributed in 42 Ohio counties, accounting for more than 90% of major soybean-growing counties in Ohio. A high-throughput RNA-Seq approach was adopted to identify all viruses in the samples that share sufficient sequence similarities with known plant viruses. To minimize sequencing costs, total RNA extracted from up to 20 samples were first pooled to make up regional pools, resulting in eight regional pools per year in both 2011 and 2012. These regional pools were further pooled into two yearly master pools of RNA, and sequenced using the Illumina's HiSeq2000 platform.

Bioinformatic analyses of sequence reads led to the identification of signature sequences of nine different viruses. The originating locations of these viruses were then mapped with PCR or RT-PCR. This study confirmed the widespread distribution of *Bean pod mottle virus*, Soybean vein necrosis virus, *Tobacco ringspot virus*, and *Tobacco streak virus* in Ohio. It additionally revealed occasional association of *Alfalfa mosaic virus*, *Bean yellow mosaic virus*, *Clover yellow vein virus*, *Soybean mosaic virus*, and *Soybean Putnam virus* with Ohio soybean. This is the first statewide survey of soybean viruses in Ohio, and provides the much-needed baseline information for management of virus diseases of soybean.

INTRODUCTION

Soybean (*Glycine max* L. Merr.) provides essential nutrients, among them oil and protein, to both humans and food animals, and is also an important source of bioenergy (Wilson 2008). Additionally, due to its relative amenability to mass-scale production and its comparative profit potential, soybean production accounts for a substantial component of farmer incomes in the United States and many other countries around the world (Wilson 2008). Like other major crops, soybean is a host of diverse microbial pathogens including many viruses that can cause serious yield losses and/or seed quality deteriorations (Ghabrial et al. 1977; Harrison et al. 2005; Giesler and Ziem 2006; Bradshaw et al. 2008). A number of viruses have been reported to infect soybeans. Among them, the bean leaf beetle-transmitted *Bean pod mottle virus* (BPMV) has been known to be a major soybean pathogen in the United States for many years and many isolates have been shown to cause diseases of varying severities (Giesler and Ziem 2006; Zhang et al. 2007). *Soybean mosaic virus* (SMV) is both seed- and aphid-transmitted, and can limit soybean yield potentials, although effective resistance sources for SMV are available (Hill et al. 2007; Gagarinova et al. 2008). Other viruses including *Alfalfa mosaic virus* (AMV), *Soybean dwarf virus* (SbDV), and *Tobacco ringspot virus* (TRSV), and *Tobacco streak virus* (TSV) have also contributed to

yield losses in soybean (Harrison et al. 2005; Giesler and Ziem 2006; Hill and Whitham 2014). More recently, Soybean vein necrosis virus (SVNV) has been characterized and subsequently reported in many states, although its impact on soybean yields has yet to be assessed (Zhou et al. 2011; Zhou and Tzanetakis 2013). Finally, *Clover yellow vein virus* (CIYVV), *Bean yellow mosaic virus* (BYMV), and *Soybean yellow mottle virus* (SYMV) have been reported to infect soybean in other countries, but their presence in soybean fields in the United States remains to be carefully assessed (Sasaya et al. 1997; Harrison et al. 2005; Campos et al. 2014; Kyrchenko 2013).

Until recently, identification of viruses in plants mostly relied on serological (e.g., enzyme-linked immune-absorbance assay [ELISA] or immune-strips) or nucleic acid-based (e.g., polymerase chain reaction [PCR] or reverse transcription [RT]-PCR) approaches, with occasional electron microscopic (EM) inspection of particle morphology (Boonham et al. 2014). Serological assays rely on availability of specific antibodies, and thus could only be used to determine the presence of specific viruses in given samples. Similarly, PCR-based methods depend on the knowledge of specific nucleotide sequences, and are therefore limited to the confirmation of known viruses as well. On the other hand, the EM approach requires expensive, high-maintenance instruments, and provides limited insights beyond virion morphology. Therefore, without additional investigations in a specialized molecular biology lab, the above methods rarely lead to the characterization of new viruses.

This bottleneck of virus discovery is now being overcome with the advent of high-throughput sequencing technologies (Boonham et al. 2014; Kehoe et al. 2014). Notwithstanding differences in

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specific designs, all high-throughput sequencing platforms use universal adaptors to tag DNA fragments of unknown sequences and anchor them to solid surfaces and resolve the sequences of these DNA fragments by walking from the adaptors into unknown sequences (Metzker 2010). This technology does not rely on previous knowledge of the DNA to be sequenced, and hence can be used to discover previously unknown viruses. More importantly, the extremely high-throughput of this technology produces sequences of billions of nucleotides (nt) in a single run. Therefore, even though the cost per run is still prohibitive, that of each sample can be greatly reduced by pooling multiple samples into a single run. Furthermore, since plant viruses regardless of the nature of their genomes synthesize mRNAs in infected cells, profiling techniques like RNA-Seq that focus solely on rRNA-depleted cellular RNAs greatly reduce sequence complexities by avoiding non-transcribed host genome elements.

Here we report the results of a cost-effective assessment of soybean virus prevalence in Ohio by taking advantage of the high throughput RNA-Seq technology. A total of 259 soybean leaf samples were collected in two soybean-growing seasons (2011 and 2012) from 42 Ohio counties, and subjected to two RNA-Seq runs in order to obtain an updated view of soybean-infecting viruses in this state. This study permitted the identification with high confidence of BPMV and SVNv as the most prevalent soybean viruses, followed closely by TRSV and TSV. Interestingly, SMV, found to be common in previous surveys (Giesler and Ziem 2006), was found in only one field used for germplasm propagation, suggesting that SMV is not a significant threat to soybeans in Ohio. We also found sporadic infection of soybeans by BYMV and CIYVV, two viruses known to infect soybeans in Asia, Europe, and South America, but have yet to be reported in the United States (Campos et al. 2014; Kyrychenko 2013). Finally, our sampling identified SPuV, a new virus associated with soybean production. These data will offer valuable guidance for management practices of soybean viruses.

COLLECTION AND PROCESSING OF FIELD SAMPLES OF SOYBEAN FOR RNA-SEQ

Depending on the number of soybean fields encountered in our scouting trips, samples were collected from one to five fields per county (Fig. 1, Tables 1 and 2). In counties in which more than two fields were chosen, the distances between any two fields were at least eight kilometers. For each field, at least two neighboring sides were scouted for at least one hundred meters, and leaves with virus-like symptoms were collected. For fields in which no plants showed virus-like symptoms, soybean leaf samples were selected without regard for appearance. Overall symptomless samples accounted for less than 10% of the total. Total RNA was extracted from each of the collected samples with the TRIpure reagent following the manufacturer's instructions (Bioline, Taunton, MA). The obtained RNA samples were then checked for concentration and quality with NanoDrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.

GENERATION AND ANALYSIS OF RNA-SEQ DATA

To minimize the cost of sequencing, we used a two-step process to construct pools of RNA samples. First, an equal amount (1 µg per sample) of RNA was withdrawn from each of the samples (up to 20) collected from fields of two to three neighboring counties, and pooled to make one regional pool. Altogether eight regional pools were generated for both 2011 and 2012. Then, 2 µg of RNA was taken from every regional pool and further pooled into two Master Pools—one for 2011 and one for 2012. The Master Pool RNAs were checked for RNA integrity, treated with DNase, and

sent to the Roy J. Carver Biotechnology Center of University of Illinois at Urbana-Champaign for ribosomal RNA (rRNA) depletion, library construction, and sequencing on a HiSeq2000 platform.

Bioinformatic analyses of the RNA-Seq data were carried out using two different approaches. For the 2011 data, reads that match soybean mRNAs and rRNAs were removed with Bowtie (Langmead et al. 2009), and the remaining reads were trimmed to remove adaptor sequence, and the trimmed reads assembled into contigs using Trinity (Grabherr et al. 2011). These contigs were then subjected to BLASTx search against the NCBI viral RefSeq virus protein database using USEARCH (Edgar 2010).

For the 2012 data, a more user-friendly software package was adopted for bioinformatic analysis. Specifically, raw reads were imported into CLC Bio Genomics Workbench v6.5.1 (Qiagen, Venlo, Netherlands) and trimmed for quality, adapter indexes, and poly(A) tails using default settings (ambiguous limit = 2, quality limit = 0.05). Reads matching soybean mRNAs and rRNAs were removed from the dataset by mapping preprocessed reads to the most recent soybean genome assembly (Glyma.Wm82.a2.v1). The unmapped reads were assembled *de novo* into contigs using two independent approaches: (i) the CLC Bio algorithm based on de Bruijn graphs and the optimized parameters of Word Size = 64, Bubble Size = 500, Length Fraction = 0.6, Similarity Fraction = 0.9; and (ii) Oases v0.2.08 with Kmer sizes of 53, 59, 65, 71, 77, 83, and 89 (Schulz et al. 2012). To obtain the set of non-redundant transcripts for each assembly, reads ≥90% sequence similarity were collapsed into clusters and the longest read retrieved using CD-HIT-EST (Li and Godzik 2006). The two independent assemblies were collapsed into a final assembly using Minimus2 (Sommer et al. 2007). Contigs of viral origin were identified using desktop-downloaded BLASTx software against the NCBI viral RefSeq virus protein database (E-value < 1e-10).

MAPPING THE DISTRIBUTION OF IDENTIFIED VIRUSES

The viruses revealed through RNA-Seq and bioinformatic analyses were then mapped to their corresponding fields using PCR or RT-PCR, using the primers listed in Table 3. Specifically, the regional pools were first examined with virus-specific primers to narrow down the specific viruses to their corresponding region(s). The individual samples in these regional pools were then tested individually to complete the final distribution mapping.

RNA-SEQ AS A ROBUST METHOD FOR SOYBEAN VIRUS SURVEYS

Our primary goal for this study was to identify most, if not all, potential viruses that pose threats to soybean production in Ohio. To this end, we chose to use RNA-Seq as the primary tool for virus discovery because it would provide viral sequence information even for previously undescribed viruses for which no detection reagents are available. More importantly, the huge amount of data generated through high-throughput sequencing meant that samples collected from multiple locations could be combined into a limited number of sequencing attempts, thus greatly reducing the per-sample costs. Indeed, we were able to pool 108 and 151 RNA samples collected in 2011 and 2012, respectively, into two yearly pools, and obtained 88,102,967 and 170,075,625 100-nt reads. BLASTx search revealed that about 0.37% of the 2011 reads (324,832) and 1.76% of 2012 reads (3,165,971) matched sequences of previously described plant viruses with a *P* value cutoff of 1e-06. These reads were further assembled into contigs and those shorter than 200 nt were excluded from further analyses. Additionally, misidentified

the 2012 virus reads matched various BPMV isolates (Gu et al. 2002). Geographically, BPMV was found in 17 (of 24) and 12 (of 22) counties in 2011 and 2012, respectively (Fig. 1; Table 2 and 3). Previous studies have classified BPMV isolates into two subgroups (Gu et al. 2002). Interestingly, our results revealed an uneven prevalence of subgroup I and II genotypes, as an overwhelming majority of contigs of both years aligned more closely with subgroup II genotypes. In fact, all of the RNA2-specific contigs 200 nt or longer aligned preferably with sequences of subgroup II isolates deposited in GenBank (>97% sequence identity). Another notable characteristic of BPMV

In both years, BPMV was found to be the most common virus. Indeed, over 93% of the 2011 virus reads, and more than 74% of

TABLE 1
County-level distribution of soybean viruses identified in 2011 samples.

Counties	No. fields	No. samples	Virus-positive	BPMV	SPuV	SVNV	TRSV	TSV
Allen	2	6	0					
Champaign	1	4	2	1		1		
Clark	2	6	3				3	
Crawford	1	4	4	4				
Darke	2	5	1	1				
Defiance	1	4	4	4				
Greene	1	4	1				1	
Hancock	1	4	4	4				
Hardin	1	4	2	2		1		
Henry	3	10	9	9				
Huron	1	4	3	3				
Madison	2	5	3	3				
Mercer	1	4	0					
Miami	1	4	4	4				2
Pauldine	1	4	3	3				
Preble	1	4	1					1
Putman	1	4	4	2	1		1	
Richland	2	6	6	6				
Sandusky	1	2	2	1		2	1	
Seneca	1	4	4	4		1	2	
Shelby	1	4	4					4
van Wert	1	4	0					
Wayne	1	4	1	1				
Wyandot	1	4	4	4		1		
Total	31	108	69	56	1	5	8	7

TABLE 2
County-level distribution of soybean viruses identified in 2012 samples.

Counties	No. fields	No. samples	Virus positive	AMV	BPMV	BYMV	CYVV	SMV	SVNV	TRSV	TSV
Ashland	2	6	5		4				2		
Ashtabula	2	6	2						2		
Champaign	1	4	3		3				1		
Clark	4	14	13		9			2	4		2
Delaware	2	6	4		1	2			3	2	
Erie	2	6			4					1	
Fulton	2	6	0								
Geauga	2	6	4						4		
Logan	2	6	4		4				3		
Lorain	2	6	4						3	2	
Lucas	2	6	5		3				2		
Marion	2	6	6		2				2	6	
Medina	2	6	5						3	2	
Morrow	2	6	6		4				5	3	
Ottawa	2	6	2		1				1		
Portage	2	6	3						3		
Sandusky	2	6	0								
Trumbull	5	15	7		1				6		
Union	2	6	5				2		5		
Wayne	3	10		2							
Williams	2	6	0								
Wood	2	6	6		2				1		3
Total	49	151	84	2	38	2	2	2	50	16	5

contigs is their extreme sequence diversity. In fact, most of the assembled contigs were under 500 nt long, as widespread single-nucleotide polymorphisms made it difficult to assemble full length genomes with sufficient confidence. These data suggest that BPMV has become associated with Ohio soybean production for some time, allowing for independent evolution of diverse field strains.

INCIDENCE OF SVN

SVNV has attracted nationwide attention since its discovery less than ten years ago (Tzanetakis et al. 2009; Zhou et al. 2011). In our samples, SVN was found in five counties in 2011 and 17 counties in 2012 (Tables 1 and 2, Fig. 2). In all, SVN was present in at least 21 counties across the state, suggesting that it is not a newly introduced virus. Rather, its presence was likely ignored in the past. Most of the 2011 reads could be assembled into three contigs of 7,551, 4,937, and 1,554 nt, corresponding to partial sequences of L, M, and S segments of the SVN genome. However, the 2012 data showed more diversity in SVN as 24 SVN-specific contigs ranging from 200 to 760 nt could not be integrated into longer assemblies because of single-nucleotide polymorphisms. Pairwise alignments suggest that at least two different SVN genotypes were present, with their L, M, and S segments sharing approximately 99%, 98%, and 96% nt level identities, respectively.

INCIDENCE OF TRSV

TRSV was the third most common virus of soybean in our samples, found in fields of five counties in 2011 (Table 1) and six counties in 2012 (Table 2; Fig. 2C). These counties were fairly evenly distributed among the Ohio soybean-producing counties, though slightly more concentrated near Lake Erie shore. A striking characteristic associated with TRSV is the extreme sequence diversity. Indeed, in spite of limited sequencing depth, three different RNA1 genotypes can be clearly discerned from 2011 samples. Four additional RNA1 genotypes were identified in 2012 samples. Furthermore, pairwise comparisons revealed that these seven RNA1 genotypes share less than 90% sequence similarity with each other within a 250-nt homologous region shared by the seven partial sequences. Indeed, different pairs of

primers had to be used to map the specific genotypes to their respective locations (see Mapping the Distribution of Identified Viruses and Table 3). These results strongly suggest that once introduced to a given location, TRSV evolved independent of each other with limited exchange of genetic materials among different genotypes.

INCIDENCE OF TSV

TSV was found in multiple Ohio soybean fields, and in both years. Specifically, it was in three counties (Miami, Preble, and Shelby) surveyed in 2011, and two counties (Clark and Wood) surveyed in 2012 (Table 1 and 2, Fig. 2D). It is interesting to note that all five TSV-positive counties are located within the western third of Ohio. It remains to be determined whether this specific geographic location contributed to TSV incidences. Another noteworthy observation was that in both years at least two distinct TSV genotypes were isolated, presumably associated with different field sites. In fact, the two genotypes isolated in 2011 were 89%, 83%, and 89% identical for RNA1, RNA2, and RNA3, respectively. The identity levels for the two 2012 genotypes were 90%, 88%, and 90%. Interestingly, although the isolates of a particular year were quite dissimilar to each other, they appear to fall into two distinct clades as year-to-year comparison revealed each of the 2011 isolates could match one of the 2012 isolates with 97 to 99% identities.

ISOLATED CASES OF AMV, SMV, BYMV, CLYVV, AND SPV

Unlike the viruses described above, these five viruses were each found in just one field. That AMV and SMV were so rare in Ohio soybean fields was surprising as both have been reported to be relatively common soybean pathogens in other states (Giesler and Ziems 2006; Malapi-Nelson et al. 2009). Closer examination revealed that the AMV-positive samples were collected from a field that was part of OARDC experimental farms, with an alfalfa plot nearby. Conversely, the SMV-positive samples were collected from a field in the OARDC Western Station used for propagation of germplasm, and hence could be caused by contaminated seed stock(s). In short, our results suggest that neither AMV nor SMV was associated with large-scale field soybeans in Ohio.

TABLE 3
DNA oligos used in the distribution studies.

Name	Sequences (5' - 3')	Use
AMVR2-1139F	CTGAAGCCCTATTAGCGATAAAGAAAC	Mapping AMV distribution
AMVR2-1757R	CTATGCGCATTAACCATAAGGTTAGA	Mapping AMV distribution
BPMVR2-1064F	GAAATTCGTCTTCTAGTGCAAG	Mapping BPMV distribution
BPMVR2-1331R	CAATCTGCACAATGGTATTCGGAA	Mapping BPMV distribution
BYMV-Fa	GAAACTTAAGTACTACGGTTTGG	Mapping BYMV distribution
BYMV-Ra	CGAGGCAACCTCACTAAGTCTT	Mapping BYMV distribution
CYVV-Fa	AGACCTATGATGGGCTTCTATCA	Mapping CYVV distribution
CYVV-Ra	AACAATGAATCCCAACCACCATA	Mapping CYVV distribution
SMV-7311F	GTGTATGTGACTGATCCTGATGA	Mapping SMV distribution
SMV-7828R	AGCATTCTCTTCCAAACCCACCA	Mapping SMV distribution
SPuV-4114F	GGTAACCCCTCAAAAGGTTTGCAA	Mapping SPuV distribution
SPuV-4881R	GTCTTTCCTCTAATAAGAGTGAGAA	Mapping SPuV distribution
SVNV-S-1734F	CCATCTTCTTTCCAGGCATTTC	Mapping SVN distribution
SVNV-S-2421R	GATTCAAGTTCAGCGAGTTCTACAA	Mapping SVN distribution
TRSVR1-1255F	GAGTGTGTGCAATTATCTGCATA	Mapping TRSV distribution
TRSVR1-1844R	CAAAGATGCCAAGAAAAGTTGCAAG	(2011) Mapping TRSV distribution
TRSVR1-1968F	GTGATGTGGTGATACCGGAAAGT	(2011) Mapping TRSV distribution
TRSVR1-2490R	CCAGCACGGACAGACTGAGTATG	(2012) Mapping TRSV distribution
TSVR1-1634F	CTCACGATTTAGACTCGGACTAC	(2012) Mapping TSV distribution
TSVR1-2138R	ACCATCCAAGATAAATCCAGACT	Mapping TSV distribution

The discovery of BYMV and CIYVV in soybean is noteworthy as neither has been reported to infect soybean in Ohio. BYMV as a soybean pathogen has been reported overseas (Campos et al. 2014). Within the United States, BYMV was reported to be present in field-grown soybeans in Illinois (<http://ipm.illinois.edu/diseases/series500/rpd505/>). Our BYMV isolate was found in a

soybean field in Delaware County in central Ohio. All BYMV-related RNA-Seq reads could be assembled into a near full-length (9,517-nt) BYMV sequence that shares 93% sequence identity with the closest BYMV isolate (GenBank Accession No. HG970850.1).

FIGURE 2

Distribution patterns in Ohio counties of (A) BPMV, (B) SVNv, (C) TRSV, and (D) other viruses. In A, B, and C, counties where the corresponding viruses were discovered are filled with gray. In D, counties where TSV were found are filled with grids of dashed lines (Miami, Preble, Shelby, and Wood), or solid lines (Clark) where SMV was also found. Counties where AMV (Wayne), BYMV (Delaware), and CIYVV (Union) were found are filled with slanted, horizontal, and vertical lines, respectively. The Putnam County where SPuV was discovered are filled with dots.

CIYVV has been associated with soybean diseases in Japan and South Korea (Sasaya et al. 1997; Shin et al. 2014). Within the continental United States, it was identified in clover and snap beans but has yet to be found in soybean. The CIYVV-positive samples were collected in a single field in Union County in central Ohio. Interestingly, two distinct CIYVV genotypes sharing 93% sequence identity could be assembled from the RNA-Seq reads. It is worth noting that both samples that tested positive for CIYVV were also positive for SVN.

Finally, the discovery of SPuV was reported earlier (Han et al. 2012). The mode of transmission, as well as potential pathology of this virus remains to be elucidated as we have so far been unsuccessful in mechanically transmitting this virus to new plants. However, its association with soybean does not appear to be isolated as it was also identified from samples collected in other states (L. L. Domier, *unpublished*).

LIMITED SCALE OF VIRUS DISEASES IN OHIO SOYBEAN FIELDS

Overall, as judged by symptoms, virus diseases were relatively uncommon in Ohio soybean fields in both years (2011 and 2012). In fact, plants with virus symptoms were often limited to a few edge rows, or sometimes even to a few plants, typically accounting for a very small fraction of the fields. The drought condition in 2012 was associated with a sharp increase in SVN incidences. Nevertheless, the symptomatic plants were still limited to mostly fewer than 10 rows on one side of the fields. We speculate that this pattern of disease distribution might be caused by arthropod vectors that migrate from weeds or wooded areas to young soybean seedlings as they emerge. While symptoms characteristic of SVN were commonly observed in mature leaves, they could have been due to infections that occurred in younger plants. Finally, the absence of SbDV in our soybean samples is consistent with reports from other states, and possibly reflects the inefficiency of the soybean aphid (*Aphis glycines* Matsumura) as a SbDV vector (Harrison et al. 2005; Damsteegt et al. 2011).

MIXED INFECTIONS OF MULTIPLE VIRUSES

Samples positive for SVN frequently contained two or more viruses. Indeed, five out of six SVN-positive samples in 2011 contained at least one additional virus: three co-infected with BPMV (from Hardin, Wyandot, and Sandusky counties); one with TRSV (Sandusky County; dark gray); and one with both BPMV and TRSV (from Seneca County; yellow). In 2012, 18 of the 50 SVN-positive samples contained other viruses: eight with BPMV; four with TRSV; and three others with both BPMV and TRSV. Additionally, SVN also co-existed with CIYVV in both CIYVV-positive samples and BYMV in one of the two BYMV-positive samples. Furthermore, two of the 2011 samples and one of the 2012 samples contained BPMV and TSV, one 2012 sample contained BPMV and TRSV, another 2012 sample contained BPMV and SMV, and yet another 2012 sample contained BPMV, SMV, and TRSV. In conclusion, although overall plants showing virus symptoms were uncommon, those that did often contained more than one virus. Therefore, it is possible that soybean plants infected with one virus might stay symptomless.

CONCLUDING REMARKS

We report an extensive sampling of soybean-infecting viruses in Ohio soybean fields during the 2011 and 2012 growing seasons. This study confirmed the widespread presence of BPMV, SVN, and TRSV in the fields, although the scale of infections was relatively limited. A substantial increase in the number of

fields positive for SVN was observed in 2012, possibly as a result of the unusually dry and hot weather that summer, which could favor the propagation of SVN-transmitting arthropod vectors (thrips). The only other virus found in more than one field, and in both years, was TSV. By contrast, AMV and SMV, which were thought to be common soybean pathogens, were only found sporadically in Ohio. Finally, isolated incidences of CIYVV, BYMV, and SPuV infections were also observed. These data are expected to offer guidance to the diagnosis and management of soybean virus problems in Ohio and other neighboring states.

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