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

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A B S T R A C T

Soybean dwarf virus (SbDV), first identified as an agricultural problem in Japan, has emerged as a growing problem in the Midwestern United States. The majority of research on SbDV had been limited to four lab maintained strains from Japan. SbDV had been found in clover in the eastern United States, but these isolates rarely emerged into soybeans. These isolates were analyzed by multiplex PCR and sequencing, revealing that some were infections of both Y and D components, including a recombinant subisolate. Phylogenetic analyses for the US isolates revealed a broad diversity of SbDV, with selection pressure greater on the movement protein than the coat protein. The field isolates from the Eastern United States showed differences in symptoms, aphid transmission and host range, demonstrating that a study of field isolates is an important complement to laboratory maintained strains in understanding the biology and evolution of plant viruses.

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Introduction

Soybean dwarf luteovirus (SbDV) is the causal agent of an economically important disease of soybean (Glycine max [L.] Merr.) in Japan (Tamada, 1975; Tamada et al., 1969; Tamada and Kojima, 1977; Terauchi et al., 2001). The causal virus has been transmitted to more than 50 species of forage legumes, pulse crops, and other broadleaf plants representing three families (Damsteegt et al., 1990, 1995; Johnstone and Guy, 1986; Makkouk et al., 1997). Although it causes economic losses only in soybean, subterranean clover (Trifolium subterraneum L.), crimson clover (T. incarnatum L.), sugar beets (Beta vulgaris L. subsp. vulgaris), broad bean (Vicia faba L.), pea (Pisum sativum L. subsp. sativum), lentils (Lens culinaris Medik.), and French bean (Phaseolus vulgaris L.) are all hosts of the virus (Tamada, 1975; Kellock, 1971; Johnstone and Munro, 1980; Ashby et al., 1979; Johnstone, 1978; Makkouk et al., 1997; Damsteegt et al., 1990).

As a member of the Luteoviridae, SbDV is transmitted only by aphids in a circulative, persistent manner. It is phloem-limited and occurs in low concentration in the plants (Tamada, 1970; Damsteegt and Hewings, 1987). The virus occurs as several distinct strains based on symptomatology in soybeans, aphid relationships, physicochemical properties, and molecular makeup (Tamada, 1973; Damsteegt et al., 1990, 1995; Rathjen et al., 1994; Smith et al., 1993, 1998; Terauchi et al., 2001). Historically, two strains were identified, based on symptoms expressed on soybeans (Tamada, 1973). Both strains, SbDV-D (dwarfing) and SbDV-Y (yellowing), were transmitted solely by Aulacorthum solani Kaltenbach. The subterranean clover red-leaf virus (SCRLV) described in Australia (Kellock, 1971) also was transmitted by A. solani. An isolate found in California, serologically related to SCRLV, (19), and other related isolates found worldwide were transmitted by Acrithosiphon pisum Harris and other aphids (McLaughlin et al., 1988; Damsteegt et al., 1995, 1999; Mikoshita et al., 1991; Honda et al., 1999; Rathjen et al., 1994) but not by A. solani. In 1986, Johnstone and Gyu suggested placing SCRLV in synonymy with the yellowing strain of SbDV (7). More recently at least 4 unique strains of SbDV have been described in Japan based on symptoms and principal vector: SbDV-DS (dwarfing, solani), DP (dwarfing, pisum), YS (yellowing, solani), and YP (yellowing, pisum) (Terauchi et al., 2001).

SbDV isolates have been found in white clover (Trifolium repens L.), red clover (T. pratense L.), alsike clover (T. hybridum L.), subterranean
clover, crimson clover, *Chenopodium* spp., and soybean from 11 states in the eastern and southeastern U.S. (Damsteegt et al., 1995; Mclaughlin et al., 1992), California (Johnstone et al., 1984), and more recently in soybeans in Illinois and Wisconsin (Harrison et al., 2005). Characterization of the eastern US strains was based on similarity of vectors, vector/virus relationships, symptom expression in common hosts, methods of purification, genomic and sub-genomic RNAs, virion morphology, immunologic relatedness using monoclonal and polyclonal antibodies, and Northern hybridization analysis of genomic and sub-genomic dsRNAs (Hewings et al., 1986; Smith et al., 1991; Damsteegt et al., 1999). To gain a better understanding of the diversity and complexity of SbDV populations in the eastern US, the coat protein genes of thirteen isolates (including 17 subisolates) from 8 different states were characterized by PCR and direct sequencing. We report here the nucleic acid and putative amino acid sequence analyses as evidence of dwarfing and yellowing SbDV strains in the US. This is the first report of mixed infections of dwarfing and yellowing strains of SbDV and the first report of a recombinant SbDV.

**Results**

The eastern US SbDV-Y isolates originated from subterranean clover or white clover, whereas the eastern US SbDV-D isolates originated from red clover, subterranean and alsike clovers, soybeans and *Chenopodium album* (Table 1). All eastern US isolates were capable of infecting soybeans. The symptoms on soybeans varied somewhat, but for the most part were less severe than the Japanese isolates. The US D-isolates caused mild stunting, with dark green leaves showing some downward curling. The US Y-isolates caused little or no symptoms in soybeans (Fig. 1). Two of the strains (MD2 and MD8) caused consistent mild yellowing in soybeans. In a limited host range experiment, all of the US isolates were able to infect peas, and several of the isolates were able to infect peanuts (all of those tested, Table 3). The US D-isolates were capable of infecting white clover, but the Y-isolates were not capable of infecting red clover when a D component was not part of the infection (Table 3). All of the eastern US SbDV isolates infected crimson clover and subterranean clover, causing stunting and red leaf margins (Fig. 1).

All eastern US isolates were transmitted by *A. pismum* and *M. persicae* (Table 3). The foxglove aphid *A. solani*, considered a non-vector from all previous research (Honda et al., 1999), was able to transmit one of two mixed D plus Y-isolated tested (VA — *A. solani* transmissible, and MD1 — *A. solani* non-transmissible). Interestingly, only the D component of VA mixed infections was transmitted by *A. solani*. However, the success of transmission was extremely low (less than 2%) but consistent in repeated experiments (six times). *N. bakerii, A.gossypii* and *A. cracciavora* were capable of transmitting all eastern US isolates tested.

The multiplex PCR assay was able to distinguish D-strains from Y-strains, and detected both strains in an artificial mixture of equal amounts of D and Y. No PCR products were amplified from healthy soybean or clover controls (Fig. 2A). Both D and Y strains were detected in the eastern US, without any particular bias based on geography. Occasionally both D and Y bands were detected in the same sample (e.g. VA), indicating that there may be mixed infections of D and Y strains (Fig. 2B). However, these results were inconsistent, suggesting that the multiplex assay was not as efficient in distinguishing mixed infections where one of the strains existed at significantly lower titers (data not shown).

Using D and Y universal primer sets, the coat protein ORF of the US isolates was amplified and sequenced directly. In addition, PCR was performed using D and Y specific primers in separate reactions for all isolates, to ensure that both isolates were found in cases of mixed infections where one strain or the other was of significantly lower titer. Ten of the isolates were either D strain (PA, SC, NY, MD3, MD7 and MD9) or Y strain (MD1, NC, KY, and MS), but three isolates contained mixed infections of both D and Y strains (MD2, MD8 and VA). The MD2 strain generated three unique sequences: a D-like sequence from the D specific primers, a Y-like sequence from the Y specific primers, and a recombinant sequence from the universal SbDV primers. The first 540 bases of the MD2 recombinant CP gene were clearly D-like, but the last 70 bases were clearly Y-like (Fig. 3). The remainder of the readthrough ORF is D-like sequence (data not shown).

The 17 coat protein nucleotide sequences (ten individual sequences for the single infections, plus two sequences each for the three mixed infections and one recombinant sequence) were aligned with the Wisc isolate and the Japanese YP, YS, DP, DS and DC isolates and analyzed phylogenetically (Fig. 4). The D-like isolate sequences clearly grouped together, as did the Y-like isolate sequences. All of the US D-isolates, including the isolate from Wisconsin, grouped with the Japanese DP isolate. All of the D-like isolates were transmitted by *A. pismum*, but not by *A. solani*. The only US isolate infrequently transmitted by *A. solani* was the VA isolate, which was actually a mixed infection of D and Y strains. The Japanese DC and DS isolates formed a separate clade. The recombinant MD2 sequence, generated by using universal primers, was distinct from the D-like and Y-like sequence groups. The US Y-like isolates grouped together separate from the YS and YP sequences, with the exception of MD8, which was most closely related to the YP and YS isolates. The other US isolates separated into two groups; one group with the MD2 Y-like and VA20 Y-like isolates and the other group containing the NC, KY, MD1 and MS isolates (Fig. 4).

The phylogenetic analyses of inferred MP and CP amino acid sequences (Figs. 5 and 6) generated different phylogenies. On the D-like side of the MP phylogeny, the MD3 and VA20 D-like amino acid sequences were in a separate group from the other US isolates. The

<table>
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<tr>
<th>Isolate</th>
<th>Type</th>
<th>Source host</th>
<th>Location</th>
<th>Contributor</th>
<th>GenBank #</th>
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<td>Nebo, KY</td>
<td>S.A. Ghabrial</td>
<td>EU306576</td>
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<td>Y-like</td>
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<td>Fort Detrick, MD</td>
<td>V. Damsteegt</td>
<td>EU306577</td>
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<tr>
<td>MD7 (1993)</td>
<td>D-like</td>
<td>Soybean</td>
<td>Prince George's Co, MD</td>
<td>A.L. Stone</td>
<td>EU306581</td>
</tr>
<tr>
<td>MS (1989)</td>
<td>D-like</td>
<td>Subterranean clover</td>
<td>Starkville, MS</td>
<td>M.R. Mclaughlin</td>
<td>EU306584</td>
</tr>
<tr>
<td>NC (1990)</td>
<td>Y-like</td>
<td>White clover</td>
<td>Rockingham Co, NC</td>
<td>C.L. Campbell</td>
<td>EU306586</td>
</tr>
<tr>
<td>VA (1990)</td>
<td>D-like and Y-like</td>
<td>Subterranean clover</td>
<td>Blacksburg, VA</td>
<td>S.A. Tolin</td>
<td>EU306590</td>
</tr>
</tbody>
</table>
Y-like isolates in the MP inferred amino acid phylogeny show the same three groups that were observed in the nucleotide phylogeny (Fig. 5). There were differences between the CP amino acid phylogeny and the nested MP amino acid phylogeny. The CP inferred amino acid sequence phylogeny lacked the definition of branches seen in the nucleotide phylogeny, essentially resembling two star phylogenies, one for US Y-like isolates and one for US D-like isolates (Fig. 6). Again, the US isolates tended to group together, distinct from Japanese isolates. There were multiple mutations in the overlapping MP and CP ORFs, but there were more non-synonymous changes in the MP ORF (cumulative 41 changes, 96.8 to 97.9% amino acid identity) than in the CP ORF (26 changes, 97.8 to 99.8% amino acid identity) (Table 4). In addition, there were fewer non-synonymous changes among the D strain coat proteins, all of which varied by a single amino acid at most (Table 4).

### Table 2

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<th>Sequence</th>
<th>NT position</th>
<th>Use</th>
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<td>TTCCATCTTTCGACTACCTT</td>
<td>1434</td>
<td>Universal reverse transcriptase primer used for Y and D sequencing and detection</td>
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<td>ShbDV-USDF</td>
<td>GTAAGTTGAAACGGATTCAG</td>
<td>2984</td>
<td>D-specific</td>
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<tr>
<td>ShbDV-USDR</td>
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<td>3493</td>
<td>D-specific</td>
</tr>
<tr>
<td>USYCPF</td>
<td>TGCAGGCAAATATACACG</td>
<td>3493</td>
<td>Y-specific</td>
</tr>
<tr>
<td>USYCPF-R</td>
<td>CTGGTATATATCTCCGCA</td>
<td>Y-specific</td>
<td></td>
</tr>
<tr>
<td>NUCP2801</td>
<td>TGTCGCTACACAAATACCTT</td>
<td>2801</td>
<td>Primers used for full length coat protein sequencing</td>
</tr>
</tbody>
</table>

### Discussion

Several soybean dwarf virus isolates from the eastern US had been partially characterized previously using antibodies and dsRNA profiles (Damsteegt et al., 1999). In that analysis the results suggested that there may be variation among US isolates, even within isolates from the same strain. Thus, a more extended characterization of the eastern US isolates was in order. Previous analyses had indicated that both Y- and D-strains were prevalent in the eastern US (Damsteegt et al., 1999). However, in these earlier studies, little molecular characterization was performed. Sequence analyses confirmed the presence of both D- and Y-isolates in the eastern US, both individually and in mixed infections. The Y-alone isolates from the eastern US only infect white clover. Both of the isolates from red clover were D-like single infections. In contrast, the isolates from white clover were either single Y-like infections or mixed infections of D-like and Y-like strains, suggesting the possibility that the mixing of isolates allows for utilization of a broader host range. Only one of the eastern isolates (MD7) was found in soybean, despite the fact that these isolates readily infect soybean in the greenhouse. It appears that soybeans are either a suboptimal host, or that there is not an efficient vector for the transmission of SbDV from clover to soybeans (Damsteegt et al., 1999). All of the SbDV isolates that have been found in soybean are either D-like or Y-like isolates (accession AB038149). Some of the SbDV populations were analyzed by cloning and sequencing to determine the levels of diversity within single populations (Table 5). The mutation frequencies for SbDV populations were within the normal range for plant RNA viruses (Roossinck and Schneider, 2006), ranging from 2.8 mutations/1000 bases to 16.9 mutations per 1000 bases. Control reactions using an in vitro RNA transcript as a template demonstrated that the experimental error rate was significantly below the mutation frequency of the viral populations. The Y-isolates were more genetically diverse within their isolates than the D-isolates, even when isolated from the same host, subterranean clover. The ratios of non-synonymous to synonymous mutations (dN/dS ratios) were similar for all populations analyzed, ranging from 0.28 to 0.41 (data not shown).
Table 3

|---------------------|---------------------|----------------|-------------------|-----------|--------|----------|----------|-----------|----------|-----------|----------|----------|----------------|----------------|-------------|-----------|----------|-----------|--------|----------|----------------|----------------|-------------|-----------|----------|----------|-----------|----------------|----------------|-------------|-----------|----------|-----------|--------|----------|-----------|----------|-----------|----------|-----------|----------------|----------------|-------------|-----------|

Fig. 2. Panel A: Multiplex assay for determining the presence of SbDV-D and Y isolates. Typical results for a multiplex assay are shown. The controls (Japanese Y alone, Japanese D alone and a 1:1 mixture of Japanese D and Y are in the middle. The predominantly Y-like US isolates are on the left side of the gel; the predominantly D-like isolates are on the right side of the gel. Sizes of the Y and D specific bands are indicated, size marker is 1 kb DNA ladder. Panel B: multiplex analysis of three US isolates that have both D-like and Y-like components. Mixed Japanese D and Y controls are shown on the right.

In general, the eastern US isolates of SbDV cause mild symptoms. The eastern US Y-isolates do not cause discernable symptoms in soybeans, and the D-isolates cause mild yellowing and/or stunting. The strongest symptoms occurred in soybeans when the isolate was a mixture of D and Y strains (VA, Fig. 1). While the eastern US isolates did not generate the severe symptoms associated with D and Y isolates from Japan, the possibility remains that a severe isolate could emerge from the existing isolates in the US. There is evidence of severe dwarfing isolates in midwestern soybeans, but they are widely scattered (Domier, personal communication). However, we have no definitive data indicating how many or what mutations separate severe isolates from milder isolates, and we can only speculate what selection pressures cause severe isolates to arise. Even if a severe isolate of SbDV arose in the US, there may be a lack of suitable vectors to colonize soybeans (Damsteegt et al., 1999). The only aphid to colonize soybeans is Aphis glycines, which is very rarely a vector of SbDV. This would appear to indicate that endemic SbDV presents a limited threat to soybeans in the US.

The multiplex PCR assay is capable of distinguishing D from Y strains and detecting mixed infections when both strains exist at relatively equal titers. The results from multiplex PCR indicated the possibility of mixed infections of D and Y strains in eastern US isolates. Sequence analysis of eastern US isolates confirmed the presence of mixed D and Y infections in three isolates: VA20, MD2 and MD8. Both Y-like and D-like strains have been found together in subterranean clover and white clover in the field, and both isolates are capable of infecting soybeans, peas and peanuts in the greenhouse. In addition, the recovery of a recombinant would indicate that both isolates at times co-exist in the same cell. This is the first report of mixed infections of D and Y strains of SbDV, as well as the first report of a recombinant SbDV isolate. This corroborates recent reports that suggest that mixed infections and recombination do occur in luteoviruses (Robertson and French, 2007; Liu et al., 2007).
Phylogenetic analyses based on nucleotide sequences clearly separate the D and Y eastern US SbDV isolates. The eastern US Y-like isolates appear to have more diversity than the eastern US D-like isolates (Fig. 4, and Table 4). It is interesting to note that the single Y-like sequences fall out as a single clade. This could be the result of a single Y isolate breaking free from an ancestral mixed infection, but this is not likely considering the broad geographic spread of the single Y-like infections. It also is possible that there is a selection for a particular variant of SbDV Y-like isolates that is more suited to single infection, but it is hard to imagine what the mechanism for this would be. This is in contrast to the D-like isolates, where the mixed infection D-like isolates do not cluster separately from the D-like single isolates. The possibility that the Y-like single isolates cluster together by chance cannot be ruled out.

The CP and MP genes of SbDV overlap, with the MP gene nested within the CP ORF in the plus-one reading frame. This makes for an interesting balance in selection pressures that are unique to viruses with overlapping ORFs. Not surprisingly, phylogenetic analyses suggest that the CP and MP amino acid sequences are under different selective constraints. The phylogeny based on the CP amino acid sequence shows star-like phylogenies, with very little resolution besides the D and Y branching. This has been demonstrated for other plant RNA viruses (Roossinck, 2002). In contrast, the phylogeny based on the MP amino acid sequence shows nearly the same clade formations that were seen in the nucleotide phylogenies. This could suggest that the selection pressures on the MP are greater than those on the CP, and that these selection pressures are driving the evolution of the virus. However, this cannot be demonstrated conclusively without the analyses of full length genome sequences.

Like many RNA plant viruses, SbDV maintains diverse populations. Sequence analyses indicated that the Y-like isolates and mixed infections of D and Y isolates maintained significantly higher levels of diversity than the D-like isolates (statistical significance determined using ANOVA). It also is interesting to note that the mixed infection populations maintained mutation frequencies that were intermediate to the exclusively D-like and Y-like isolates, although the differences between mixed infections and exclusively Y-like isolates were not statistically significant. It is important to note that data from the mixed D + Y population are only presented for the Y component, so the mutation frequency is not sequence dependent. A possible explanation for the lower mutation frequency might be the potential that in mixed infections the D replicase is amplifying the Y template. However, additional tests would need to be done to confirm this. Differences in population diversity between strains of the same virus have been noted for other viruses (Schneider and Roossinck, 2001), but the mechanisms that cause this are not completely understood. There appeared to be differences in diversity between SbDV isolates from different hosts. However, because no single isolate was compared across multiple hosts.
it is impossible to say whether or not the host plays a role in determining levels of genetic diversity in SbDV. It is clear that SbDV maintains diverse populations, as do many other plant RNA viruses.

From the molecular analyses of the eastern US SbDV isolates, we conclude that both Y-like and D-like strains exist in the US, that they form mixed infections in clover and soybeans that occasionally result in the production of viable recombinants, and that Y-like isolates maintain higher levels of diversity in single infections and on a broad geographic scale. This study exclusively used SbDV isolates directly from the field (Table 1), in contrast to the majority of SbDV studies that feature work done on isolates maintained for years in greenhouse and laboratory settings. The isolates characterized directly from the field violate some of the assumptions and dogma generated by years of greenhouse maintained SbDV work. For example, SbDV field isolates that are Y strains by sequence can cause dwarfing symptoms, and D strains that cause Y-like symptoms. The strict limitations observed in clover host range (D strains in red clover, and Y strains in white clover) do not hold up in field isolates from the eastern US. Perhaps most significantly, a Virginia field isolate was transmissible by A. solani and A. pisum, (Table 3), which is in direct contrast to all previously published reports for SbDV (Tamada, 1970; Terauchi et al., 2001). These differences are likely an artifact of researchers intentionally selecting for and maintaining the isolates that cause only the most severe symptoms on the agricultural host of interest, most likely using a single aphid species.

This work serves to corroborate the recent emphasis on studying viral ecology (Wren et al., 2006; Fargette et al., 2006), not as a replacement for laboratory and greenhouse based studies, but as a means to augment and extend our knowledge of plant viruses. Our understanding of SbDV biology and evolutionary potential, particularly in regard to recombination and its role in luteovirus emergence (Smith et al., 2000; Salem et al., 2008; Silva et al., 2008), would be significantly reduced if field isolates were not included in the scope of this research. Clearly laboratory and greenhouse maintenance is necessary for obligate parasites such as viruses, but it’s important to compare the results of experiments performed with isolates long exposed to these selective regimes with the results of field based experiments to identify potential selection pressures introduced by the artificial environment. It is entirely possible that the bias towards lab maintained isolates is a significant limitation for the study of many vectored viruses, both plant and animal.

Materials and methods

Virus isolates and insect vectors

Thirteen SbDV isolates were obtained from eight eastern states from a variety of source plants (Table 1). All isolates were transferred into subterranean clover and pea (P. sativum L cv. Puget) by the pea aphid A. pisum and maintained in subterranean clover. Routine transmissions were accomplished by the pea aphid although several aphid species were tested as potential vectors including the green peach aphid Myzus persicae Sulzer, cowpea aphid Aphis craccivora Koch, foxglove aphid Aulacorthum solani Kaltenbach, yellow clover aphid Thrips tabaci L, and the short-beaked clover aphid Neoraphis Controlled. All aphids were given a 48 h acquisition access period (AAP) on source plants and a 48 h inoculation access period (IAP) on test plants (Damsteegt et al., 1999). Inoculated plants...
Mean amino acid identity for Y strain movement proteins = 96.8% ± 2.4%.

Mean amino acid identity for Y strain coat proteins = 97.8% ± 1.1%.

Mean amino acid identity for D strain movement proteins = 97.9% ± 0.8%.

Mean amino acid identity for D strain coat proteins = 99.8% ± 0.2%.

Table 4
Amino acid identities for coat proteins and movement proteins of US SbDV isolates, Japanese YP and DP strains are provided as a reference. The amino acid identity levels (in percent) for coat proteins are shown on the top right, for the movement protein (shaded boxes) on the bottom left.

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Table 5
Mutation frequencies for SbDV populations. Mutation frequencies (defined as number of mutations per 1000 bases) were determined for four exclusively D strain populations, three exclusively Y strain populations and two mixed infections. The mutation frequency for the RT-PCR was determined by in vitro transcriptions as a template (labeled as control). In the case of mixed infections, the mutation rate was determined using the Y strain template only. ANOVA analysis indicates that the mutation frequency for D populations is significantly lower than the mutation frequency for Y populations and mixed populations.

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<th>isolate</th>
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<th>total mutations</th>
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<td>5</td>
<td>0.4</td>
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<td>VA</td>
<td>control transcript</td>
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were checked for the presence of virus using the NUCPF and NUCPR primers (Table 2) in PCR as described below.

Viral RNAs

Viral RNA was extracted from each isolate by three different methods: 1) total RNA was extracted from 200 mg of infected and healthy subterranean clover tissue using a phase lock centrifuge tube kit and guanidine isothiocyanate protocol from Perfect Prep Kit (5′–3′, Boulder, CO); 2) double-stranded RNA (dsRNA) was extracted from 80 g of infected subterranean clover tissue by CF-11 column chromatography (Smith et al., 1991); and 3) genomic RNA was obtained from purified viros by extraction with 1:1 phenol/chloroform and elution (Damsteegt et al., 1999; Smith et al., 1988).

Multiplex PCR assay

Sequence alignments of the open reading frame (ORF) 1 & 2 regions from previously sequenced SbDV isolates (Wisc3, DP, DS, DC, YS, and YP) were used to design a multiplex PCR assay for the detection of D and Y strains (Table 2). Conserved areas were identified and strain-specific primers were selected using Primer 3 Input program (Rozen and Skaletsky, 2000) with the following parameters: melting temperature range of 57 to 62 °C, primer length 18 to 24 and GC content 40 to 70%. Primer sequences were analyzed using NCBI blastn program (National Center for Biotechnology Information, Bethesda, MD) for specificity. Reverse transcription was performed using 2.0 μg RNA in a 20.0 μl reaction that contained 11.7 μl nuclelease-free H2O, 4.0 μl 5 × 1st strand buffer (Invitrogen, Carlsbad CA), 1.0 μl 0.1 M DTT (dithiothreitol – Invitrogen, Carlsbad CA), 0.8 μl 5 μM 1434 MUR reverse primer (5′-CGTGGGAAAAGAATGAGG-3′), 0.4 μl 10 mM dNTPs and 0.1 μl SuperScript II Reverse Transcriptase (1 unit) (Invitrogen, Carlsbad, CA) at 42° for 1 hour 30 min followed by 72° for 5 min. PCR was performed in a 30.0 μl reaction using 2.0 μl cDNA template, 16.8 μl nuclelease-free H2O, 4.0 μl 10 × PCR buffer (Qiagen, Valencia, CA), 2.2 μl 25 mM MgCl2, 1.9 μl 5 μM 1434 MUR reverse primer, 1.0 μl 5 μM 1134 MDF (5′-GGCTGCTAAAATCGTCCAA-3′) forward primer, 1.0 μl 10 mM dNTPs, 0.9 μl 5 μM 882 MYF (5′-AGAGCCTGAACCCAAAATG-3′) forward primer, and 0.2 μl Taq DNA Polymerase (0.2 μl units) (Qiagen, Valencia, CA). Amplification consisted of initial denaturation at 94° for 3 min, 30 cycles of 94° for
30 s, 58°C for 30 s and 72°C for 45 s with a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose gel. The expected size of the D specific product is approximately 300 base pairs, and the expected size of the Y specific product is approximately 550 base pairs.

Direct sequencing

First strand cDNA synthesis was performed using Invitrogen Superscript II reverse transcriptase according to manufacturer specifications (Invitrogen, Carlsbad, CA). The first strand cDNA was used as a template for PCR amplification with a variety of ShdBV primers. The primers were a combination of D-specific, Y-specific and universal primers, allowing for amplification of all possible viral products (Table 2). The primers flanked the coat protein ORF (the movement protein is nested within the coat protein in a different reading frame). PCR products were cleaned using Exo-Sap DNA cleanup according to manufacturer specifications (USB, Cleveland, OH) prior to direct sequencing. The amplified products were directly-sequenced using Applied Biosystems Big-Dye reactions (Applied Biosystems, Foster City, CA) to determine the consensus sequence of the viral isolates. The final cleanup was performed using a Dyex spin column according to manufacturer specifications (Qiagen, Valencia, CA). The products were analyzed on an ABI 310 sequencer (Applied Biosystems, Foster City, CA). All sequences have been deposited into Genbank (Table 1).

Cloning and sequencing

In order to do a more detailed analysis of ShdBV populations, the amplified PCR products of some ShdBV populations were cloned and sequenced. RT-PCR products representing the coat protein were generated as described for direct sequencing, with modifications made for high fidelity (Schneider and Roossinck, 2000). The coat protein gene of nine distinct isolates were cloned using either the pT7Blue vector kit (Novagen) and DH5 alpha competent cells (BRL, Bethesda, MD) or Topo TA cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer specifications. Five to twenty-three clones were sequenced per isolate, and mutation frequencies were calculated as previously described (30). As a control, in vitro transcripts were generated from a clone containing the ShdBV coat protein from the South Carolina isolate and used as a template for RT-PCR and cloning, to estimate the basal error rate of the experimental process (Schneider and Roossinck, 2000). Statistical differences between mutation frequencies were determined using ANOVA, as previously described (Schneider and Roossinck, 2000).

Sequence analysis

Complete coat protein nucleotide sequences, coat protein and movement protein amino acid sequences were aligned as three distinct assemblies using the Clustal W analysis function of Codon-Code Aligner according to manufacturer specifications (CodonCode Corp., Dedham, MA). These alignments were used to determine mutation frequencies, where bases differing from the consensus sequence were designated as mutations. The Japanese YS (Genbank ID# AB038147), VP (Genbank ID# AB038148), DS (Genbank ID# AB038149), DP (Genbank ID# AB038150) and DC (Genbank ID# AB076038) sequences and the sequence of an isolate from Wisconsin (Wisc3, Genbank ID# DQ145545) were included for comparison. Bean leafroll virus (BLRV) was used as the outgroup. Phylogenetic trees were assembled using PAUP version 4.10b for UNIX with the maximum likelihood analysis (100 bootstraps), maximum parsimony analysis and minimum evolution programs. Branches with bootstrap values less than 65 were collapsed. Branches with bootstrap values between 65 and 70 were maintained if all three analyses generated the same branch pattern, and the bootstrap values were above 70 in at least two of three analyses. Branches with bootstrap values above 70 for all three types of analyses were maintained.

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References


