HHS Public Access

Author manuscript

Virus Res. Author manuscript; available in PMC 2020 May 01.

Published in final edited form as:

Virus Res. 2019 May ; 265: 80–87. doi:10.1016/j.virusres.2019.02.008.

Beta vulgaris mitovirus 1 in diverse cultivars of beet and chard

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Abstract

Recent results indicate that mitoviruses, which replicate persistently in host mitochondria, are not restricted to fungi, but instead are found also in plants. Beta vulgaris mitovirus 1 (BevuMV1) is an example first discovered in sugar beet cultivars. For the current study, complete coding sequences of 42 BevuMV1 strains were newly determined, derived from not only sugar beet but also fodder beet, table beet, and Swiss chard cultivars of *Beta vulgaris*, as well as wild sea beet. BevuMV1 is thus a common phytobiome component of this valuable crop species. Most of the new BevuMV1 sequences originated from RNA extracted from *B. vulgaris* seed clusters, consistent with vertical transmission of this virus. Results suggest that BevuMV1 entered the *B. vulgaris* lineage prior to human cultivation and also provides a marker for tracing the maternal ancestry of *B. vulgaris* cultivars. Especially notable is the monophyletic relationship and limited sequence divergence among BevuMV1 strains from cultivars that are thought or shown to share the “Owen” trait for cytoplasmic male sterility, which is transmitted by maternal mitochondria and has been broadly established in commercial breeding lines of *B. vulgaris* since the mid-20th century.

Keywords

Beta vulgaris; cytoplasmic male sterility; mitochondrion; *Mitovirus*; *Narnaviridae*; plant virus

1. Introduction

Mitoviruses (genus *Mitovirus*, family *Narnaviridae*) have small RNA genomes and, in most or all cases, are thought to replicate persistently in the mitochondria of their host cells (Hillman and Cai, 2013; Nibert, 2017; Polashock and Hillman, 1994; Rogers et al., 1987). They had been earlier reported to infect only fungi (Hillman and Cai, 2013; Nibert, 2017), but recent evidence indicates that they also infect a wide range of land plants (Goh et al., 2018; Nerva et al., 2019; Nibert et al., 2018). Reported sequences to date suggest genome lengths of 2.7 to 3.0 kb for plant mitoviruses, with each genome encompassing a single long open reading frame (ORF) that encodes a deduced protein of 760 to 821 aa in length (Goh et al., 2018; Nerva et al., 2019; Nibert et al., 2018) and with conserved motifs reflective of a

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viral RNA-dependent RNA polymerase (RdRp) (Bruenn, 2003). Plant mitoviruses, like fungal ones, are thought not to form virions and to persist instead as replicating, intramitochondrial ribonucleoprotein complexes, which are transmitted to daughter cells during cell division, as well as vertically through presence in seeds in the case of plant mitoviruses (Nerva et al., 2019; Nibert et al., 2018). Possible effects of plant mitoviruses on plant health, etc., remain to be investigated. Transmission of an ancestral mitovirus from plant to fungus or *vice versa* appears possible based on phylogenetic analyses and other considerations (Bruenn et al., 2015; Marienfeld et al., 1997; Nibert, 2017; Nibert et al., 2018; Roossinck, 2018; Shackleton and Holmes, 2008).

Recent evidence indicates that several sugar beet cultivars (i.e., members of the sugar beet crop type of flowering plant (core eudicot) *Beta vulgaris* L. subsp. *vulgaris* (Lange et al., 1999)) are persistently infected with a plant mitovirus that we have named Beta vulgaris mitovirus 1 (BevuMV1) (Nibert et al., 2018). Sugar beet is an important crop, grown on an industrial scale for production of table sugar (sucrose) in many countries worldwide (Draycott, 2006; Ford Lloyd, 1995; McGrath and Panella, 2018). Interestingly, other familiar vegetables represent different crop types of the same subspecies, namely table beet and Swiss chard, as well as leaf beet, and yet another crop type of the same subspecies, fodder beet, is grown for livestock feed (Ford Lloyd, 1995; Francis, 2006; Lange et al., 1999). All 5 of these crop types of *B. vulgaris* L. subsp. *vulgaris* are thought to have been ultimately derived from Mediterranean wild sea beet, which is designated as a distinct subspecies, *B. vulgaris* L. subsp. *maritima* (L.) Arcang. (Francis, 2006; Letschert et al., 1994; McGrath and Panella, 2018).

Given the array of different cultivated and wild plants belonging to species *B. vulgaris*, as well as reported evidence to date for the presence of BevuMV1 only in sugar beet, we undertook this study to survey more widely for the presence of BevuMV1 in other crop types of *B. vulgaris*. New results reveal the broad distribution of BevuMV1, not only in sugar beet, fodder beet, table beet, and Swiss chard cultivars but also in wild sea beets. BevuMV1 thus appears likely to have entered the *B. vulgaris* lineage prior to human cultivation. Further evidence for presence in seeds as well as predominantly maternal transmission/inheritance of BevuMV1 has also been obtained, suggesting its utility as a marker for tracing the maternal ancestry of *B. vulgaris* cultivars.

2. Materials and methods

2.1. Database searching and transcript assembly

Sequence Read Archive (SRA) transcriptome data for *B. vulgaris* analyzed for our original report on plant mitoviruses (Nibert et al., 2018) were registered as BioProjects at NCBI most recently in late 2015. SRA transcriptome data for *B. vulgaris* registered as BioProjects since that time (PRJNA328353, PRJNA343692, PRJNA358634, and PRJEB21674) were subjected to Discontiguous MegaBLAST searches for BevuMV1-matching reads for the current report (Table 1). In addition, SRA transcriptome data from BioProjects PRJNA73561 and PRJNA41497 were reanalyzed in the same manner in an effort to extend BevuMV1 sequences from 3 sugar beet cultivars previously reported to be BevuMV1-positive, but for which coding-complete BevuMV1 sequences had not yet been assembled.

Methods for assembling transcript contigs for BevuMV1 from SRA data (Table S1) were the same as used previously (Nibert et al., 2018) except that terminal residues represented by single reads were trimmed only if they diverged from the overall consensus. Program CAP3 was used for contig assembly as implemented at http://doua.prabi.fr/software/cap3, http://biosrv.cab.unina.it/webcap3/, or https://galaxy.pasteur.fr/. See Table 1 for GenBank accession numbers of the reported sequences. The complete set of sequence reads that mapped to each final contig were analyzed using the "Map Reads to Reference" tool in CLC Genomics Workbench 7 to determine the coverage values at each nt position in the contig, which were in turn used to calculate the mean coverage value and range (Table S2). In addition, the number of matching sequence reads for each final contig and the total sequence reads in the original SRA data set(s) were used to calculate the fraction of total (Table S2).

2.2. Sanger sequencing of BevuMV1 strains

Seeds of fodder beet, sugar beet, table beet, and Swiss chard cultivars were purchased from retail suppliers at local stores or online (Tables 2 and S3). Plantlets of table beet Merlin were purchased at a local store. The hybrid status of each cultivar (OP or F_1) was specified by the retail supplier and checked for consistency with other online references. Plantlets of table beet Taunus and Swiss chard Red Magic were grown from seeds in a home garden (M.L.N.).

Seed clusters (0.5 g, ~20 count) were ground into a fine powder at room temperature using a coffee grinder. Plantlets were frozen in liquid nitrogen and then ground into a fine powder using a mortar and pestle. In each case, the ground material was then resuspended in 0.5 mL TRIzol (Invitrogen). RNA extraction, cDNA synthesis, and PCR amplification were performed largely as described previously (Nibert et al., 2018). The chief exception was that random hexamers were sometimes used instead of BevuMV1-specific primers for cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen). When random hexamers were used for cDNA synthesis, different aliquots of that reaction were then used in separate PCR reactions with EconoTaq PLUS (Lucigen) to amplify 4 different, overlapping regions of the BevuMV1 genome for Sanger sequencing, as obtained with 4 different BevuMV1-specific primer pairs. For determining terminal sequences of BevuMV1-VDH66156, rapid amplification of cDNA ends (RACE) was performed as described previously (Depierreux et al., 2016).

For sequencing, PCR amplicons were visualized by agarose gel electrophoresis and then purified from excised gel fragments as described previously (Nibert et al., 2018). Sanger sequencing was performed at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Reads were obtained from each PCR amplicon using one primer for each strand. In this manner, each nt position in the respective BevuMV1 sequence was read from both strands, except for a short stretch (≤28 nt) at each end of the overall sequence that was read from only one strand. Terminal primers bracketing the reported sequence region of each Sanger-sequenced BevuMV1 strain were GTGAGTGATCACTCTCCGTG (forward) and TTTGGTAGGCCATCAACGTAC (reverse), and these primer-fixed residues were excluded from the reported sequences. See Table 2 for GenBank accession numbers of the reported sequences.

2.3. Sequence alignments and phylogenetic analyses

The BevuMV1 RNA sequences were first trimmed to shared termini as dictated by the shortest sequences (trimmed length, 2581 aligned nt positions); all of these trimmed sequences still fully encompassed the single long ORF for the viral RdRp. Pairwise alignments were then performed using EMBOSS Needleall as implemented at http://www.bioinformatics.nl/cgi-bin/emboss/needleall. Pairwise alignments of the deduced BevuMV1 RdRp sequences were also performed using EMBOSS Needleall. Multiple sequence alignments of the BevuMV1 RNA and RdRp sequences were performed using MAFFT (L-INS-i) v7 as implemented at https://mafft.cbrc.jp/alignment/server/ (Katoh and Standley, 2013).

Multiple sequence alignments of the trimmed BevuMV1 RNA sequences described in the preceding section were used for phylogenetic analyses using IQ-TREE as implemented at https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html (Trifinopoulos et al., 2016). The “Find best and apply” option was used for identifying the best-fit substitution model according to Bayesian information criterion (BIC) scores with ModelFinder (Kalyaanamoorthy et al., 2017) (identified model for Fig. 2, TPM2+R2). Branch support values were determined using the ultrafast bootstrap method UFBoot (Minh et al., 2013) with 1000 replicates.

2.4. Owen CMS mitochondrial markers

Based on findings by Satoh et al. (2004), we used the mitochondrial genome sequences of sugar beet cultivars TK81-O (GenBank BA000009.3; nonsterile, wild type) and TK81-MS (GenBank BA000024.1; Owen CMS) to identify sequences that are unique to Owen CMS mitochondria within transcripts from genes *Satp6*, *Scox2-2*, and *Sorf324* (the initial *S* in each name designates the gene form/allele present in *s*terile Owen CMS mitochondria; as used below, *N* designates the gene form/allele present in *n*onsterile non-Owen mitochondria). For this purpose, we settled on residues 301–600 of the TK81-MS *Satp6* coding region, the 3′ -terminal 300 nt of the TK81-MS *Scox2-2* coding region, and the 3′-terminal 300 nt of the TK81-MS *Sorf324* coding region. We also chose the 3′-terminal 300 nt of the *N Scox1-1* coding region as a control shared by both TK81-O and TK81-MS. We then used these 4 marker sequences as queries for Discontiguous MegaBLAST searches of the SRA transcriptome libraries for the 4 SUAS sugar beet cultivars (BioProject PRJNA358634), followed by transcript contig assembly from the identified hits for SUAS-R1, SUAS-S1, and SUAS-S2; SUAS-R2 was found to be negative for the Owen-specific markers.

A PCR assay for the Owen CMS form of mitochondrial gene *atp6* (i.e., *Satp6*) was performed for sugar beet cultivars BCHS and SSC and table beet cultivar Burpee’s Golden, as well as for negative and positive control cultivars (table beets Ruby Queen and Taunus, respectively). The amplification employed a forward primer (GGCGTATCTCATGTGTATCCC) targeting the upstream region unique to Owen *atp6* and a reverse primer (TTCGTTTACCAGGTTCAGCAC) targeting the downstream region conserved between Owen and wild-type *atp6*. Amplicon purification and confirmatory

Sanger sequencing were then performed as described for BevuMV1-derived amplicons (see GenBank accession MK423956 for these *Satp6* sequences).

3. Results

3.1. New BevuMV1 sequences from sugar beet transcriptomes

We reexamined the SRA database at NCBI in an effort to assemble coding-complete BevuMV1 sequences from transcriptome (RNA-Seq) data from other sugar beet cultivars of *B. vulgaris* subsp. *vulgaris*, in addition to the 4 that we have previously reported (Nibert et al., 2018). By allowing for a single short gap in each assembled sequence, we were able to extend the BevuMV1 sequences from 3 additional sugar beet cultivars, previously reported to be BevuMV1-positive (Nibert et al., 2018), to encompass the complete coding region of the virus. We were also able to assemble sequences encompassing the complete coding region of BevuMV1 from 6 other sugar beet cultivars by using SRA data that had been released since the cut-off date for our previous study. These new efforts bring the total number of coding-complete BevuMV1 sequences assembled from transcriptome data of distinct sugar beet cultivars to 13 (Table 1). Features and comparisons of these new sequences are described below.

3.2. New BevuMV1 sequences from wild sea beet transcriptomes

While examining the SRA database at NCBI for the efforts described in the preceding section, we discovered SRA data from two recent transcriptome studies of wild sea beet *B. vulgaris* subsp. *maritima*. One of these libraries (ERX2099280 from BioProject PRJEB21674) is negative for BevuMV1-matching reads, but the other (SRX1924131 from BioProject PRJNA328353; Capistrano-Gossmann et al., 2017) is strongly positive. In fact, we were able to assemble two related but distinct coding-complete BevuMV1 sequences from separate subsets of RNA-seq runs (representing separate plants) deposited under SRX1924131 (one from SRR3823656–SRR3823657, and the other from SRR3823689–SRR3823692; Table 1). These two sequences are designated BevuMV1-WB-KalundborgA and BevuMV1-WB-KalundborgB, and exhibit single-nucleotide polymorphisms (SNPs; no indels) at 53 out of a total of 2769 shared nt positions (98.1% identity; 9 deduced aa substitutions in the encoded RdRps). Other features and comparisons of these sequences are described below.

3.3. New BevuMV1 sequences from open-pollinated fodder beet, sugar beet, table beet, and Swiss chard cultivars

We were interested to address whether other crop types of *B. vulgaris* subsp. *vulgaris* might also carry BevuMV1. We therefore purchased seeds of reportedly open-pollinated (OP) cultivars of fodder beet, leaf beet, sugar beet, table beet, and Swiss chard from retail suppliers. After extracting RNA from the seed clusters, we performed cDNA synthesis followed by PCR with BevuMV1-specific primers designed from available sequences. Sanger sequencing was then performed on appropriate amplicons. In this manner, we were able to assemble coding-complete BevuMV1 sequences from 1 OP fodder beet, 2 OP sugar beet, 4 OP table beet, and 2 OP Swiss chard cultivars (Table 2). A number of other BevuMV1-positive OP cultivars of fodder beet, table beet, and Swiss chard have also been

partially sequenced as of the time of this writing; in other words, those shown in Table 2 are not the only BevuMV1-positive OP cultivars of these crop types. Features and comparisons of these new sequences are described below.

3.4. New BevuMV1 sequences from F_1-hybrid sugar beet, table beet, and Swiss chard cultivars

Modern sugar beet cultivars that are grown at industrial scales for sugar production are marketed for sale as F_1-hybrid seeds, in which the so-called Owen CMS trait (Owen, 1945) and other genetic attributes allow remarkable consistency and control in breeding, production, and sales (Bosemark, 2006; Ford Lloyd, 1995; McGrath and Panella, 2018; Mikami et al., 2011). This strategy has also been transferred to other crop types of *B. vulgaris*, for table beet most notably via a breeding program at the University of Wisconsin, in which the Owen CMS trait from sugar beet has been combined with the table beet nuclear genome in breeding lines (Goldman, 1996; Goldman and Navazio, 2003). As a result, seeds and plants of F_1-hybrid fodder beet, table beet, and Swiss chard cultivars are now widely available for purchase. For the current study, we purchased seeds or plantlets of reportedly F_1-hybrid table beet and Swiss chard cultivars from retail suppliers and then subjected those to analysis as described for OP cultivars in the preceding section. In this manner, we were able to assemble coding-complete BevuMV1 sequences from 19 F_1-hybrid table beet and 3 F_1-hybrid Swiss chard cultivars (Table 2).

F_1-hybrid table beet cultivars Alto and Taunus were found to be unique in exhibiting an ~1:1 mixture of A and G residues at one position (the same position in both). Our method for obtaining BevuMV1 sequences from seeds was first to extract RNA from multiple seed clusters in order to obtain sufficient amounts of RNA for robust RT–PCR amplification and sequencing. As a result, the ~1:1 mixture of A and G residues at one position in Alto and Taunus might be explained in two ways. (1) In ~50% of seed clusters, the virus has A at this position, whereas in the other ~50% of seed clusters, the virus has G. By combining multiple seed clusters before extraction, we ended up with an ~1:1 mixture of A and G in the results. (2) An ~1:1 mixture of viruses is already present in each seed cluster, ~50% with A and ~50% with G at this position, which again yielded an ~1:1 mixture of A and G in the results. We reasoned that we should be able to distinguish between these possibilities by examining individual seed clusters; however, to obtain enough RNA for robust amplification, we instead grew individual seed clusters into plantlets, from which RNA was then extracted one plantlet at a time. The results revealed an ~1:1 mixture of A and G residues again in this position, from plantlets arising from two different seed clusters, suggesting that the mixture of viruses is already present in each seed cluster.

We have previously reported the coding-complete sequence of BevuMV1-VDH66156, from F_1-hybrid sugar beet cultivar VDH66156 (GenBank MG721540.1; Nibert et al., 2018). For the current study, we attempted to determine the extreme terminal sequences of this BevuMV1 reference strain by RACE. For unknown reasons, we failed to obtain a RACE amplicon for the 3′ plus-strand terminus of BevuMV1-VDH66156. We succeeded for the 5′ plus-strand terminus, however, and Sanger sequencing results from the RACE amplicon

extended the sequence of this strain by 57 nt, for a new overall genome length of 2750 nt (GenBank accession MG721540.2).

3.5. Basic features of BevuMV1 sequences

The 31 new coding-complete BevuMV1 sequences that we obtained by Sanger sequencing range in length from 2693 to 2696 nt, as delimited by the terminal primers used for PCR amplification. The 11 new or revised coding-complete BevuMV1 sequences assembled from public transcriptome data range in length from 2643 to 2817 nt, as likely explained by differential coverage of the BevuMV1 genome termini in the different SRA libraries. In fact, the extreme 5′-terminal sequences of BevuMV1-VDH66156 that we newly determined by RACE allowed us to compare those with ones from the several other BevuMV1 strains from sugar beet cultivars whose transcriptome-derived sequences extend into this extreme 5′-terminal region. Results of the alignment reveal that to the extent of coverage provided by the transcriptome data, the sequence of this region is devoid of indels and almost entirely conserved (Fig. 1). Only the 5′-terminal A residue observed by RACE in BevuMV1-VDH66156 is not represented in any of the transcriptome-derived sequences to date.

Two of the new BevuMV1 sequences assembled from transcriptome data, those from sugar beet cultivars SD13289 (2662 nt) and KWS230-DH1440 (2643 nt), are more substantially truncated at their 5′ ends than those from other cultivars, and we therefore eliminated those two sequences for the preceding and following comparisons. The other new and previously reported BevuMV1 sequences shown in Tables 1 and 2 align across a shared region of 2696 nt positions, corresponding to the region subjected to Sanger sequencing for some cultivars. Notably, no indels are present in the central long protein-coding region or the 3′ nontranslated region (NTR) of any of these aligned sequences. In the 5′ NTR, however, three indels are present in a few of the aligned sequences, all located within a 57-nt region near the 5′ terminus: the two BevuMV1 sequences from wild sea beet have a unique 5-nt deletion at the end of this region; the BevuMV1 sequence from sugar beet cultivars KWS2320 and SUAS-R2 plus table beet cultivar Lutz Green Leaf share a unique 1-nt insertion at the start of this region; and the BevuMV1 sequence from table beet cultivars Crosby’s Egyptian and Ruby Queen share a unique 2-nt insertion at the start of this region (Fig. S1). The significance of these indels remains to be determined.

All of the BevuMV1 sequences in Tables 1 and 2 encompass a single long ORF utilizing the universal genetic code (code 1) common to plant mitochondria, chloroplasts, and cytosol (Jukes and Osawa, 1990), and encoding a 793-aa protein with regions of strongest similarity to mitovirus RdRps, especially plant mitovirus RdRps. Upon alignment of the BevuMV1 RdRp sequences, no indels are observed, and substitutions are found at only 48 of the 793 aa positions (Fig. S2).

3.6. Pairwise comparisons and phylogenetic analyses of BevuMV1 sequences

We used EMBOSS Needleall to perform pairwise comparisons with all of the new and previously reported coding-complete BevuMV1 sequences. Pairwise identities were found to be ≥92.9% among the BevuMV1 RNA sequences and ≥95.3% among the deduced BevuMV1 RdRp sequences (Fig. S3). Excluding the most divergent sequences, those from

wild sea beet, pairwise identities were found to be ≥95.3% among the BevuMV1 RNA sequences and ≥97.5% among the deduced BevuMV1 RdRp sequences (Fig. S3).

The RNA sequences of viruses in Tables 1 and 2 were subjected to multiple sequence alignment using MAFFT followed by maximum likelihood phylogenetic analysis using IQ-TREE (see Materials and methods for additional details). A midpoint-rooted phylogram of the results is shown in Fig. 2. These findings point to three subclades of viruses in this analysis: subclade I containing solely wild beet viruses BevuMV1-WB-KalundborgA and BevuMV1-WB-KalundborgB, subclade IIa containing BevuMV1-SB-BS02 and two other viruses, and subclade IIb containing all other viruses. A mixture of fodder beet and sugar beet viruses is present in subclade IIa, and a mixture of sugar beet, table beet, and Swiss chard viruses is present in subclade IIb. A compact cluster of viruses including all of those from reportedly F_1-hybrid cultivars with Owen CMS mitochondria (red in Fig. 2) is notably located at the distal end of subclade IIb. Additional details and significance are discussed below.

3.7. Owen CMS mitochondrial markers in cultivars not reported to be F_1 hybrids

In Fig. 2, the BevuMV1 results for 3 of the 4 SUAS sugar beet cultivars, the reportedly OP sugar beet cultivars BCHS and SSC, and the reportedly OP table beet cultivar Burpee's Golden (orange labels) are potentially surprising in that all map to the same compact cluster of viruses as those from F_1-hybrid *B. vulgaris* cultivars. The nature of the SUAS sugar beet cultivars is not specified in the metadata for BioProject PRJNA358634; however, the results for the SUAS viruses in Fig. 2 suggest that SUAS-R1, SUAS-S1, and SUAS-S2 are likely F_1 hybrids with Owen CMS mitochondria, whereas SUAS-R2 is likely not. To address this suggestion directly, we used the SRA transcriptome data from PRJNA358634 to search for sequence regions unique to Owen CMS mitochondria from mitochondrial genes *Satp6*, *Scox2*-2, and *Sorf324* (Satoh *et al.,* 2004) (see Materials and methods for more details). The results revealed that SUAS-R1, SUAS-S1, and SUAS-S2 are positive for these Owen CMS markers whereas SUAS-R2 is not (Table 3). We therefore conclude that sugar beet cultivars SUAS-R1, SUAS-S1, and SUAS-S2 carry Owen CMS mitochondria, explaining why their viruses fall within the Owen-linked cluster in Fig. 2.

And how about the reportedly OP sugar beet cultivars BCHS and SSC and reportedly OP table beet cultivar Burpee's Golden? For these, since we had seeds available for testing, we devised a PCR assay to screen for presence of the Owen CMS form of mitochondrial gene *atp6* (i.e., *Satp6*), which we then confirmed by amplicon sequencing. The results indicate that these 3 reportedly OP cultivars indeed also carry Owen CMS mitochondria (Fig. 3 and GenBank accession MK423956 for *Satp6* sequences). A possible explanation for this finding is that these cultivars, assuming that they are indeed OP, carry at least one of the dominant nuclear restorer-of-fertility alleles for Owen CMS (Goldman, 1996; McGrath and Panella, 2018; Mikami et al., 2011; Owen, 1945), which allows them to produce fertile pollen for maintenance by open pollination.

4. Discussion

The phytobiome concept has emerged as an important one for the future of crop management (Bean, 2017). Viruses are common components of the phytobiome, including persistent viruses that are not readily identifiable as pathogens but could be expected to have at least some demonstrable effects, either negative or positive (Roossinck, 2018), on the plants that they infect. For a crop species such as *B. vulgaris*, even minor effects of persistent viruses on the rate of plant growth, tolerance of environmental stressors, etc., could have major economic consequences. In this study, we showed that a persistently infecting plant mitovirus, BevuMV1, is prevalent among diverse crop types and cultivars of *B. vulgaris* and therefore represents a common phytobiome component of this valuable crop species, worthy of further scrutiny.

Altogether in this study, we determined coding-complete BevuMV1 sequences from 40 cultivars of *B. vulgaris* subsp. *vulgaris* as well as from wild seed beet *B. vulgaris* subsp. *maritima*. In fact, as noted in Table 2 but not described above, we determined coding-complete BevuMV1 sequences two times each from 7 of the 40 cultivars, using seeds obtained from two different suppliers. For each of the twice-sequenced cultivars, the BevuMV1 sequences obtained from seeds from the two suppliers matched 100%, demonstrating the reproducibility of our sequence determinations.

The high levels of nt and aa sequence similarity observed in the pairwise comparisons (nt, ≥92.9%; aa, ≥95.3%; see Fig. S3) suggest that it is reasonable to identify all of these virus strains as members of the same species, previously suggested to be named “Beta vulgaris mitovirus 1” (Nibert et al., 2018). On the other hand, the virus strains from wild sea beet are clearly distinguished as belonging to a separate subclade by phylogenetic analyses (see Fig. 2, subclade I). At present, nonetheless, considering the high levels of sequence similarity, we consider it appropriate to assign all of these virus strains to the same species.

The mixtures of *B. vulgaris* crop types from which the viruses in subclades IIa and IIb derive may seem surprising at first but is consistent with the frequent crossbreeding between crop types that is known to have occurred during the historical search for desired properties among these vegetables (Ford Lloyd, 1995). For example, the development of sugar beet as a new crop type of *B. vulgaris*, in late-18th century Europe, is thought to have involved crosses between fodder beet and Swiss chard cultivars (Fischer, 1989; Ford Lloyd, 1995; McGrath and Panella, 2018).

The finding of BevuMV1 strains in wild sea beet is consistent with a conclusion that this virus entered the *B. vulgaris* lineage prior to cultivation by humans starting several thousand years ago (Ford Lloyd, 1999; Francis, 2006; Goldman and Navazio, 2003; McGrath and Panella, 2018), and perhaps much earlier. BevuMV1 sequences from additional wild sea beet populations may be useful for addressing whether *B. vulgaris* subsp. *maritima* was indeed the source of BevuMV1 ancestral to all of the strains of this virus now present in *B. vulgaris* subsp. *vulgaris*. *Beta macrocarpa* Guss. is a distinct species related to *B. vulgaris* (Letschert et al, 1994; Lange et al., 1999; Kadereit et al., 2006). SRA data from two *B. macrocarpa* transcriptome studies are available at NCBI (BioProjects PRJNA229835 and

PRJNA394607) but are negative for BevuMV1-matching reads. De novo generation of shotgun transcriptome assemblies from these SRA libraries also failed to identify more distantly related mitoviruses. Data from other *B. macrocarpa* isolates, other *Beta* species, and other species in subfamily *Betoideae* might be useful to shed light on the deeper ancestry of BevuMV1.

One especially notable element of the current results is the compact monophyletic relationship among BevuMV1 strains from sugar beet, table beet, and Swiss chard cultivars that are thought or shown to contain Owen CMS mitochondria (red and orange labels in Fig. 2). CMS is a maternally transmitted, mitochondrial trait found in many flowering plants (Chen et al., 2017), and although the molecular mechanism determining the Owen CMS trait in *B. vulgaris* subsp. *vulgaris* seems not to be wholly understood (Mikami et al., 2011), it is best known to be associated with an extended coding region for mitochondrial core gene *atp6* (Onodera et al., 1999; Satoh et al., 2004; Yamamoto et al., 2005). The Owen CMS trait was originally identified in sugar beet cultivar US1 (Owen, 1945), but since has been crossed into table beet cultivars via a breeding program at the University of Wisconsin (Goldman, 1996; Goldman and Navazio, 2003). As a result of the latter program and licensing of those breeding lines by commercial breeders, most or all F_1-hybrid table beet cultivars currently available for retail purchase are thought to have a maternal lineage that traces back to cultivar US1 (Goldman and Navazio, 2003). Indeed, the sequencing results for BevuMV1 strains from F1-hybrid table beet cultivars in this study are consistent with that conclusion, in that all are monophyletic (Fig. 2), and share a high degree of sequence identity (Fig. S3), with the two known F1-hybrid sugar beet cultivars for which BevuMV1 sequences have been reported to date (SD13829 and VDH66156). As evident in Fig. 2, the viruses from 3 F_1-hybrid Swiss chard cultivars are also members of this monophyletic cluster. This last finding indicates that the Owen trait has been additionally crossed into Swiss chard cultivars, even though that fact appears to be less well documented in the literature, probably for proprietary reasons.

Limited sequence divergence among the BevuMV1 strains from F_1-hybrid table beet and Swiss chard cultivars is further highlighted in Fig. 4, where it is seen that SNPs at only 7 nt positions distinguish these virus strains. Moreover, these nt substitutions appear to have accumulated in a stepwise manner, with each step shown in Fig. 4 characterized by only 1 or 2 new substitutions and probably reflecting the progressive breeding heritage of these cultivars.

With few exceptions, paternal mitochondria in the pollen-associated cells of flowering plants are efficiently excluded from fertilized ova (Mogensen, 1996). Thus, in almost all cases, mitochondria within the seed-associated embryos of flowering plants are thought to be exclusively maternal in origin. The compact cluster of BevuMV1 strains with Owen CMS mitochondria identified in this study indeed supports this expectation, in that no evidence for paternally introduced BevuMV1 strains from outside this cluster has been found among the F_1-hybrid cultivars examined to date. These findings are therefore consistent with a proposal that the mitoviruses of flowering plants have been transmitted largely by vertical descent through the maternal mitochondria in seed-associated embryos over the course of flowering plant evolution, with occasional loss from some flowering plant lineages and possibly

horizontal gain by uncommon means in some others (Nibert et al., 2018). A corollary expectation is that the mitoviruses of flowering plants are unlikely to undergo efficient transmission by pollen, either vertically or horizontally, unlike the case for some other plant viruses (Jones, 2018). Replication of plant mitoviruses in mitochondria remains to be established directly by biochemical means, but the evidence in this report for predominantly maternal transmission/inheritance of BevuMV1 is consistent with that conclusion. Evidence very recently reported by Nerva et al. (2019), for a novel plant mitovirus in crop plant *Chenopodium quinoa*, also strongly supports the mitochondrial localization of that virus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Irwin L. Goldman, University of Wisconsin–Madison, for his kind gift of Pacemaker 3 seeds. We also thank Dr. Goldman and Dr. Karen K. Fugate, USDA–ARS Fargo, for very helpful discussions and comments on the manuscript. M.V. was supported in part by NIH Grant T32 GM007598 to the doctoral program in Molecules, Cells, and Organisms at Harvard University. A.M. was supported in part by NIH Grant T32 AI007245 to the doctoral program in Virology at Harvard University. W.G. was supported by the Microbial Sciences Initiative at Harvard University.

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Highlights

- Previous recent results indicate that mitoviruses infect plants as well as fungi
- Here we report coding sequences for 42 further strains of Beta vulgaris mitovirus 1
- The strains derive from diverse crop types of *B. vulgaris* as well as wild sea beet
- Strains from cultivars with “Owen” mitochondria form a compact monophyletic cluster

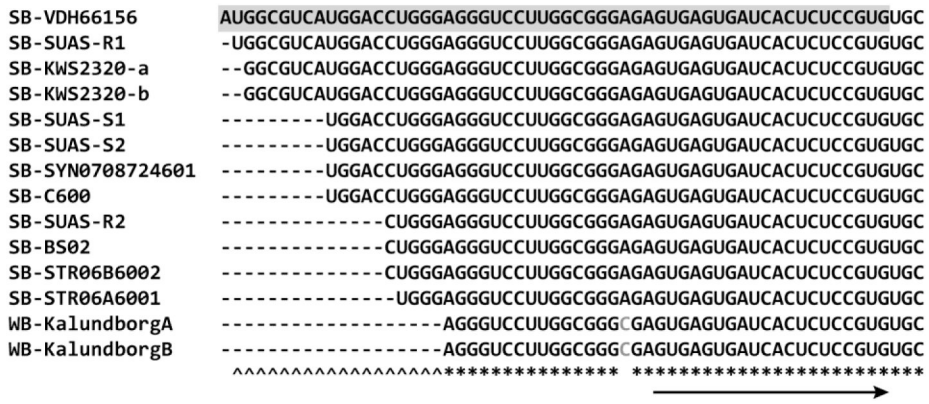

Fig. 1.

Extreme 5′-terminal sequences of BevuMV1 strains. Gray shading highlights the complete 5′-terminal plus-strand sequence newly determined by RACE for BevuMV1-SB-VDH66156. The other sequences shown in the MAFFT alignment are those for other BevuMV1 strains whose sequences as determined to date from public transcriptome data extend into this 5′-terminal region. Asterisks, conserved residues represented in all 14 sequences; carets, conserved positions represented in at least two of the sequences; gray text, one SNP found in this region. The arrow represents the position of the forward primer flanking the 5′ end of the sequences determined for other BevuMV1 strains by Sanger sequencing in this report.

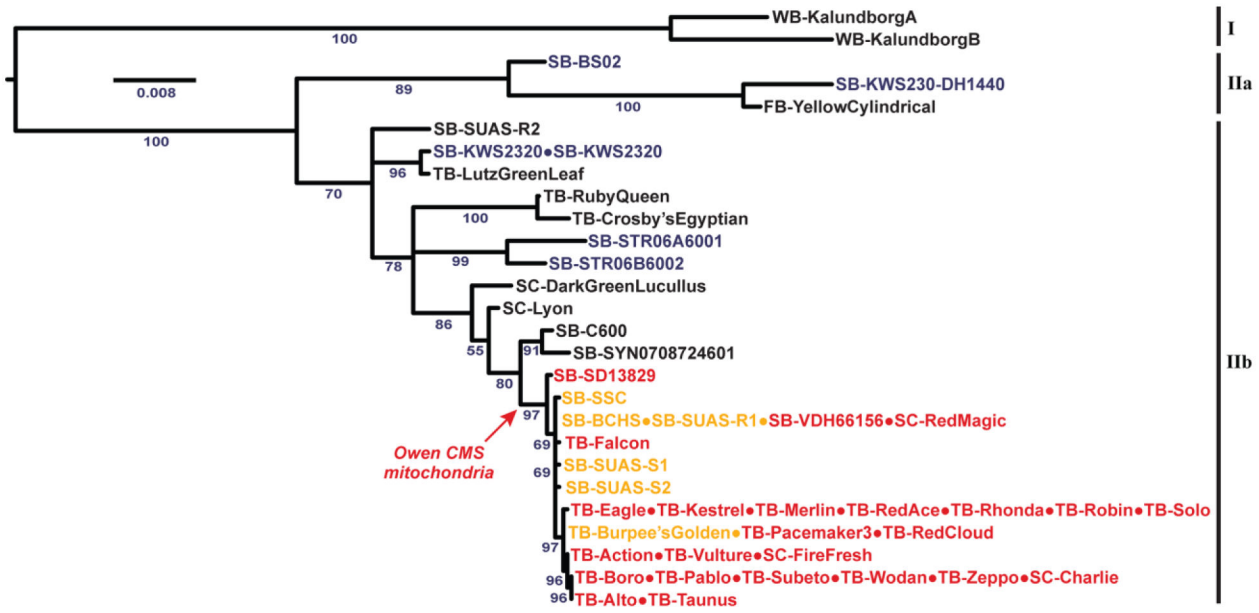

Fig. 2.

Phylogenetic analysis of BevuMV1 sequences. RNA sequences were used, trimmed to shared termini as described in Materials and methods. Multiple sequence alignment was performed with MAFFT, followed by maximum-likelihood phylogenetic analysis with IQ-TREE. Results are displayed as a midpoint-rooted rectangular phylogram (scale bar, mean substitutions per nt position). Some branch tip labels indicate two or more BevuMV1 strains that have identical sequences (FB, fodder beet; SB, sugar beet; SC, Swiss chard; TB, table beet; WB, wild sea beet). Branch support values (shown in %) were calculated by ultrafast bootstrap method; branches with <50% support have been collapsed to the preceding node. BevuMV1 strains from sugar beet, table beet, or Swiss chard cultivars with Owen CMS mitochondria are labeled in red and orange; see text for further explanations. Apparent subclades of BevuMV1 strains are labeled at right (subclade I, viruses from *B. vulgaris* subsp. *maritima*; subclades IIa and IIb, viruses from *B. vulgaris* subsp. *vulgaris*).

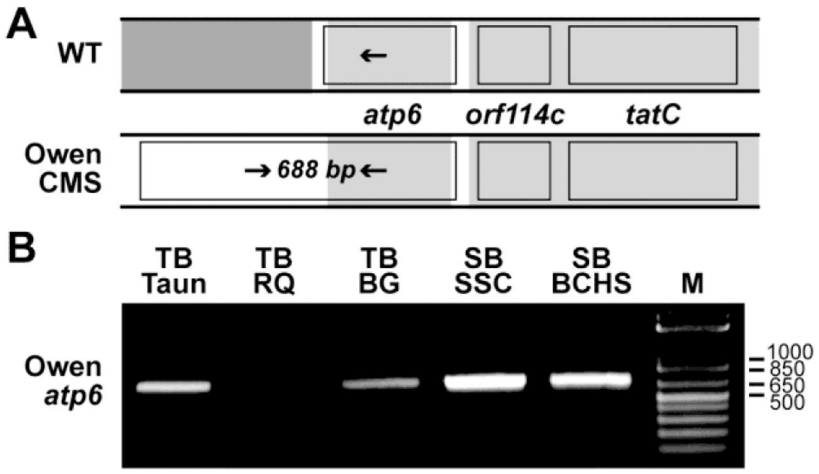

Fig. 3.

Analysis of mitochondrial gene *atp6* in reportedly OP cultivars of *B. vulgaris* that were found to harbor BevuMV1 strains that unexpectedly map to the compact cluster of viruses with Owen CMS mitochondria. (A) Diagram of the *atp6* region of both wild-type (non-Owen) and Owen mitochondrial genomes, modified from Satoh et al. (2004). ORFs of the *atp6*, *orf114c*, and *tatC* genes are shown as boxes. The ORF of Owen *atp6* (i.e., *Satp6*) is substantially extended relative to that of wild type. Gray shading indicates regions conserved between the two genomes; the darker gray region is conserved elsewhere in the Owen genome. Arrows indicate approximate positions of PCR primers, yielding the amplicon of indicated length. (B) Agarose gel electrophoresis of PCR amplicons as diagrammed in A and further described in the text. BG, Burpee's Golden; RQ, Ruby Queen; Taun, Taunus; see text and tables for other abbreviations.

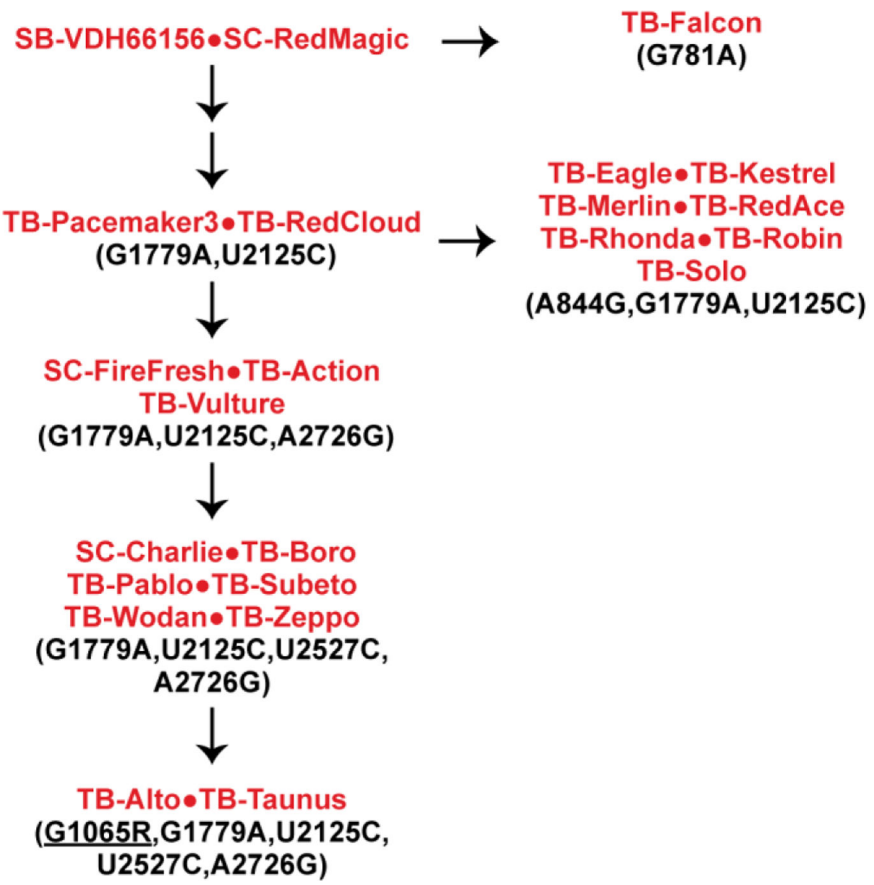

Fig. 4.

Limited stepwise sequence divergence among BevuMV1 strains from F_1-hybrid table beet and Swiss chard cultivars. BevuMV1-SB-VDH66156 (GenBank MG721540.2) is used as the standard for assigning nt-position numbers. The unique mixed nt position in BevuMV1-TB-Alto and BevuMV1-TB-Taunus (G1065R) is underlined for emphasis (see Results text for explanation). The directions of the arrows are consistent with the phylogenetic ordering suggested in Fig. 2.

Table 1

Coding-complete BevuMV1 sequences assembled from public transcriptome data

Crop type of *B. vulgaris*	**NCBI BioProject**	**Cultivar**	**GenBank no. for BevuMV1**[1]
Sugar beet (SB)	PRJNA41497	KWS2320	BK010442.1[2]
		STR06A6001	BK010440.1[2]
		STR06B6002	BK010439.1[2]
		KWS230-DH1440	BK010708.1
		SYN0708724601	BK010709.1
	PRJNA73561	C600[3]	BK010424.2[4]
	PRJNA254489	KWS2320	BK010441.1[2]
	PRJNA343692	BS02	BK010710.1
		SD13829	BK010711.1
	PRJNA358634	SUAS-R1	BK010712.1
		SUAS-R2	BK010713.1
		SUAS-S1	BK010714.1
		SUAS-S2	BK010715.1
Wild beet (WB)	PRJNA328353	KalundborgA	BK010716.1
		KalundborgB	BK010717.1

[1] These GenBank accessions are all in the form of Third Party Annotation (TPA) sequences since derived from primary sequence data previously reported by others.

[2] From Nibert et al. (2018).

[3] This transcriptome was generated from a mixture of RNA samples from sugar beet cultivars C600 (inbred line) and Roberta (F_1 hybrid). According to the associated publication (Mutasa-Göttgens et al., 2012), two-thirds of the RNA samples that gave rise to the transcriptome were from C600, and we have now assigned the associated BevuMV1 sequence to that cultivar.

[4] This is a new revision (3′-extension) of the sequence originally reported in BK010424.1.

Table 2

Coding-complete BevuMV1 sequences from Sanger sequencing of amplicons

Crop type of *B. vulgaris*	**Hybrid status**[1]	**Cultivar**	**GenBank no. for BevuMV1**[2]
Fodder beet (FB)	OP	Yellow Cylindrical	MK423949.1
Sugar beet (SB)	OP	BCHS	MK423942.1
		SSC	MK423943.1
	F_1	VDH66156	MG721540.2[3]
Table beet (TB)	OP	Burpee's Golden	MK423951.1
		Crosby's Egyptian	MK423945.1
		Lutz Green Leaf	MK423946.1
		Ruby Queen[4]	MK423944.1
	F_1	Action	MK423953.1
		Alto	MK423955.1
		Boro	MK423954.1
		Eagle	MK423952.1
		Falcon[4]	MK423950.1
		Kestrel	same as TB-Eagle
		Merlin[5]	same as TB-Eagle
		Pablo	same as TB-Boro
		Pacemaker 3[4]	same as TB-Burpee'sGolden
		Red Ace	same as TB-Eagle
		Red Cloud	same as TB-Burpee'sGolden
		Rhonda[4]	same as TB-Eagle
		Robin	same as TB-Eagle
		Solo	same as TB-Eagle
		Subeto	same as TB-Boro
		Taunus[4]	same as TB-Alto
		Vulture[4]	same as TB-Action
		Wodan	same as TB-Boro
		Zeppo[4]	same as TB-Boro
Swiss chard (SC)	OP	Dark Green Lucullus	MK423948.1
		Lyon	MK423947.1
	F_1	Charlie	same as TB-Boro
		Fire Fresh	same as TB-Action
		Red Magic[5]	same as SB-BCHS

[1] As reported by each supplier: OP, open pollinated; F_1, F_1 hybrid.

[2] These are all regular GenBank accessions since derived from primary sequence data determined by the authors.

[3] This is a new revision (5′-extension by RACE) of the sequence originally reported in MG721540.1.

[4] Seeds from two different retail suppliers (see Table S1) were used for sequencing BevuMV1.

[5] Due to trouble extracting adequate RNA from Red Magic seeds, we grew these seeds into plantlets. For Merlin, we purchased plantlets from a local nursery. The plantlets of these two cultivars were then used for extracting RNA, RT–PCR, and sequencing of BevuMV1.

Table 3

Owen CMS mitochondrial markers in SRA transcriptome data from BioProject PRJNA358634

Mitochondrial gene query[1]	Query-matching reads for sugar beet cultivar (SUAS[2]):			
	R1	R2	S1	S2
Scox2-2	50	0	28	34
Sorf324	27	0	31	31
Satp6	198	0	257	249
N/Scox1-1	284	233	206	253

[1] See text for further explanation. Choice of these sequences was based on findings from Satoh et al. (2004).

[2] SUAS, Swedish University of Agricultural Sciences: R, resistant genotype; S, sensitive genotype.