Characterization of *Blueberry shock virus*, an Emerging *Ilarvirus* in Cranberry

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Abstract

Blueberry shock virus (BShV), an *Ilarvirus* sp. reported only on blueberry, was associated with scarring, disfigurement, and premature reddening of cranberry fruit. BShV was detected by triple-antibody sandwich enzyme-linked immunosorbent assay and reverse-transcription polymerase chain reaction, and isometric virions of 25 to 28 nm were observed in cranberry sap. The virus was systemic, although unevenly distributed in plants. The coat protein of BShV from cranberry shared 90% identity compared with BShV accessions from blueberry on GenBank. Phylogenetic analysis of isolates of BShV from cranberry collected from Wisconsin and Massachusetts did not indicate grouping by state. BShV was detected in cranberry pollen, and seed transmission of up to 91%

was observed. Artificial inoculation of cranberry flowers by pollination did not cause virus transmission. In some *Nicotiana* spp., rub inoculation of leaves with homogenized BShV-positive cranberry flowers resulted in systemic infection. Cranberry plants recovered from symptoms the year after berry scarring occurred but continued to test positive for BShV. The virus caused significant reduction in the average number of marketable fruit and average berry weight in symptomatic cranberry plants but recovered plants yielded comparably with healthy plants. Although recovery may limit the immediate economic consequences of BShV, long-term implications of single- or mixed-virus infection in cranberry is unknown.

Cranberry (*Vaccinium macrocarpon* Aiton, family *Ericaceae*), a vegetatively propagated perennial crop for which plantings can remain in production for several decades, has had few virus disease problems since it was first cultivated in the late 1800s. Three viruses—*Tobacco streak virus* (TSV), *Blueberry scorch virus* (BIScV), and *Blueberry red ringspot virus*—have been described on cranberry but symptoms were generally minor or absent (Jones et al. 2001; Polashock et al. 2009; Wegener et al. 2004). Although TSV was not initially associated with symptoms (Jones et al. 2001), in 2012, TSV was associated with berry scarring in commercial cranberry marshes in Wisconsin (Wells-Hansen et al. 2016). Surveys in Wisconsin in 2013 and 2014 revealed a second virus associated with berry-scarring symptoms, *Blueberry shock virus* (BShV), on 69% of samples of older cranberry cultivars (Wells-Hansen et al. 2016). Because BShV has been studied only on highbush blueberry (*V. corymbosum* L., family *Ericaceae*) (Bristow and Martin 1999, 2001; MacDonald et al. 1991), there are many knowledge gaps in the biology and economic impact of this recently discovered virus on cranberry.

Wisconsin and Massachusetts together account for >80% of the world's cranberry production. Cranberry is the leading fruit crop in Wisconsin, valued at \$146 million in 2015, with a harvested area of 8,175 ha (20,200 acres) (USDA-NASS 2016). Therefore, the association of BShV with berry scarring in Wisconsin in 2013 and Massachusetts in 2014 necessitated this study which, excepting preliminary reports (Thomas-Sharma et al. 2015, 2016), is the first investigation of BShV on cranberry.

BShV is a typical member of the genus *Ilarvirus* (family *Bromoviridae*), with a tripartite genome of positive-sense, single-stranded RNA, and is quasi-isometric in shape (MacDonald et al. 1991). Genomic RNA is directly translated into protein except for coat protein (CP), where a subgenomic RNA is produced from a bicistronic RNA3. The virus was initially detected on blueberry in the Pacific Northwest region of the United States (MacDonald et al. 1991) and has since been detected in other blueberry-growing regions in Canada and the United States, including Michigan, New York,

Nova Scotia, and Pennsylvania (Martin et al. 2012). BShV can be detected using nucleic acid (Tzanetakis and Martin 2008) and serological (MacDonald et al. 1991; Sedegui and Osterbauer 2009) detection methods. The virus has a narrow herbaceous host range and causes ringspot-like symptoms on some tobacco varieties artificially inoculated with homogenized blueberry flowers (MacDonald et al. 1991). BShV has never been found in native vegetation, making the origins of the virus puzzling, and movement of infected planting stock is suspected to introduce disease into new areas (MacDonald et al. 1991).

Like other ilarviruses, BShV is pollen-transmitted in blueberry (Bristow and Martin 1999), making the disease particularly challenging to manage. Infection of blueberry occurs specifically during flowering, with the aid of honeybees, and symptoms are noticeable within 1 to 2 years on entire plants or a few branches (Bristow and Martin 1999). In early summer, foliage and flowers of infected blueberry plants appear blighted, eventually dropping off and leaving bare branches. By the end of summer, however, infected plants have a new flush of growth and form reproductive buds for the next year (Bristow and Martin 2002). BShV-infected blueberry plants become asymptomatic 1 to 3 years after the occurrence of symptoms, a phenomenon referred to as “symptom recovery” or just “recovery” (Bristow and Martin 2002). Thus, although 34 to 90% yield losses can occur in the year of blighting, recovery limits losses in subsequent years (Bristow and Martin 2002). Recovered plants, however, continue to test positive for the virus and can serve as inoculum sources in the field (Martin et al. 2006).

On cranberry, the symptoms associated with BShV are similar to those associated with TSV, making visual differentiation of the diseases difficult (Wells-Hansen et al. 2016). Cranberry shoots that arise vertically from prostrate runners, hereafter referred to as “uprights,” do not consistently show foliar symptoms, and scarred berries that turn prematurely red are the main symptom of the disease (Fig. 1A). Similar to BShV on blueberry, TSV infection on cranberry results in symptom recovery (i.e., uprights that have symptomatic fruit in one year bear asymptomatic fruit the following year), and recovered uprights yield comparably with healthy uprights (Wells-Hansen and McManus 2016). If recovery occurs in BShV-infected cranberry, the immediate economic consequences of the virus may be limited. The objectives of this study were to (i) detect BShV and determine virus localization in cranberry uprights, (ii) study phylogenetic relationships of BShV in cranberry, (iii) investigate recovery and the impact of BShV on yield components of cranberry, and (iv) identify

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inoculum sources and routes for disease transmission. We relate our findings on BISHV of cranberry to previously described ilarviruses of fruit crops and discuss implications for disease management.

Materials and Methods

Detection and imaging of BISHV. Serological detection. Plant tissue (maximum of 0.1 g), combined with 600 μ l of general extraction buffer (GEB, provided in a commercial kit; Agdia, Inc., Elkhart, IN), was macerated with 1 or 2 chrome-steel beads in a Mini-BeadBeater (BioSpec Products, Inc., Bartlesville, OK). An aliquot of the sample (100 μ l) was then tested with a triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) kit, according to manufacturer instructions. In every 96-well plate, two samples each of controls were included: buffer only, positive control provided in the kit, and leaves from known BISHV-negative cranberry plants. A test sample was considered positive if the absorbance at 405 nm, measured on a Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA), was three times the average of the BISHV-negative plant samples.

Reverse-transcription polymerase chain reaction (RT-PCR). Leaves (0.1 g) from TAS-ELISA-positive plants were harvested from field samples and stored at -80°C until RNA extraction. The leaves were homogenized in liquid nitrogen in a mortar with a pestle, and total RNA was extracted using the Qiagen RNeasy Plant Mini kit, according to manufacturer instructions (Qiagen, Valencia, CA). The RNA was converted to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). PCR amplification was then carried out using the GoTaq Green Master Mix (Promega Corporation, Madison, WI) in a thermal cycler (Mastercycler gradient; Eppendorf, Westbury, NY) using primers for RNA3 that yield a 417-bp product (Tzanetakis and Martin 2008). The cycling protocol included a 3-min initial denaturation at 95°C , followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were resolved in a 1% agarose gel in Tris-acetate EDTA that was stained with SYBR Safe (Life Technologies, Grand Island, NY) and viewed on a UV transilluminator.

Transmission electron microscopy. To image virus or virus-like particles, 2- to 5-g leaf samples were ground in liquid nitrogen and extracted with 20 ml of standard extraction buffer, consisting of 500 mM sodium phosphate (pH 7.4), 1 M urea, 5% (wt/vol) polyvinyl pyrrolidone (40,000 molecular weight [MW]), and 0.25% (vol/vol) 2-mercaptoethanol. The extract was filtered through polyester

fabric and centrifuged at $20,000 \times g_{\text{max}}$ for 15 min at 10°C . The supernatant (17 ml) was layered over 5 ml of 30% (wt/vol) sucrose in 100 mM sodium phosphate (pH 7.2) and centrifuged at $109,000 \times g_{\text{max}}$ for 2.5 h at 10°C . The resulting pellet was resuspended in 100 μ l of 100 mM sodium phosphate (pH 7.2), extracted with an equal volume of chloroform, and centrifuged for 10 min. The upper aqueous phase constituted the partially purified tissue extract. Carbon-coated transmission electron microscopy (TEM) grids were floated for 2 to 5 min on 10 μ l of an aqueous solution of poly-L-lysine (1,000 to 5,000 MW) at 250 $\mu\text{g/ml}$, blotted, and floated for 2 to 5 min on 10 μ l of sample consisting of 8 μ l of partially purified tissue extract and 2 μ l of 25% EM-grade glutaraldehyde. For TEM examination, sample grids were then negatively stained by three rinses on 10 μ l drops of 2% (wt/vol) sodium phosphotungstate (pH 7.0) or uranyl formate.

Localization of BISHV in cranberry and potential sources of inoculum in the field. Cranberry plant samples (cultivar Stevens) were collected throughout the growing seasons of 2014 to 2016 from three commercial cranberry farms in Wisconsin where BISHV had been identified previously. Plant samples were stored at 4°C until processing. Various plant parts, including apical buds, flowers, previous- and current-season leaves, asymptomatic berries, symptomatic berries, and roots, were tested for the presence of BISHV by TAS-ELISA. Berries and leaves of wild blueberry (*V. angustifolium*, $n = 35$) and leatherleaf (*Chamaedaphne calyculata*, $n = 27$), ericaceous weeds in and around cranberry beds on the three farms, were tested for the presence of BISHV by TAS-ELISA. Cranberry pollen samples ($n = 21$ in 2014 and $n = 17$ in 2015) were collected from areas in cranberry beds where the incidence of BISHV was known to be high and tested by TAS-ELISA. An individual sample consisted of pollen pooled from six to eight flowers. Pollen samples were agitated briefly in GEB and then centrifuged to separate pollen grains from the pollen wash. Washing was repeated three to five times until, finally, the pollen was macerated in GEB to obtain pollen extract (Wells-Hansen et al. 2016). All pollen washes and extracts were tested separately.

Phylogenetic studies. Full-length coat protein sequence. RNA3 sequences of BISHV from blueberry (GenBank accessions KF031039.1 and KF030142.1 from the Pacific Northwest region of the United States) were used to design primers to obtain the full-length CP gene of BISHV from cranberry. The forward and reverse primers were 5'-TATTCCGG CAGTTCGATAAC-3' and 5'-TCGGGGCATCTAATTCCTTC-3', respectively. RNA extraction, cDNA synthesis, and PCR were conducted as described above on plants previously determined as BISHV-positive by TAS-ELISA. The 993-bp PCR product was cloned into the pGEM-T Easy vector system (Promega Corporation) and the plasmid was used to transform *Escherichia coli* competent cells. Plasmids were extracted from transformed *E. coli* using the Wizard Plus Miniprep DNA purification system (Promega Corporation), and the presence of the insert was confirmed by PCR. The plasmid with insert was then sequenced using the forward and reverse primers 5'-CGCCAGGGTTTCCAGTCAC GAC-3' and 5'-TCACACAGGAACAGCTATGAC-3', respectively. Three such insert sequences (deposited to GenBank, accessions numbers KY927862, KY927863, and KY927864) obtained from two different cranberry plants were subjected to BLAST searches on National Center for Biotechnology Information and protein translation tools (ExPASy; Swiss Institute of Bioinformatics, Lausanne, Switzerland). Using the pairwise sequence alignment tool Needle (EMBL-EBI, Cambridgeshire, UK), the nucleotide and amino acid sequences of the CP open reading frame (ORF) were compared with the Pacific Northwest accessions of BISHV from blueberry.

Diversity of CP in BISHV isolates. RNA was extracted from the leaves of 17 symptomatic uprights collected from three farms each in Wisconsin and Massachusetts. The CP gene of each isolate was amplified by RT-PCR and sequenced directly. The positive control provided in the TAS-ELISA kit, which originated from blueberry leaf samples from the United States, was cloned and two plasmids with inserts were sequenced and deposited in GenBank (accessions numbers KY927865 and KY927866). The nucleotide sequences of the CP ORF of KY927865 and KY927866 along with the 17 isolates

Fig. 1. Symptoms associated with *Blueberry shock virus* (BISHV). **A**, Symptomatic, scarred berries that turned prematurely red, observed in cranberry beds in Wisconsin. **B**, Recovery in BISHV. Symptomatic uprights were tagged in the year when berry scarring occurred. These uprights bore asymptomatic berries for the remainder of the 3-year study but continued to test positive for the virus.

from cranberry were included in a multiple sequence alignment with the BISHV accessions from blueberry from the Pacific Northwest. *Prunus necrotic ringspot virus* (PNRSV, JN416776) was included as the outgroup. The multiple sequence alignment (using ClustalW) and phylogenetic trees were constructed using MEGA, version 6 (Tamura et al. 2013). A maximum-likelihood method was used to construct the phylogenetic trees and maximum-composite-likelihood method was used to compute the distances. A bootstrap test of 1,000 pseudoreplicates was used to assign a confidence value to the branches.

Artificial inoculation of cranberry and herbaceous host plants.

Flowers of potted Stevens cranberry plants ($n = 14$ pots in 2014 and $n = 22$ pots in 2015), determined by TAS-ELISA to be BISHV-negative, were inoculated by pollination using pollen collected from BISHV-positive plants in the field. Flowers of two or three plants in each experiment were self-pollinated and served as negative controls. Symptom development on fruit was monitored for approximately 3 months, after which all berries and a sample of leaves from each plant were tested by TAS-ELISA. The plants were overwintered in a cold frame, and the new leaves were tested for BISHV the following year.

Six species or cultivars of herbaceous hosts—*Nicotiana benthamiana*, *N. sylvestris*, *N. tabacum* ‘Samsun’, *N. tabacum* ‘Havana’, *N. clevelandii*, and *Helianthus annuus*—were maintained in a growth chamber and were inoculated when they were 6 to 8 weeks old. Inoculum was prepared by grinding either BISHV-positive cranberry leaves or flowers (approximately 20 to 30 each) in 1 ml of buffer (MacDonald et al. 1991; Spiegel and Cohen 1985; Wells-Hansen et al. 2016). Inoculations with macerated leaves and flowers were performed by sprinkling a small amount of diatomaceous earth onto three or four leaves per plant and then rubbing the prepared inoculum onto the leaves with a gloved finger. In each experiment, one or two control plants for each species were mock inoculated with buffer or with macerated leaves of TAS-ELISA-negative cranberry plants. Inoculated plants were observed twice weekly for symptoms that could be indicative of virus infection and tested by TAS-ELISA 2 to 3 weeks after inoculation.

Recovery of cranberry from BISHV symptoms. In summer 2013, 2014, and 2015, 20 to 30 symptomatic cranberry uprights were tagged with twist ties at two (2013 and 2014) or three (2015) farms in Wisconsin. Smaller areas were demarcated as sites within farms. In the following years, we monitored the tagged uprights for symptom development on berries, and collected leaves and berries for testing by TAS-ELISA. Percent return bloom was calculated as the percentage of the originally tagged uprights that produced flowers in successive years (Wells-Hansen and McManus 2016).

Effect of BISHV on yield components. In early August 2015, 100 to 150 uprights with symptomatic berries were tagged using twist ties at three farms in Wisconsin. In September 2015 (2 weeks before commercial harvest), about 70 to 85 of these symptomatic uprights along with about 300 to 500 uprights with asymptomatic berries were collected from each site. On each asymptomatic upright, current-season leaves, previous-season leaves, and one asymptomatic berry were tested by TAS-ELISA. An upright was considered infected if at least one of the plant parts tested positive for BISHV. These results were used to classify asymptomatic uprights as either healthy

(ELISA-negative) or recovered (ELISA-positive). Individual uprights were evaluated by counting and weighing the total marketable berries, and counting the number of aborted berries, small berries, and pedicels (as a late-season indicator of flowering). The data were used to calculate the response variables: fruit set, marketable berry number, and individual berry weight. Fruit set was calculated as the sum of marketable berries and small berries divided by the number of pedicels, and expressed as a percentage. Individual berry weight was calculated by dividing the weight of marketable berries by the number of marketable berries. Analysis of variance was conducted on fruit set (natural log transformed after converting zeros to 0.0001) and individual berry weight whereas for marketable fruit number, a Quasipoisson generalized linear model was fitted with pedicel number as the offset value. For all response variables, upright status (i.e., healthy, recovered, or symptomatic) served as the explanatory variable and analysis was carried out separately for each farm. When upright status had a statistically significant result ($P < 0.05$), Tukey’s honestly significant difference test was used for mean separation. All statistical analyses were conducted in R version 3.3.2 (R Core Team 2015).

Transmission of BISHV through vegetative and sexual progeny.

In January 2016, dormant cranberry vine cuttings were collected from the field. Leaves of individual uprights were tested by TAS-ELISA and the subtending runners were accordingly classified as healthy or infected. Vine cuttings (6 to 8 cm) were planted and maintained in the greenhouse. New leaves from the cuttings were tested for BISHV by TAS-ELISA 1 to 2 months later.

Seed were extracted from surface-disinfested ripe berries (Wells-Hansen et al. 2016) of uprights classified as healthy, symptomatic, or recovered collected from each of the aforementioned three farms in Wisconsin. For germination, seed were treated overnight in gibberellic acid at 2,000 ppm, washed three times in sterile water, and pressed gently into water agar in 36-cm² tissue culture boxes (Magenta, Chicago). After incubation in a south-facing window of the laboratory, seedlings emerged in 3 to 4 months. Individual seed or seedlings were crushed in 300 μ l of GEB and tested by TAS-ELISA. Virus incidence in seed and seedlings that grew from seed in each of the upright categories was calculated separately for each farm.

Results

Detection and imaging of BISHV. BISHV was detected in cranberry leaves by complementary serological and nucleic acid-based detection methods. Commercial TAS-ELISA kits successfully detected BISHV and were used for routine detection throughout this study. RT-PCR primers (designed for BISHV in blueberry) gave a product of expected size and sequence from TAS-ELISA-positive BISHV samples. Transmission electron micrographs showed the presence of isometric particles of 25 to 28 nm, consistent with size descriptions of BISHV (MacDonald et al. 1991).

Localization of BISHV in cranberry and potential sources of inoculum in the field. BISHV was detected in different parts of cranberry plants, including apical buds, leaves (both current- and previous-season), flowers, and berries (symptomatic and asymptomatic) (Table 1). In infected cranberry uprights (i.e., uprights in which at least one plant part tested positive), detection of BISHV in all plant parts was often <100% (Table 1). BISHV was detected in 62% ($n = 34$) of root samples taken

Table 1. Detection of *Blueberry shock virus* (BISHV) by triple-antibody sandwich enzyme-linked immunosorbent assay in different parts of infected cranberry uprights

Time of testing	Presence of symptoms ^b	Percent incidence of BISHV in plant part ^a					
		Apical buds	Flowers	Previous-season leaves	Current-season leaves	Asymptomatic berry	Symptomatic berry
Before bloom	...	70 (44)	...	82 (67)	100 (21)	...	...
During bloom	...	...	91 (64)	87 (63)	84 (62)	...	...
After fruit set	Asymptomatic	...	...	91 (226)	89 (312)	28 (337)	...
	Symptomatic	...	...	82 (239)	61 (243)	28 (25)	94 (117)

^a An upright was considered infected if at least one plant part tested positive. Numbers in parentheses indicate sample size.

^b Berry scarring is the only symptom of the disease.

from runners before bloom and after fruit set. BISHV was not detected in either wild blueberry or leatherleaf. Incidence of BISHV in pollen extracts was 76 and 53% in 2014 and 2015, respectively. Pollen washes were consistently negative for the virus.

Phylogenetic studies. The cranberry and blueberry isolates shared 89.9 to 90.8 and 93.4 to 93.9% amino acid identity and similarity, respectively, and 89.7 to 90.1% nucleotide similarity. The length of the CP ORF was 677 to 687 among the 17 isolates of BISHV from cranberry. The cranberry isolates grouped into a single clade and did not group based on geography (Fig. 2).

Artificial inoculation of cranberry plants and other herbaceous hosts. None of the healthy cranberry plants inoculated by pollination with BISHV-positive pollen developed symptomatic berries or tested positive for BISHV. None of the herbaceous plants that

were inoculated with macerated cranberry leaves tested positive for BISHV or developed symptoms. Three species of *Nicotiana* inoculated with macerated cranberry flowers developed systemic infection by BISHV (Table 2). Two *N. benthamiana* plants tested positive for BISHV but were asymptomatic, while all other infected plants developed etching symptoms (Fig. 3).

Recovery from BISHV symptoms. Symptomatic uprights that were tagged in one season produced asymptomatic fruit (when they produced fruit) the following years for the remainder of the study (Fig. 1B) but their leaves continued to test positive for BISHV. The return bloom on these uprights varied by year and site and ranged from 5 to 79 and 11 to 68% in the first and second years after symptom observation, respectively (Table 3).

Effect of BISHV on yield components. Yield components were variable among farms (Fig. 4). The average fruit set was comparable across upright categories, except at farm 2, where symptomatic uprights had significantly lower fruit set (Fig. 4B). The average marketable berry numbers and the average berry weight were significantly lower in symptomatic uprights at all three farms. None of the farms showed a statistically significant difference between recovered and healthy uprights with regard to the average marketable berry number and the average berry weight.

Natural transmission of BISHV through vegetative and sexual progeny. A high incidence of BISHV (97% of $n = 194$) was observed in new leaves of plants propagated from vine cuttings of infected cranberry. A low incidence of BISHV (4.6% of $n = 194$) was observed in plants propagated from vine cuttings classified as healthy (i.e., leaves from the uprights themselves were BISHV-negative or the runner subtended only negative uprights). BISHV incidence was high in both seed and seedlings from healthy, symptomatic, and recovered uprights (Table 4). Seed originating from uprights that were classified as healthy had 1 to 4% incidence of BISHV, whereas seedlings originating from these same uprights had 46 to 72% incidence.

Discussion

We detected BISHV in cranberry plants by TAS-ELISA and RT-PCR and observed isometric virions in cranberry sap by TEM. Originally reported on cranberry in association with fruit-scarring symptoms in Wisconsin (Wells-Hansen et al. 2016), BISHV has since been identified on cranberry in British Columbia, Massachusetts, New Jersey, and Oregon (Polashock 2017). The occurrence of BISHV in major cranberry production regions across North America underscores the importance of the current study, which fills several knowledge gaps concerning the biology and epidemiology of this emerging pathogen.

Using TAS-ELISA, we detected BISHV in all cranberry plant parts, thereby confirming the systemic nature of the virus. The incidence of BISHV in parts of infected cranberry uprights was often <100%, indicating uneven virus distribution. Such uneven distribution of viruses in planta has been observed in many pathosystems and can affect virus detection and transmission (Kominek et al. 2009; Kritzman et al. 2001). In our work, uneven BISHV distribution

Fig. 2. Inferred phylogenetic tree of 17 *Blueberry shock virus* (BISHV) isolates on cranberry in relation to other members of family *Bromoviridae* sequenced separately or available in GenBank. Isolates in cranberry begin with "C" and were from Wisconsin (preceded by WI) or Massachusetts (preceded by MA). Isolates BISHV_KY927865 and BISHV_KY927866 were obtained by cloning and sequencing the positive control supplied in the triple-antibody sandwich enzyme-linked immunosorbent assay kit (originally isolated from blueberry leaves from the United States). Published sequences from GenBank were included as representative sequences for BISHV on blueberry (BB BISHV: KF031042.1 and KF031039.1) and *Prunus necrotic ringspot virus* (PNRSV: JN416778), the latter serving as the outgroup. Numbers at the branches are bootstrap values (1,000 pseudoreplicates).

Table 2. Artificial inoculation of herbaceous plants to determine *Blueberry shock virus* (BISHV) host range

Plant species inoculated	Number of plants testing positive for BISHV following inoculation with macerated leaves or flowers in two buffers (total number inoculated) ^a		
	Macerated leaves		Macerated flowers
	Potassium phosphate	Potassium phosphate	Sodium phosphate
<i>Nicotiana benthamiana</i>	0 (14)	4 (5)	1 (4)
<i>N. sylvestris</i>	0 (6)	0 (2)	0 (2)
<i>N. tabacum</i> 'Samsun'	0 (8)	2 (4)	1 (4)
<i>N. tabacum</i> 'Havana'	0 (14)	0 (2)	0 (4)
<i>N. clevelandii</i>	0 (7)	0 (3)	1 (4)
<i>Helianthus annuus</i>	0 (6)	0 (5)	0 (5)

^a Leaves of BISHV-positive cranberry plants maintained in the greenhouse or flowers collected from infected plants in the field were macerated in buffer and used to rub inoculate leaves of herbaceous hosts. One to three control plants of each species or cultivar that were mock inoculated with water did not develop symptoms and tested negative for BISHV by triple-antibody sandwich enzyme-linked immunosorbent assay. Data are combined over two trials and the number in parentheses is combined sample size. Not every species or cultivar was included in both trials.

resulted in false negatives, because we sometimes detected the virus upon retesting parts of cranberry uprights that were previously classified as healthy (based on a negative TAS-ELISA result). In cranberry infected with TSV, systematic sampling through the growing season revealed that the distribution of TSV was relatively uniform in recovered uprights but uneven in symptomatic uprights (Wells-Hansen and McManus 2016). Thus, when screening for viruses in cranberry, testing both leaves and berries is advised to avoid false-negative results.

BIShV on cranberry shared only 90% CP identity with blueberry accessions in GenBank. Phylogenetic trees indicated that BIShV from blueberry and BIShV from cranberry formed distinct clades by host. Taken together, these observations suggest that BIShV on cranberry is distinct from BIShV on blueberry. There are no specified levels of sequence similarity to differentiate strains and species of ilarviruses (Roossinck et al. 2005). Because extant field isolates of BIShV from blueberry were not included in this study, more sampling is necessary to conclusively establish host-based strain differences and the evolution of BIShV on *Vaccinium* hosts.

BIShV isolates from cranberry did not group based on their geographic origin, similar to what was observed for TSV on cranberry (Wells-Hansen et al. 2016). Among virus isolates collected from Wisconsin, the pairwise identity of CP nucleotide sequences was 90.9 to 99.9% for TSV and 96.1 to 100% in BIShV, indicating less diversity in BIShV. The similarity of BIShV isolates from Wisconsin and Massachusetts is possibly because of the narrow host range of the virus (Table 2) (MacDonald et al. 1991) and suggests a relatively recent introduction of BIShV into cranberry. However, the origins of BIShV in cranberry remain elusive, because the ericaceous weeds (wild blueberry and leatherleaf) that were abundant in and near infected beds in Wisconsin tested negative for the virus.

We detected BIShV in extracts of crushed cranberry pollen but not in pollen washes. This internalization of the virus differs from BIShV on blueberry (Bristow and Martin 1999), TSV on cranberry (Wells-Hansen et al. 2016), and PNRSV in apricot (Amari et al. 2007), where the viruses were detected in both washes and extracts of pollen. The presence of BIShV in cranberry pollen grains prompted our studies on the role of pollen in horizontal and vertical transmission of the virus.

Horizontal (plant-to-plant) transmission of pollen-borne viral diseases can occur when infected pollen transmits disease to healthy maternal tissue during fertilization (Card et al. 2007). In *Torenia* plants inoculated with raspberry pollen infected with *Raspberry bushy dwarf virus* (genus *Idaeovirus*), the virus was found to infect the stigma and style during pollen tube elongation (Isogai et al. 2014).

Fig. 3. Symptoms that developed 2 to 3 weeks after mechanical transmission of *Blueberry shock virus* to **A** and **B**, *Nicotiana tabacum* 'Samsun' and **C**, *N. benthamiana*.

In BIShV on blueberry, the presence of honeybees during bloom resulted in transmission of the virus to healthy trap plants, implying that honeybees are the vectors of infected pollen (Bristow and Martin 1999). However, when we hand-pollinated healthy cranberry plants with BIShV-infected pollen, we failed to reproduce berry-scarring symptoms, and neither fruit nor leaves of the pollinated plants tested positive for BIShV. The unsuccessful transmission of BIShV to cranberry plants probably cannot be attributed to low infectivity of the virus, because when flowers from BIShV-infected fields were used as inoculum, *N. benthamiana* became systemically infected and developed symptoms. Lack of transmission by pollination might have been because the inoculated flowers were not emasculated, allowing them to self-pollinate with healthy pollen. Alternatively, pollination might not be a significant mode of BIShV horizontal transmission in cranberry.

Vertical transmission of plant viruses occurs when the embryo within a seed is infected during fertilization (i.e., because of infection in pollen or ovule) or during embryo development, postfertilization (Card et al. 2007). A very low rate of BIShV transmission (1.2%) was observed in blueberry seed (Bristow and Martin 1999), and no transmission of TSV was observed in cranberry seed (Wells-Hansen et al. 2016). By contrast, we observed vertical transmission of BIShV at rates up to 91% in cranberry seed. Cranberry seed can germinate in the field and, thus, if animals or humans move infected cranberry fruit, the establishment of infected seedlings could introduce the disease to new areas. Although the reasons for variability of BIShV incidence in ungerminated seed are unclear, the health of the seedlings is independent of the health of the mother plant (healthy, recovered, or symptomatic). The availability of more tissue in seedlings and virus replication in such tissues possibly reduced the occurrence of false negatives and gave a true representation of seed transmission.

Commercially, cranberry is vegetatively propagated from dormant or rooted vine cuttings. In our studies, 97% of cuttings taken from infected runners tested positive for BIShV. Because there are no industry-wide certification programs established for cranberry propagation material, the unrestricted movement of infected planting material is potentially an important source of inoculum. Thus, ensuring that the vines used for propagation are not sourced from infected beds can delay disease introduction, but given the pollen-borne nature of BIShV and high seed transmission rate, new introductions cannot be eliminated. Similarly, bed management practices such as killing flowers of newly established beds by withholding frost protection or reflooding bearing beds after harvest to remove remaining berries that might contain infected seed can also reduce inoculum and delay disease development.

BIShV-infected symptomatic cranberry uprights recovered from symptoms, similar to the recovery phenomenon described for TSV in cranberry (Wells-Hansen and McManus 2016) and BIShV in blueberry (Bristow and Martin 2002). Recovered cranberry uprights

Table 3. Percent return bloom on symptomatic uprights 1 or 2 years after observation of initial symptoms associated with *Blueberry shock virus*^a

Year of symptoms	Site ^b	First year after symptoms	Second year after symptoms
2013	1	5 (22)	68 (19)
	2	17 (17)	47 (15)
2014	3	10 (20)	57 (7)
	4	29 (21)	36 (11)
	5	13 (15)	11 (9)
2015	6	79 (28)	...
	7	58 (19)	...
	8	26 (30)	...

^a Return bloom was calculated as the percentage of the originally tagged uprights that produced flowers in successive years. The number in parentheses indicates sample size.

^b Sites were smaller areas demarcated each year within the three commercial cranberry farms in Wisconsin.

continued to bloom at rates considered normal given the variability in return bloom reported previously for cranberry (DeVetter et al. 2013; Wells-Hansen et al. 2016). Studies on the mechanism of recovery in annual plants has indicated that transcriptome reprogramming, including RNA silencing, is responsible for recovery from virus infection (Bengyella et al. 2015). Similar mechanisms may be at play in cranberry. We have isolated BISHV-specific small interfering RNA (siRNA; data not shown) in recovered cranberry leaves, suggesting that RNA silencing was triggered.

Because BISHV is a new disease problem for the cranberry industry, there was a need to determine impact of the virus on yield. Given the inherent variability in bed-wide cranberry yield (Pozdnyakova et al. 2005), we measured yield components on individual cranberry uprights, as conducted previously (Roper et al. 1993; Wells-Hansen and McManus 2016). For this, we classified uprights as recovered, healthy, and symptomatic based on fruit appearance and TAS-ELISA tests. Because the time between infection and symptom expression is not known, it is possible that uprights classified as recovered included latently infected uprights that had not shown symptoms previously. Despite this caveat, our results indicate that a reduction in the number of marketable berries on symptomatic uprights and average weight of symptomatic berries are the major detriments to yield. Recovered uprights, however, were comparable with healthy uprights for all yield components measured. Thus, similar to observations of cranberry yield recovery from TSV infection (Wells-Hansen and McManus 2016) and blueberry yield recovery from BISHV infection (Bristow and Martin 2002), recovery also limits the immediate economic consequences of BISHV on cranberry.

In this study, we have documented substantial similarities between BISHV and TSV on cranberry with regard to symptomatology, virus

distribution within plants, symptom recovery, and impact on yield. The apparent simultaneous emergence of the two ilarviruses as disease concerns in cranberry raises questions about the future importance of virus diseases in this long-lived perennial crop. In the current study (unpublished data) and previous work (Wells-Hansen et al. 2016), we did not detect TSV and BISHV on the same cranberry upright, and siRNA sequencing and assembly did not reveal sequences of other viruses (unpublished data). However, in some blueberry varieties, dual infection by BISHV and BISCV causes a characteristic red leaf spot symptom (Martin 2017). If mixed virus infections involving BISHV in cranberry were to evolve, interactions between viruses might result in greater economic impact than losses incurred by single infections.

Table 4. Incidence of *Blueberry shock virus* (BISHV) in seed and seedlings of cranberry

Plant material tested	Farm number	Percent incidence of BISHV in seeds or seedlings from different upright categories ^a		
		Healthy	Recovered	Symptomatic
Seed	1	1 (75)	75 (75)	12 (73)
	2	4 (57)	26 (57)	29 (58)
	3	4 (100)	67 (100)	11 (100)
Seedlings	1	46 (56)	54 (41)	56 (41)
	2	64 (39)	79 (38)	86 (43)
	3	72 (32)	85 (33)	91 (44)

^a Seeds were extracted from cranberry uprights that were classified as healthy (if asymptomatic and BISHV-negative), recovered (if asymptomatic and BISHV-positive), and symptomatic. The number in parentheses is sample size.

Fig. 4. Effect of *Blueberry shock virus* (BISHV) on yield components of cranberry from three farms in Wisconsin. Cranberry uprights were classified as healthy (if asymptomatic and BISHV-negative by triple-antibody sandwich enzyme-linked immunosorbent assay [TAS-ELISA]), recovered (if asymptomatic and BISHV-positive by TAS-ELISA), and symptomatic. Yield components calculated for each of the upright categories were **A**, average fruit set (number of marketable fruit + number of small fruit/number of pedicels $\times 100$); **B**, average berry weight (weight of total marketable berries/number of marketable berries); and **C**, average marketable berry number per upright. Bars represent standard errors. Statistical significance is represented by *** above the bars ($P < 0.001$).

The findings reported here will provide a basis for ongoing efforts to manage BISHV and monitor its effect on cranberry health.

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