BREWTON, RANDOLPH G., LAWRENCE LEE and CHARLES M. RUSH, Texas A & M Agricultural Research Center, 6500 Amarillo Blvd W., Amarillo, TX 79106. **Detection of genetic diversity in beet soilborne mosaic virus (BSBMV) in the central United States; identification of single-strand conformation polymorphisms (SSCP).**

Introduction: Beet necrotic yellow vein virus (BNYVV) and beet soilborne mosaic virus (BSBMV) are two closely-related soilborne viruses that infect the roots of sugar beets (*Beta vulgaris*) by utilizing the fungus *Polymxya betae* as a vector. Both BNYVV and BSBMV possess a divided genome consisting of four, polyadenylated, single-stranded RNAs. Because of numerous distinct features, these viruses were recently separated from the Furovirus genus and placed in the new Benyvirus genus. BNYVV is the cause of rhizomania and has been identified in most sugar beet growing areas of the world. Relatively little genetic variability has been reported for BNYVV; the principal global populations comprising two 'strain groups' (Koenig et al., 1995, 1997; Kruse et al., 1994).

BSBMV has been confirmed only in the United States. BSBMV often moves systemically and causes distinct foliar symptoms that include chlorosis, necrosis and leaf crinkling. While the roots of most BSBMV-infected sugar beets appear normal, we have found numerous examples of plants that display rhizomania-like symptoms. Serological assays indicate that these plants are infected with BSBMV but not BNYVV suggesting that BSBMV may have the potential to cause rhizomania-like symptoms.

Our objectives for this study were: 1) determine the degree of genetic diversity in BSBMV isolates from a wide geographic area, 2) identify candidate genetic markers that may link to disease phenotypes; particularly those that include the roots, and 3) clarify the evolutionary relationship between BNYVV and BSBMV.

Materials and Methods: Sugar beets displaying foliar and/or root symptoms were collected from fields in Colorado, Minnesota, Nebraska, Texas and Wyoming. Fragments of symptomatic sugar beet leaves (0.8-1.0 gm) were homogenized in 4.5 ml TRIzol Reagent (Life Technologies, Gaithersburg, MD). Total RNA was isolated according to manufacturer's recommendations with the following modification. In order to minimize polysaccharides contamination, RNA was precipitated with 0.25 ml isopropanol followed by 0.25 ml of a high salt precipitation solution (1.2 M sodium citrate and 0.8 M sodium chloride) per 1 ml of TRIzol used for homogenization. Soil samples were air-dried and archived.

First strand cDNA was made from total RNA using a commercial kit (Life Technologies) under the following conditions: 0.5 μg oligo-dT in 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT. Superscript II reverse transcriptase was used to synthesize cDNA at 42° C for 50 min. 1 μl aliquots of cDNA was used for the polymerase chain reaction (PCR) using unique and RNA-specific 18-mer oligonucleotide primers in 50 μl reaction volumes using Elongase DNA polymerase mixture (Life Technologies) and the 'hotstart' method (Chou et al, 1992). Typical cycling parameters were 94° C for 30 sec., 56° C for 30 sec., and 68° C for 1 min. 30 sec., for 35 cycles.

PCR products were analyzed by SSCP (single-strand conformation polymorphism) essentially as described (Orita et al., 1989; Koening et al., 1995). PCR products (typically 5 μl) were denatured for 5 min. at 70° C in an equal volume of formamide containing 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene-cyanol and immediately cooled on ice. Samples were

loaded to 10% polyacrylamide gels and electrophoresed at room temperature overnight at 65 volts in a Hoefer Scientific SE600 apparatus with 0.5x TBE buffer (45 mM Tris, 45 mM borate, 1mM EDTA, pH 8.3). Initial buffer temperature was 4° C. After electrophoresis, the gels were silver-stained as described (Bassam et al., 1991).

Results and Discussion: In preliminary experiments, primer pairs that generated PCR products ranging in size from 166-740 bp were analyzed by SSCP. Under the conditions used for our analyes, PCR products of approximately 400 bp were most informative, although some larger PCR products, in particular, on RNA 2 were informative (Table 1).

Table 1. Summary of variation identified in RT-PCR products subjected to SSCP analysis.

BSBMV RNA	Product	Amplified Region (nt)	Size of PCR Product	Results
2	A	3685-4292	608	Not informative
	B	2970-3448	479	Not informative
	C	2443-3122	696	Informative (three groups)
	D	2137-2832	696	Minor variation
	E	1527-1953	427	Not informative
	F	583-1322	740	Informative (2+ groups)
	G	251-762	512	Informative (2 groups)
3	1	1338-1709	372	Informative (four groups)
	2	1089-1418	416	Minor variation
	3	788-1195	408	Not informative
	4	484-891	408	Informative (two+ groups)
	5	177-585	409	Minor variation
4	1	3-398	396	Informative (two groups)
	2	328-745	413	Minor variation
	3	3-745	743	Not informative

To date, we have analyzed 56 samples (19 from Colorado, 11 from Minnesota, 6 from Nebraska, 13 from Texas, and 7 from Wyoming) using the primers listed above that cover the majority of RNAs 2, 3 and 4. Our results clearly demonstrate that SSCP is a useful tool for detecting multiple migration patterns of single-stranded DNA fragments isolated from various BSBMV isolates. When this methodology was applied to BNYVV, two primary 'strain groups' were identified (Kruse et al., 1994; Koening et al., 1995). For BNYVV, the grouping was consistent, regardless of which RNA was being analyzed. This means that if a PCR product derived from one BNYVV RNA was subjected to SSCP analysis, and gave rise to two groups, other PCR products from one of the other BNYVV RNAs gave rise to the same two groups.

The tendency for viral isolates to fall into a limited number of stable groups was not observed for BSBMV. Instead, based on SSCP results, BSBMV appears to exhibit greater

genetic diversity than BNYVV. More than two distinct migration patterns were observed with several of the PCR products analyzed, including products C and F on RNA 2, and products 1 and 4 on RNA 3. Figure 1 shows some of the SSCP patterns observed for PCR products derived from RNA 2.

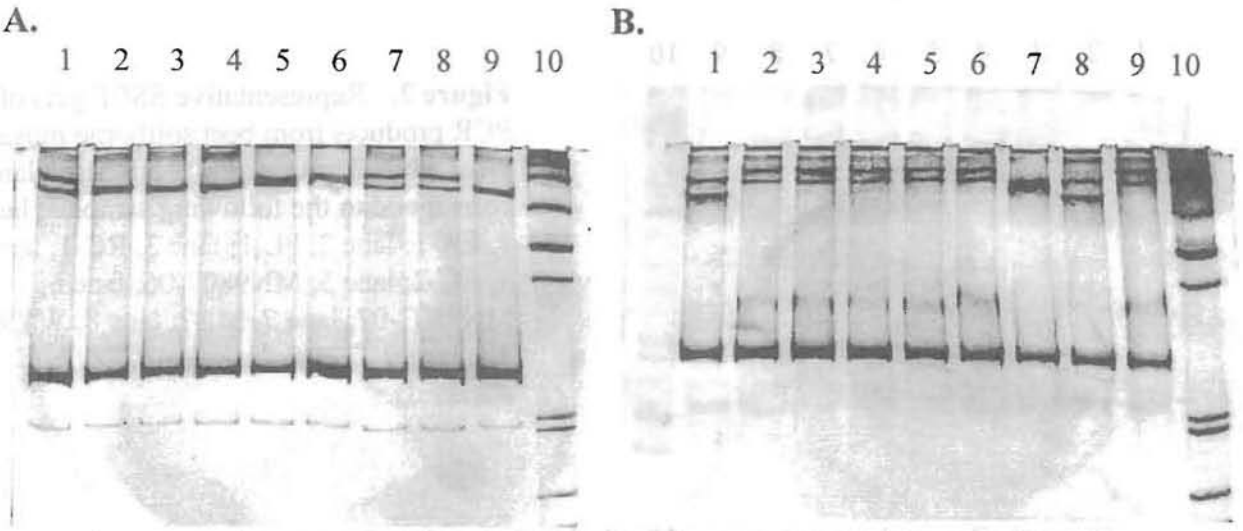

Figure 1. Representative SSCP gels of PCR products from beet soilborne mosaic virus RNA 2, product F (panel A.), and product C (panel B.). Numbered lanes correspond to the following samples: lane 1, EA-1; lane 2, PL-1; lane 3, RC-1; lane 4, RC-2; lane 5, MN9807-06; lane 6, MN9807-07; lane 7, MN?; lane 8, WY96-3B; lane 9, CO96-44, lane 10, DNA standards.

In Figure 1A, two patterns are readily identified. The first pattern, found in lanes 2, 3, 4, 5, 6 and 9, exhibits a closely spaced upper doublet that may appear as a solid band at the magnification shown in Figure 1. The second pattern, found in lanes 1, 7 and 8, exhibits a more widely spaced upper doublet. In Figure 1B, three patterns are seen. In the first pattern, an upper doublet in seen in lanes 2, 3, 4, 5, 6 and 9. This group of samples corresponds to the first pattern seen in Figure 1A and includes samples from Texas (PL-1 in lane 2), Colorado (RC-1, RC-2 and CO96-44 in lanes 3, 4 and 9, respectively), Minnesota (MN9807-06, MN9807-07 and MN? in lanes 5, 6 and 7, respectively), and Wyoming (WY96-3B in lane 8, respectively). The second pattern, found in lanes 1 and 8, exhibits a more widely spaced and faster migrating doublet. The samples showing this pattern includes a sample from Colorado (EA-1 in lane 1) and Wyoming (WY96-3B in lane 8). The third pattern is a unique closely spaced doublet in lane 7, which is a sample labeled from Minnesota. Therefore, when we consider the two PCR products shown in Figure 1, we see two principle 'groups' with the exception of the isolate in Figure1B, lane 7.

The relatively simple grouping of viral isolates seen in Figure 1 is lost when we include samples from RNA 3 (Figure 2). For this particular set of samples, we can also observe two distinct patterns. The first pattern consists of a closely spaced upper doublet and is found in lanes 1, 2, 3, 6 and 7. In addition to the upper doublet, the second pattern exhibits an additional band that is found in lanes 4, 5, 8 and 9. However, the RNA 3 markers bear no relation to the distribution of markers on RNA 2. Of particular interest is the fact that more than one marker is present in the same field. Lanes 3 and 4, illustrate the case of two RC samples that originated from the same field in Colorado. RC-1 in lane 3 exhibits only the upper doublet while RC-2

also exhibits the additional, uppermost band. A similar example is illustrated in lanes 5 and 6, where two different BSBMV isolates collected from different sugar beets in the same Minnesota field differ with regard to the uppermost band. MN9807-06 exhibits the uppermost band while MN9807-07 lacks the uppermost band.

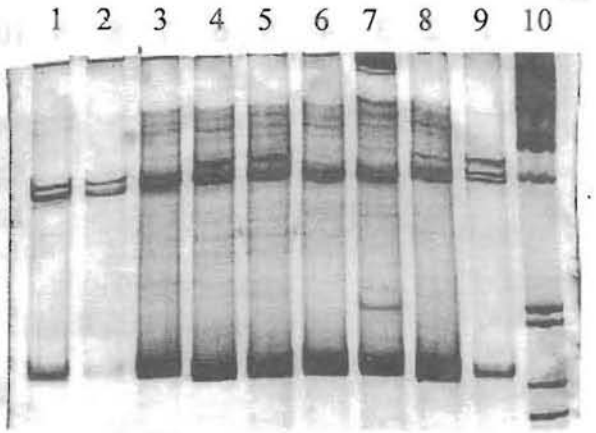

Figure 2. Representative SSCP gels of PCR products from beet soilborne mosaic virus RNA 3, product 4. Numbered lanes correspond to the following samples: lane 1, EA-1; lane 2, PL-1; lane 3, RC-1; lane 4, RC-2; lane 5, MN9807-06; lane 6, MN9807-07; lane 7, MN?; lane 8, WY96-3B; lane 9, CO96-44, lane 10, DNA standards.

Since similar markers are observed in viral isolates taken from widely separated locations, it is important to demonstrate that similar appearing markers are also highly similar at the nucleotide level. Therefore, the 408 base pair PCR product 4 from RNA 3 was subcloned and sequenced for five BSBMV isolates: PL-1 from Texas, RC-1 and RC-2 from Colorado, and MN9807-06 and MN9807-07 from Minnesota. These samples correspond to lanes 2-6 in Figure 2. The results clearly indicate that the presence or absence of the uppermost band is determined by five nucleotide changes (data not shown). For the isolates that exhibit the uppermost band, DNA sequencing demonstrated the substitution of G's for A's at positions 153, 156 and 321, while C's were substituted for T's at positions 276 and 297. Additional single base changes were observed in MN9807-06 at position 84 (a T for C substitution), as well as at position 329 (an A for G substitution). These results confirm that viral isolates from Texas, Colorado and Minnesota (PL-1, RC-1 and MN9807-07) exhibit essentially identical nucleotide sequence in the case of RNA 3, product 4. Viral isolates from Colorado and Minnesota (RC-2 and MN9807-06) also exhibit essentially identical nucleotide sequences.

This ongoing investigation has demonstrated that, in contrast to BNYVV, which is relatively stable from the genetic point of view, BSBMV isolates exhibit significant genetic diversity that can be identified using SSCP. Different BSBMV isolates possess a variety of genetic markers on different RNAs that can be found in many combinations. Rather than try and classify BSBMV into a limited number of 'strains' or 'strain groups' we propose to refer to BSBMV isolates as distinct genotypes. Different markers can be found in the same field and can also be found across wide geographic areas. The makers we have identified may be useful in attempts to obtain linkage between specific markers and disease phenotypes.

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