Complete Genome Characterization and Coat Protein Genealogy of Isolates of *Maize dwarf mosaic virus* from Johnsongrass and Maize in Oklahoma and Missouri

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Abstract

Maize dwarf mosaic virus (MDMV) significantly affects maize production worldwide, including the United States. This study describes the distribution and biological and molecular characterization of MDMV isolates from Johnsongrass and maize. A total of 262 samples (symptomatic = 214, asymptomatic = 48) were collected in Oklahoma and Missouri during 2016, 2017, and 2019 growing seasons. Based on a dot-immunobinding assay (DIBA), the average incidence of maize dwarf mosaic disease varied from ~71% (79/111) in 2016, ~76% (81/106) in 2017, and 62% (28/45) in 2019. Sixty-five DIBA-positive samples for MDMV were further confirmed by RT-PCR, and the complete coat protein (CP) gene was cloned and sequenced. Phylogenetic analysis of 132 isolates (This study = 65; GenBank = 67) revealed two main groups (G1 and G2) of MDMV isolates. All 65 MDMV isolates contained a 39-nucleotide insertion in the N-terminal region of CP genes and clustered in G1 which were different from the isolates in G2, without 39-nucleotide

insertion. The first complete genome (9,563 nucleotides) of a MDMV isolate (Bixby1) from Johnsongrass was sequenced, which was distantly related to eight previously reported MDMV isolates from maize. The dN/dS ratio showed mostly purifying selection on each of cistrons except 6K1 being subjected to the diversifying selection. Further analyses revealed three putative recombination events between MDMV-Bixby1 and MDMV isolates from other countries. The successful mechanical and aphid transmission of MDMV-Bixby1 onto maize cultivars was achieved. Altogether, this information showed that Johnsongrass harbors genetically diverse MDMV isolates, which could pose a threat to cultivated crops such as maize and sorghum.

Keywords: *Maize dwarf mosaic virus*, Johnsongrass, coat protein, phylogenetic analysis, pathogen diversity, viruses and viroids, field crops, cereals and grains

The United States is the largest producer of maize (*Zea mays* L.), accounting for ~33% of the world's ~1.13 billion metric tons maize production in 2017 (FAOSTAT 2018). Apart from being a staple crop in many countries, maize also provides indirect calories through its byproducts such as sweeteners, including corn syrup. In addition, maize-based ethanol production in the U.S. has increased eight-fold during 2000 to 2010 (Awika 2011). Thus, the demand of maize for ethanol production and other usages further increased up to six million bushels in 2017 (USDA NASS 2018).

More than 50 viruses are known to infect maize worldwide (Lapierre and Signoret 2004). Maize dwarf mosaic virus (MDMV; genus *Potyvirus*, family *Potyviridae*) is one of the major viruses that infects several cereal crops, particularly maize and sorghum (*Sorghum bicolor* L.) (Achon et al. 2011; Brunt et al. 1996; Gell et al. 2010). MDMV typically produces yellow streaks and mild mosaic on leaf blades and overall dwarfing of infected plants. Susceptible cultivars of maize and sorghum show severe yield reduction and less overall fitness of plants. Apart from its major cultivated hosts, Johnsongrass (*Sorghum halepense* L. Pers.) is a well-known natural overwintering wild host for MDMV, which acts as a reservoir of inoculum (Achon et al. 2011). MDMV is

transmitted in a nonpersistent manner by 23 species of aphids (Knoke et al. 1974, 1983; Toler 1985).

In the United States, MDMV was first reported in the 1960s from maize in southern Ohio, and since then, it has been reported in more than 13 states (Janson and Ellett 1963; Singh and Gordon 1980; Szirmai and Paizsne 1963; Toler 1985; Tosic et al. 1990; Williams and Alexander 1965). Severe MDMV infection can cause total yield reduction up to 42 to 75% (Forster et al. 1980; Mikel et al. 1981a, b). MDMV also infects sorghum, which can reduce its yield by 15 to 69% (Janson and Ellett 1963).

MDMV has flexuous filamentous particles that range from 650 to 900 nm in length (McDaniel and Gordon 1989) and contain a positive sense, single-stranded RNA genome of approximately 9,500 nucleotides (nt). MDMV genome is translated into a single long 338 kDa polypeptide, which is then proteolytically cleaved into 10 different protein products and additionally contains a conserved short ORF known as Pretty Interesting Potyviridae ORF (PIPO) within the P3 cistron (Chung et al. 2008; Kong and Steinbiss 1998). The coat protein (CP) gene is involved in virus systemic movement, aphid transmission, and genome encapsidation (Achon et al. 2011; Gell et al. 2010).

The CP gene of MDMV has been used in a few evolutionary studies to understand the genetic diversity among the Spain, Hungarian, and Bulgarian isolates of the virus (Achon et al. 2011, 2012; Gell et al. 2010; Tóbiás and Palkovics 2004; Tóbiás et al. 2007). In 2004, a study reported an unusual duplication event (39 nt [13 amino acids (aa)] insertion) in the N-terminal region of the CP of a Hungarian MDMV isolate (Dallas) from maize that increased the length of its CP gene to 915 nt (304 aa) (Tóbiás and Palkovics 2004). Subsequently, Gell et al. (2010) also reported sequence diversity in the CP gene among several Hungarian MDMV isolates, resulting in the detection of three different lengths of the CP gene (i.e., <876, 888, and 915 nt; 292, 296, and 305 aa, respectively). Currently, the CP genes of only six MDMV isolates (one from Argentina and five from Hungary) contain the 39-nt insertion (Supplementary Table S1). Similarly, there are only eight complete MDMV genome sequences obtained from maize which are reported from six countries (Supplementary

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Table S2). However, there are no reports of the complete genome sequence for MDMV from Johnsongrass.

Recently, we reported MDMV for the first time from Johnsongrass in Oklahoma (Wijayasekara and Ali 2017). A few studies exist that reported comprehensive analysis of the genetic diversity of MDMV isolates from maize and Johnsongrass using restriction fragment length polymorphism (RFLP) of the CP gene and sequencing of P1 and HC-Pro genes (Achon et al. 2011, 2012). Similarly, another study from Ohio State analyzed MDMV isolates from maize and Johnsongrass by next generation sequencing using pooled samples and confirmed the presence of MDMV (Stewart et al. 2014), but none of the partial sequences of MDMV from Johnsongrass are available in GenBank, and no comprehensive sequence analysis of MDMV isolates exist from Oklahoma and Missouri. In addition, a lack of the complete genome sequence of the MDMV isolate from Johnsongrass also hinders the understanding of natural mutation accumulation and its ability to cause disease in cultivated hosts. Therefore, this study was conducted to determine the prevalence, biological and molecular characterization of MDMV. In addition to obtaining the complete genome of MDMV from Johnsongrass, the genetic diversity of the virus was also assessed based on analysis of the coat protein gene of a large number of MDMV isolates from Johnsongrass and maize from Oklahoma and Missouri relative to corresponding sequences of the available global MDMV isolates.

Materials and Methods

Virus sources and collection of field samples. Field surveys were conducted from March to July in the 2016, 2018, and 2019 growing seasons in six different counties (Cherokee, Kay, Muskogee, Payne, Osage, and Tulsa) in Oklahoma and McDonald County in Missouri (Fig. 1A). The minimum distance between Tulsa County and other survey points was 47, 68.7, 70, 76, 110, and 137 miles to Muskogee, Cherokee, Payne, Osage, Kay, and McDonald counties, respectively, while the maximum distance was 246 miles between Kay and McDonald counties. During 2016 and 2017 growing seasons, 10 to 30 Johnsongrass plants in each county were chosen randomly based on characteristic MDMV symptoms as well as a few asymptomatic plants. One of the Johnsongrass plants showing typical MDMV symptoms in Tulsa County, OK (known as MDMV-Bixby1 isolate) was collected in 2018 and confirmed by RT-PCR. Virus-like preparations (VLP) were made according to Ali et al. (2012) from this isolate and used for biological characterization and complete genome sequencing. In 2019, 45 leaf samples were randomly collected from maize plants in Cherokee and Kay counties of Oklahoma.

Mechanical inoculation. To test the infectivity of isolate MDMV-Bixby1, symptomatic leaf tissues (0.5 g) were homogenized in 1 volume (wt/vol) of phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCL, 8 mM Na₂HPO₄, and 1 mM KH₂PO₄, pH 7.4) (Ali and Randles 1997). The extracted sap was centrifuged briefly, and the supernatant was rub-inoculated onto the first two leaves (pre-dusted with Carborundum) of 2-week-old seedlings of commercial maize cultivars Steller and Jubilee (six seedlings/cultivar). For the healthy control, only PBS was used to inoculate the maize seedlings. All inoculated and control maize seedlings were kept in growth chambers at 25°C with a photoperiod of 8 to 10 h for symptom development. Symptoms were recorded weekly for 8 weeks. At 8 weeks postinoculation, 0.5 g of leaf tissues from the inoculated and negative control maize plants were used for total RNA extractions and assayed by RT-PCR for the presence of MDMV as described below.

Aphid transmission assay. To confirm the vector transmission of MDMV-Bixby1, experiments were conducted using corn leaf aphids (*Rhopalosiphum maidis*), which were collected from Johnsongrass in Tulsa County. The aphids were reared for 3–4 days on healthy and then MDMV-Bixby1-infected Johnsongrass, and 8 to 10 aphids were selected randomly to confirm the infectivity of MDMV with RT-PCR. Viruliferous aphids (10 to 15 aphids/seedling) were transferred to four 2-week-old seedlings of maize cv. Steller and left overnight in a cage. Next day, the maize seedlings were sprayed with lawn and

yard insect killer (Lowe's), and the plants were kept for symptom development in the greenhouse. At 8 weeks postinoculation, total RNA was extracted and the presence of MDMV was confirmed by RT-PCR as shown below.

Serological detection. All the Johnsongrass and maize leaf samples collected in the 2016, 2017, and 2019 growing seasons were tested by dot immuno-binding assay (DIBA) as described previously (Ali and Randles 1997). All samples were tested against polyclonal antisera of MDMV (Nano Diagnostics, AR, U.S.A.).

RNA extraction, RT-PCR, cloning and sequencing. Total RNA was extracted as reported previously (Ali et al. 2012) from approximately 100 mg of symptomatic leaf tissue of 65 DIBA-positive MDMV isolates (58 from Oklahoma and 7 from Missouri) representing seven counties in two states. The RNA pellets were dissolved in 50 µl of RNase-free water and stored at –20°C. Two-step RT-PCR was performed with MDMV-specific primers targeting the CP gene or the complete virus genome (Supplementary Table S3). The RT step for each reaction includes 5 µl of total RNA, 4 µl RT buffer, 2 µl of 10 µM dNTPs; 1.0 µl of 50 µM of reverse primer, 0.5 µl of RNase inhibitors, and 0.5 µl MMLV reverse transcriptase according to the manufacturer's instructions (Genscript, Piscataway, NJ). PCR was carried out in a 25-µl reaction using 1.5 U of Taq DNA polymerase (Genscript, Piscataway, NJ), 1× Taq buffer, 0.2 mM dNTPs, 0.2 µM each primer, and 2 µl of template cDNA. Thermal cycling conditions were 35 cycles of 95°C for 30 s; annealing appropriate temperature for each primers pair (Supplementary Table S3) ranged from 48 to 55°C for 1 min, and extension 72°C for 30 s. The PCR products were run on 1% agarose gel and DNA bands of the expected sizes were purified using gel extraction kit (Promega, U.S.A.). Complementary DNA from virus-free Johnsongrass was used as the negative control in each reaction.

The complete genome of MDMV-Bixby1 was amplified using nine primers pairs (Supplementary Table S1) as described above. The 5' and 3' ends of the MDMV genome was sequenced using 5' and 3' RACE protocols as described by Deng et al. (2008) (Takara Bio Inc.).

Purified PCR products of each amplified DNA fragment were cloned using pGEM-T Easy Vector (Promega, U.S.A.) and transformed into *Escherichia coli* DH5α competent cells (New England BioLabs, U.S.A.). At least three recombinant plasmid DNA clones for each fragment were sequenced in both directions using Big-Dye terminator cycle sequencing (Applied Biosystems, 3130 Genetic Analyzer, U.S.A.) at the core facility of the Department of Biological Science, The University of Tulsa, OK, U.S.A. Consensus sequence was obtained using Seqman software (DNASTar, Madison, WI, U.S.A.).

Sequence analysis. The complete CP nt/aa sequences of 65 MDMV isolates were aligned with 67 other MDMV CP gene sequences (Supplementary Table S1) using ClustalW (Thompson et al. 1994). Phylogenetic analysis was performed using MEGA6 software to construct a Maximum Likelihood (ML) tree with GTR+G+I model and 1,000 bootstrap replications (Tamura et al. 2013). The CP gene sequence of *Sorghum mosaic virus* (Accession no. U57358) was used as an outgroup.

The complete genome and the polyprotein nt/aa sequence of MDMV-Bixby1 was assembled from the consensus sequences of all nine overlapping genome fragments using the Seqman software (DNASTar, Madison, WI, U.S.A.) and aligned with corresponding sequences of eight other MDMV isolates (Supplementary Table S2) available in GenBank using the ClustalW program. Based on the best model selection obtained with MEGA6, an ML tree was constructed using the Tamura Nei model with 1,000 bootstrap replicates.

To further understand the selection intensity among the coding regions of the CP gene and the complete genomes of MDMV isolates, the ratio of nonsynonymous (dN) to synonymous (dS) substitutions was calculated using DnaSP 5.10 (Librado and Rozas 2009). Site-specific selection pressure was measured for each codon site using different codon-based maximum-likelihood methods, SLAC, FEL, and MEME for positive selection with $P = 0.1$ available in DataMonkey online version www.datamonkey.org (Pond et al. 2005). Neutrality tests such as Tajima's D (Tajima 1989), and Fu

and Li's D (Fu and Li 1993) were also performed in DnaSP5.10. for verifying polymorphism among the phylogroups (Rozas et al. 2003, 2017). Nucleotide diversity (π) within and between MDMV populations was estimated according to Nei (1987), while the mean values for the genetic distance (d) were calculated using MEGA6 (Nei 1972).

Test for recombination. The complete genome sequences of MDMV-Bixby1 and eight other complete genomes from GenBank (Supplementary Table S2) were aligned and used in the recombination analysis by RDR4 v 4.69 (Martin et al. 2015). Seven different recombination detection algorithms (RDP, GENECONV, Chimaera, MaxChi, Bootscan, SISCAN, and 3Seq) were implemented using

Fig. 1. Map of Oklahoma and Missouri states where samples of Johnsongrass and maize were collected during 2016–2019 growing seasons. (A) MDMV-infected Johnsongrass and maize samples were collected in seven counties indicated with a black circle in both states. (B) Symptoms on leaves of Johnsongrass sampled showed typical yellow streaking and mild mosaic of MDMV, (I) Healthy Johnsongrass from Muskogee County in 2016, (II) virus-infected Johnsongrass from Muskogee County in 2017, (III) Payne County in 2017, (IV) Osage County in 2017, (V) Tulsa County in 2017, (VI) McDonald County in 2017, and (VII) virus-infected maize from Kay County in 2019. (C) Transmission of MDMV-Bixby1 isolate, healthy control maize seedling, mild streaking on leaf of maize seedling (cv. Steller) at 8 weeks post mechanical inoculation, severe streaking and mosaic on leaf of maize seedling (cv. Steller) at 8 weeks post aphid's inoculation.

default parameters. Recombination events that were detected by at least five of the seven detection methods with significant statistical support and Bonferroni-corrected *P* value cutoff of 0.05 were considered acceptable.

Results

Field symptoms of maize dwarf mosaic disease. Common field symptoms of MDMV in Johnsongrass and maize include yellow streaks and mild to severe mosaic on the leaf blades (Fig. 1B, II–VII) compared with healthy-looking asymptomatic leaves (Fig. 1B, I). There were no apparent differences among the field symptoms of MDMV in Johnsongrass plants collected from different counties in Oklahoma and Missouri. However, symptoms were apparently more severe in maize compared with Johnsongrass (Fig. 1B, VII).

Serological detection of MDMV. A total of 262 samples (111 in 2016, 106 in 2017, and 45 in 2019), including symptomatic (*n* = 214) and asymptomatic (*n* = 48), were processed by DIBA. Out of 111 Johnsongrass samples collected in 2016, only the 79 symptomatic samples reacted positively to the MDMV antisera; while the remaining nine symptomatic and 23 asymptomatic samples were negative. In 2017, 81 out of the 106 Johnsongrass samples were positive for MDMV, while the remaining 25 asymptomatic samples were negative by DIBA. In 2019, 28 out of 45 maize samples were positive for MDMV, while the remaining 17 symptomatic samples were negative. Thus, approximately 89% (188 positive out of 214 symptomatic samples) were positive to MDMV. The incidence of MDMV in Johnsongrass at 22 sites in five counties in two states ranged from 71.1 to 77.14% in 2016 and 2017, while the incidence of MDMV in maize at four sites in two counties (Cherokee and Kay) in Oklahoma ranged from 62 to 100% during the 2019 growing seasons.

Symptoms in the mechanically inoculated and vector-transmitted maize seedlings. The inoculated maize seedlings (cultivars Steller and Jubilee) that were mechanically inoculated with MDMV-Bixby1 did not show any symptoms from 1 to 3 weeks postinoculation. However, mild mosaic was observed on systemically infected leaves of cv. Jubilee at 4 weeks postinoculation, while no symptom was observed on seedlings of cv. Steller for the same time period. At 8 weeks postinoculation, mild streaking or chlorosis was observed on the systemically infected leaves of cv. Steller (Fig. 1C) and cv. Jubilee (data not shown). A total of three seedlings (two from cv. Jubilee and one from cv. Steller) out of the 12 inoculated plants (6/cultivar) showed obvious MDMV symptoms.

Aphid-inoculated maize cv. Steller seedlings also did not show any symptoms until 4 weeks postinoculation. Two out of four seedlings infected by viruliferous aphids developed mild symptoms at 4 weeks postinoculation, and the symptoms became severe at 8 weeks postinoculation (Fig. 1C). Both mechanical and aphid transmission assays were repeated and the infectivity of MDMV was achieved.

When all inoculated maize seedlings (mechanically or by aphids) and the healthy control plants were tested against MDMV by CP-specific RT-PCR, only the three symptomatic seedlings (two from cv. Jubilee and one from cv. Steller) from mechanical inoculation and two symptomatic seedlings cv. Steller with aphid inoculation were positive, while all of the remaining inoculated asymptomatic seedlings and control seedlings were negative. Sequences of the CP gene of MDMV revealed that isolates from all positive seedlings of maize retained the 39-nt insertion in the N-terminal region of the CP gene that was present in the genome of the source plant harboring MDMV-Bixby1. All three mechanically infected maize plants were kept until physiological maturity R6 stage. None of the infected plants produced commercially acceptable corn cob compared with the uninfected maize plants of the same cultivars, which produced normal corn cobs under the same growth conditions (data not shown).

Analysis of the CP gene sequences. The size of the CP gene for all MDMV isolates from Johnsongrass or maize in this study was 915 nt (Supplementary Table S4) compared with the CP of global MDMV isolates which ranged in size from 745 to 915 nt (Supplementary Table S1). A 39-nt insertion (nt position 152–190 and aa position 54–69 aa) in the N-terminal region of the CP gene of all the MDMV isolates from Johnsongrass and maize in this study was

observed, as reported previously for some Hungarian MDMV isolates (Gell et al. 2010). In pairwise comparison, the CP gene sequences of 65 MDMV isolates from Johnsongrass and maize shared 95.5–100% nt and 96.5–100% aa sequence identity, among each other, and 83.8–99.8% nt and 89.7–99.5% aa sequence identity with 67 MDMV isolates obtained from GenBank (Supplementary Table S1).

The ML phylogenetic tree based on the CP gene nt sequences of MDMV isolates (this study = 65; GenBank = 67) revealed two main groups (Fig. 2A) named G1 (with 39-nt insertion except for isolate AJ542536) and G2 (without 39-nt insertion). Phylogroup G1 contained 72 MDMV isolates including all the 65 MDMV isolates from Johnsongrass and maize, along with one MDMV isolate from Argentina, and six Hungarian MDMV isolates. Five of the MDMV isolates from Hungary contained the 39-nt insertion in the N-terminal of the CP gene (Fig. 2A), while the remaining one isolate did not contain the 39-nt insertion. The size of the CP gene of MDMV isolates in G1 group ranged from 888 to 915 nt (Supplementary Tables S1 and S4).

Phylogroup G2 contained 60 MDMV isolates including 28 from Spain, 20 from Hungary, five from Bulgaria, two from Iran, two from the United States, one isolate each from Israel and Italy, and one isolate of unknown origin.

Within G1, further segregation was observed based on the geographical origins of the 65 MDMV isolates (Fig. 2A). In addition, some isolates that were sampled from the same county segregated in various clusters (Fig. 2A). For example, 10 MDMV isolates (MK1–MK10) from Muskogee County that were collected in 2016 formed two separate well-supported (>93%) subclusters (Fig. 2A). The remaining 11 isolates (MK11–MK21) from the same county that were collected in 2017 also formed two separate well-supported (>67%) subclusters distinct from those formed by the 2016 isolates (Fig. 2A). Similarly, seven isolates from McDonald County, MO formed three separate well-supported (>90%) subclusters indicating a pattern of seasonal and location-based CP gene genetic of MDMV. The remaining MDMV isolates from Osage, Payne, and Tulsa counties clustered according to their locations without any further within-location subclustering (Fig. 2A). MDMV isolates (KA1–KA10) from maize clustered together from the rest of MDMV isolates, while Cherokee isolate obtained from maize was closely related to isolates from Kay County.

Diversity of MDMV population. The nucleotide diversity (π) was 0.0856 for G1 and 0.1072 for G2 phylogroup (Table 1). The genetic distance (*d*) among the MDMV isolates in G1 was 0.194 ± 0.067 and G2 was 1.065 ± 0.366 . In both phylogroups, the dN/dS ratio was <1 (Table 1), while DataMonkey analysis showed that a large number of codons were under negative selection in both phylogroups (Table 1). The pairwise *FST* value between the two phylogroups was 0.34, indicating infrequent gene flow, which was above the threshold of *FST* > 0.33 value (Rozas et al. 2003, 2017) between G1 and G2 phylogroups.

The highest nucleotide diversity (π) between the MDMV isolates collected from various counties in this study was 0.126 (for comparison between Kay and MacDonald counties) and the lowest was 0.014 (for comparison between Osage and Payne counties), while the genetic distance (*d*) ranged from 0.012 ± 0.001 to 0.090 ± 0.006 (Table 2). Similarly, the highest nucleotide diversity (0.104) and the genetic distance (0.088 ± 0.005) was observed between the MDMV isolates from the United States and Spain, while the lowest π 0.067 and “*d*” 0.066 ± 0.005 was observed between Hungary and Bulgaria (Table 2).

The pairwise *FST* values based on the CP gene nt sequences among the MDMV isolates from various counties within the United States or different countries (Table 2) indicated infrequent gene flow between these populations as *FST* value was >0.33 except from Muskogee and MacDonald counties (*FST* > 0.25). Further pairwise comparison of 65 MDMV isolates from the United States and other countries (Bulgaria, Hungary, and Spain) also showed *FST* value >0.33, confirming infrequent gene flow except MDMV isolates from Hungary and Bulgaria with a *FST* value of 0.17.

Comparison of CP gene aa. The aa of the complete CP gene of 48 out of 65 MDMV isolates were selected and compared with six MDMV isolates that contained the 39 nt insertion in their CP and two MDMV isolates that did not contain the 39 nt insertion. The

CP gene was subdivided into three regions: N-terminal region (1–204 nt/1–68 aa), core region (205–716 nt/69–239 aa), and C-terminal region (717–915 nt/240–304 aa). Most of the aa substitutions were observed in the N-terminal region of the CP gene, which

(Continued on next page)

Fig. 2. (A) Maximum likelihood (ML) trees showing evolutionary relationships between the 65 MDMV isolates from Johnsongrass and 67 MDMV isolates (downloaded from GenBank) (accession and abbreviation listed in Supplementary Table S1). The tree was constructed using complete coat protein gene sequences in MEGA6 program using ML tree principal, GTR+G+I model, and 1,000 bootstrap replications. The GenBank accession number of each isolate is followed by name of the country. Three collapsed clusters are denoted as darker triangle in G2. Cluster one contained five isolates from Spain, second cluster contained 23 isolates from Spain, and third cluster contained 20 isolates from Hungary. **(B)** Comparison of amino acids (only shown from 1–100 aa) in the N-terminal region of coat protein (CP) genes among the representative MDMV isolates from Johnsongrass and maize and seven other (one from Argentina and five from Hungary) MDMV isolates that contained the 39 nucleotides insertion in the N-terminal of the CP gene and one isolate from Italy that does not contain 39 nt insertion. The first box (6–8 aa) indicates the DAG motif while the second box (55–68 aa) indicates the region of 39 nt insertion.

also includes the 39-nt insertion that translates into 13 aa (TTEKTGDTSTGK) (Fig. 2B). A total of 34 unique aa substitutions were observed only among the selected 48 MDMV isolates when compared with the global MDMV isolates from Argentina, Hungary, and Italy. Eighteen out of 34 substitutions, 18 substitutions occurred in more than one isolates while the remaining occurred once. Nine out of 34 unique aa substitutions among the MDMV isolates from Johnsongrass and maize were observed within the 13 aa (from 39-nt insertion) at positions 57, 59, 60, 61, 62, 63, 64, and 66 (Fig. 2B).

There were 23 and 12 single aa substitutions in the core region and the C-terminal regions of the 53 MDMV isolates, respectively (data not shown). However, all of these aa substitutions were observed

only once at different positions in MDMV isolates except at 165, 182, 220 aa in the core regions, and one at position 292 in the C-terminal regions that occurred in two or more MDMV isolates from Johnsongrass (data not shown).

Complete genome characterization of MDMV isolate from Johnsongrass. The complete genome of the Johnsongrass isolate MDMV-Bixby1 from Tulsa County, OK was amplified as nine overlapping fragments, and its 5' and 3' ends were verified by RACE assays. The complete genome sequence of the MDMV-Bixby1 isolate was determined to be 9,563 nt long (MK282423). Thus, MDMV-Bixby1 from Johnsongrass has the longest genome length among all currently sequenced MDMV isolates (Supplementary Table S2). It contained a 5'-UTR (146 nt), open reading frame (ORF)

Fig. 2. (Continued from previous page)

(9,165 nt) and 3'UTR (252 nt). The ORF of MDMV-Bixby1 isolate translated into a polyprotein of 3,054 aa. The length of the CP gene of the MDMV-Bixby1 isolate was 915 nt (8,397–9,311 nt) and also contained the 39-nt insertion. The CP gene was translated into 304 aa (2,750–3,053 aa). The 39-nt insertion in the N-terminal region of the CP gene (8,548–8,588) translated into 13 aa (2,803–2,815). None of the other eight complete MDMV genomes (Supplementary Table S2) contain the 39-nt insertion in their CP genes except one MDMV isolate from Hungary (FM883164) (Supplementary Table S2).

The sequence identity of the complete ORF of the MDMV-Bixby1 isolate with eight other MDMV isolates ranged from 86 to 90% nt and 94 to 97% aa levels (Supplementary Table S5). Sequence identities among the individual genes of these MDMV isolates showed that the P3 gene has the lowest sequence identities (86 to 89% nt and 91 to 94% aa) compared with the other genes (Supplementary Table S5). An MDMV isolate from Italy showed the highest sequence identity with MDMV-Bixby1 isolate in all the 10 encoded proteins (88 to 96% nt and 92 to 100% aa).

Phylogenetic analysis of the nine complete genome nucleotide sequences of MDMV isolates (Fig. 3) showed that MDMV-Bixby1 isolate from Johnsongrass was distantly related to other global MDMV isolates obtained from maize.

Selection pressure (dN/dS ratio) on individual genes among the 9 MDMV isolates showed that (dN) values were lower than (dS) values in all genes, indicating purifying selection because dN/dS ratio was <1 (Table 3). Strongest purifying selection was found for CI (0.02), while HC-Pro and NIa Pro also had second highest ratio

(0.03), indicating strong purifying selection, while 6KI have the highest ratio (0.98) also showing purifying selection.

The above results were further confirmed by DataMonkey analysis where HC Pro, Nib, and CI were identified to contain largest number of codons under negative selection by SLAC and FEL, indicating high selection pressure for these coding regions. 6KI and 6K2 had the lowest number of codons under negative selection compared with other genes. P1, HC-Pro, and CP contain the largest number of codons under positive selection compared with other genes based on MEME algorithm (Table 3). Overall, the number of positively selected codons was less compared with negatively selected codons. According to the neutrality tests by Tjima's D, Fu and Li's D&F, the overall values for all nine complete genomes of MDMV were negative, which indicates low frequency polymorphisms (data not shown).

Recombination analysis of complete genome. A total of 20 recombination events were detected when the complete genome sequences of MDMV-Bixby1 (in this study) and eight other MDMV isolates were used in the RDR4 v 4.69 software. However, seven recombination events were found to be significant and acceptable among various MDMV isolates because they were detected by at least five or more RDP-implemented programs with significant statistical support ($P < 0.05$) (Table 4). Only three of the seven significant recombination events involved MDMV-Bixby1 from Johnsongrass with potential MDMV isolates from Italy and Spain. These recombination events were observed in different parts of the genome, including the 5' end, 3' end, and also in the middle of the genome.

Table 1. Selection pressure analysis based on the coat protein gene among the MDMV isolates

Population	ENC ^a	dN ^b	dS ^b	dN/dS ^b	<i>FST</i> value	Number of negatively selected codons ^c				Number of positively selected codons ^c				Nucleotide diversity (π) ^d	Mean value for genetic distances (d)
						SLAC	MEME	BUSTED	FEL	SLAC	MEME	BUSTED	FEL		
G1 phylogroup	304	0.49	2.13	0.23	0.34	49	0	0	82	0	5	1	1	0.0856	0.194 ± 0.067
G2 phylogroup	304	0.18	5.61	0.03		148	0	0	182	0	3	1	0	0.1072	1.065 ± 0.366

^a ENC: Effective number of codons.

^b dN, dS, and the dN/dS ratio were determined by DnaSP6.

^c Number of negatively and positively selected codons, obtained using the SLAC, FEL, BUSTED, and MEME methods implemented in DataMonkey.

^d According to Nei (1987).

Table 2. Gene flow (*FST* analysis) between MDMV isolates based on coat protein gene sequences from different geographical locations

Country	State	Locations (counties)	Phylogroups	No. of isolates	<i>F_{st}</i>	Nucleotide diversity (π) ^a	Mean value for genetic distance (d)
USA	Oklahoma	Muskogee versus Osage	G1	21 versus 12	0.34	0.015	0.024 ± 0.002
		Muskogee versus Payne	G1	21 versus 10	0.54	0.016	0.014 ± 0.001
		Muskogee versus MacDonald's	G1	21 versus 7	0.25	0.018	0.022 ± 0.002
		Muskogee versus Tulsa	G1	21 versus 4	0.35	0.033	0.090 ± 0.006
		Osage versus Payne	G1	12 versus 10	0.74	0.014	0.013 ± 0.001
		Osage versus MacDonald's	G1	12 versus 7	0.39	0.015	0.021 ± 0.002
		Osage versus Tulsa	G1	12 versus 4	0.50	0.038	0.088 ± 0.006
		Payne versus MacDonald's	G1	10 versus 7	0.55	0.017	0.012 ± 0.002
		Payne versus Tulsa	G1	10 versus 4	0.68	0.043	0.075 ± 0.005
		Kay versus Muskogee	G1	10 versus 21	0.91	0.109	0.022 ± 0.002
		Kay versus Osage	G1	10 versus 12	0.93	0.121	0.021 ± 0.002
		Kay versus Tulsa	G1	10 versus 4	0.59	0.094	0.083 ± 0.005
		Kay versus Payne	G1	10 versus 10	0.94	0.125	0.012 ± 0.001
		Kay versus MacDonald's	G1	10 versus 7	0.91	0.126	0.020 ± 0.002
	Missouri	MacDonald's versus Tulsa	G1	7 versus 4	0.37	0.059	0.083 ± 0.005
Hungary		USA versus Hungary	G1 versus G2	65 versus 26	0.53	0.100	0.088 ± 0.004
Spain		USA versus Spain	G1 versus G2	65 versus 28	0.54	0.104	0.088 ± 0.005
Bulgaria		USA versus Bulgaria	G1 versus G2	65 versus 5	0.64	0.095	0.080 ± 0.005
		Hungary versus Spain	G2	26 versus 28	0.37	0.092	0.077 ± 0.005
		Hungary versus Bulgaria	G2	26 versus 5	0.17	0.067	0.066 ± 0.005
		Spain versus Bulgaria	G2	28 versus 5	0.41	0.085	0.072 ± 0.006

^a According to Nei (1987).

Event one involved genomic region from CI to 6K2 genes with the potential major parent Italy isolate and minor parent Bixby1 (Table 4). Event two involved partial 5'-UTR and partial P1 gene where Bixby1 was a major parent while Italy isolate was the minor parent. Similarly, event three involved 3'-UTR of Bixby1 and Spain isolates where Spain isolate was the major parent and Bixby1 was the minor parent. All these events were supported by at least six

RDP4-implemented programs with significant *P* values (Table 4). In addition, percent nt and aa identities of these regions of the genomes were higher among these isolates (Supplementary Table S5).

Discussion

The significance of Johnsongrass as a reservoir of MDMV cannot be ignored due to its presence near many commercial maize growing

Fig. 3. Maximum likelihood (ML) tree showing phylogenetic relationship between MDMV-Bixby1 isolate and eight other MDMV isolates downloaded from GenBank (Supplementary Table S2). The tree was constructed using complete genome nucleotide sequences in MEGA6 based on ML tree principal, TN model and 1,000 bootstrap replications. The complete genome sequence of sorghum mosaic virus (accession no. U57358) was used as an outgroup.

Table 3. Analysis of genetic parameters of different coding regions in the complete genomes of nine MDMV isolates

Genomic region	ENC ^a	dN ^b	dS ^b	dN/dS ^b	No. of negatively selected codons ^c			No. of positively selected codons ^c		
					SLAC	FEL	MEME	SLAC	FEL	MEME
P1	233	0.20	2.3	0.09	40	80	0	0	1	4
HC-Pro	460	0.08	2.73	0.03	127	258	0	0	0	4
P3	347	0.14	1.81	0.08	51	144	0	0	1	0
6K1	67	2.27	2.31	0.98	11	35	0	0	0	0
CI	638	0.04	2.5	0.02	188	388	0	0	0	1
6K2	53	0.10	2.08	0.05	7	23	0	0	0	0
NIa-VPg	189	0.13	2.8	0.05	43	98	0	0	0	0
NIa-Pro	242	0.06	2.3	0.03	67	117	0	0	0	1
NIb	521	0.09	2.5	0.04	121	273	0	0	0	0
CP	304	0.09	1.87	0.05	44	107	0	0	1	4

^a ENC: Effective number of codons.

^b dN, dS, and the dN/dS ratio were determined by DnaSP6.

^c Number of negatively and positively selected codons, obtained using the SLAC, FEL, and MEME methods implemented in DataMonkey.

areas in the United States. Thus, it is important to gain a better understanding of MDMV isolates from both Johnsongrass and maize, compare their genetic diversities with each other and their potential roles in the epidemiology of the disease. This is the first study to provide the complete genome of an MDMV isolates from Johnsongrass in the United States.

Although field symptoms of MDMV isolates were closely similar across fields assessed during the three growing seasons, the grouping of isolates based on the CP genes indicates that they clustered according to the geographical locations. These locations where 65 MDMV isolates were collected had a minimum distance of at least 47 to 246 miles from each other, which indicates that natural vector-mediated spread is unlikely between the different counties because MDMV is nonpersistently transmitted by its aphid vectors (Knoke et al. 1983).

Another important feature of this study was that all the 65 MDMV isolates from both Johnsongrass and maize contained 39-nt insertions in the CP genes which was previously reported in only six MDMV isolates (Supplementary Table S1). The presence of 39-nt insertions in the eight CP gene sequences that were obtained from pooled RNA from Johnsongrass in Ohio was reported (Stewart et al. 2014), but the sequences are not available in the GenBank database at the time of this analysis. It was therefore not possible to use them for comparison in this study. In addition, the CP gene of the two reported U.S. MDMV isolates (OH-1 and OH-2) (Supplementary Table S1) is 819 nt (Stewart et al. 2014), which is much shorter than the one reported from other countries (Gell et al. 2010; Tóbiás and Palkovics 2004; Tóbiás et al. 2007) and did not contain the 39-nt insertion. Based on the 39-nt insertion in the CP gene, all 65 isolates and six other global MDMV isolates (Supplementary Table S1) clustered together in G1 group, indicating that these isolates are unique from all other MDMV isolates without 39-nt insertion. MDMV isolates obtained in this work have high diversity in the N-terminal region of the CP compared with the core and C-terminal regions of the CP gene.

The N-terminal region of the CP was reported to be highly variable in length and sequence identity across the potyviral group of plant viruses (Shukla and Ward 1988). More importantly, both N- and C-terminal regions of the CP are known to be surface located in rod shaped virions. Notably, the N-terminal region of the CP is known to contain major antibody binding epitopes and is the most immunodominant region of the genome (Shukla and Ward 1988; Shukla et al. 1989). Similarly, most aa variation was observed in the N-terminal region of the 65 MDMV isolates from Johnsongrass and maize as compared with the core and C-terminus, which indicates that N-terminus is more informative compared with the other regions of the CP. However, biological characterization is needed to further compare host specificity among these MDMV isolates. In addition, MDMV isolates from Johnsongrass segregated based on their geographical locations (Fig. 2A), which provides variation in nature and could potentially infect maize by aphid transmission as confirmed in this work. Furthermore, our results showed that 11 isolates obtained from maize in Cherokee and Kay counties also contained the 39-nt insertion which supports the hypothesis that Johnsongrass acts as a reservoir of the virus for possible aphid-mediated transmission to maize. The symptoms produced by 11 MDMV maize isolates in the field were more severe than those induced by MDMV-Bixby1 from Johnsongrass in the greenhouse. A possible reason could

be different maize cultivars, stage of infection, and MDMV isolates (Fig. 2A). However, experimental evidence is needed in future studies to compare symptom severity between MDMV isolates from maize and Johnsongrass.

In the N-terminus region of the CP gene, the DAG motif (at position 6-9 aa) was mutated to DVG in all the 65 MDMV isolates except for two isolates (OS2 from Johnsongrass and CH1 from maize) collected from Osage and Cherokee counties, which have DVR and NVG, respectively. Similar mutations in the DAG motif were also observed in the CP of seven other MDMV isolates from the Hungary and Italy, while the Argentina isolate contained NVG instead of DAG (Fig. 2B). Similarly, DAG motif in the complete genomes of the MDMV-Bixby1 isolate and MDMV isolates from Hungary and Italy changed to DVG. Based on our aphid transmission experiment with MDMV-Bixby1 isolate, two out of four maize seedlings cv. Stellar showed typical MDMV symptoms at 8 weeks postinoculation (Fig. 1C) when inoculated with corn leaf aphid. Corn leaf aphid has been reported to be one of the most common vectors of MDMV (Nault and Bradley 1969). We observed that aphid transmission of Bixby1 isolate was not affected with the presence of DVG instead of DAG in the N-terminal region of CP gene. In addition, it has been shown that not all aphid-transmissible potyviruses contain the DAG motif and that aphid-transmissibility involves both CP and HC-Pro (Lopez-Moya et al. 1999).

Phylogenetic analysis, nucleotide diversity (π), pairwise comparison (F_{ST} value), and mean genetic distance (d) showed that MDMV isolates in G1 phylogroup are distantly related to isolates in G2 phylogroup (Tables 1 and 2; Fig. 2A). In both phylogroups, the dN/dS ratio was <1 (Table 1), which indicates a strong purifying selection in these populations and could restrict the variability of the MDMV isolates at the CP gene.

Selection pressure on the CP as calculated by SLAC and FEL (Table 1) showed a large number of negatively selected codons in both G1 and G2 phylogroups which indicated high evolutionary constraints on the CP gene. However, the number of negatively selected codons in G1 phylogroup was significantly lower than the G2 phylogroup (Table 1). One of the reasons for this difference could be the host, because in G2 all MDMV isolates were obtained from maize except one from Johnsongrass, while in G1, 53 out of 65 MDMV isolates were from Johnsongrass. From our results, it was also observed that 11 isolates collected from maize in Kay County during 2019 showed high nucleotide diversity and genetic distances compared with the MDMV isolates collected from Johnsongrass in other counties.

In this study, the MDMV-Bixby1 isolate showed low infectivity on maize cultivars when inoculated mechanically or by aphids at two-leaf stage but still retained the DVG motif, which shows the stability of the DVG instead of DAG even after infection of maize. A previous study showed that the stage of maize growth affected MDMV infectivity by mechanical inoculation, where lowest and highest infectivity of MDMV on maize was obtained at three- and five-leaf stages, respectively (Rosenkranz and Scott 1978). However, a future experiment shall be focused to inoculate maize seedling at five-leaf stage to obtain maximum infectivity. Similarly, the use of infectious clones of the MDMV-Bixby1 isolate will further determine the function of the 39-nt insertion in the CP gene and the effects on virus life cycle.

Table 4. Putative recombination events among the nine complete genomes of MDMV isolates detected by RDP4.92 software. Only three recombination events that involved MDMV-Bixby1 isolates are shown in this table which were detected by at least five of the seven suits implemented in the RDP4.92 with a good statistical support ($P \leq 0.05$) are shown.

Event no.	Putative recombinant	Putative parents		Location in the genome	Detected by ^a	P value ^b
		Major	Minor		R G B M C S T	
1	Italy isolate	Italy isolate	US-Bixby1 isolate	4,786–5,573	R - B M C S T	4.374×10^{-09}
2	US-Bixby1 isolate	US-Bixby1 isolate	Italy isolate	117–1,398	R G B M C S T	2.76×10^{-23}
3	Spain isolate	Spain isolate	US-Bixby1 isolate	9,547–9,572	R G B M C S -	8.11×10^{-16}

^a R = RDP, G = GENECONV, B = BootScan, M = MaxChi, C = Chimaera, S = SiScan, T = 3Seq.

^b P values are shown only for the algorithm indicated as a “bold” letter.

This study provides the first complete genome sequence of MDMV-Bixby1 isolate from Johnsongrass, which was never reported before and shows some distant evolutionary relationship (Fig. 3) with other MDMV complete genomes from the United States and other countries. Due to recombination events among the MDMV isolates, it is difficult to accurately determine diversity between different groups based on the complete genome analysis. To determine comprehensive evolutionary relationships among the MDMV isolates, more complete genome sequences of MDMV from Johnsongrass and cultivated crops such as maize and sorghum are required in the future.

In conclusion, the results obtained in this work will be useful for managing MDMV in cultivated crops such as maize and sorghum. Particularly, the prevalence of Johnsongrass grown naturally in these states will be of concern because of its potential as a virus source for infection of maize crops. In future management strategies, it is vital to minimize the presence of Johnsongrass near maize or sorghum crops so that horizontal transmission of the virus can be avoided, and ultimately minimize crop losses due to infections from MDMV.

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