Identification of *Tobacco streak virus* in Cranberry and the Association of TSV with Berry Scarring

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Abstract

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Cranberry plants bearing disfigured, scarred fruit were reported by growers in the major cranberry-growing region of central Wisconsin in July 2012. Plants bearing scarred fruit have since been observed in Massachusetts and New Jersey. Three complementary methods provided evidence of *Tobacco streak virus* (TSV) in symptomatic plants: (i) leaves and scarred berries tested positive for TSV by double-antibody sandwich enzyme-linked immunosorbent assay; (ii) quasi-isometric particles approximately 33 nm in diameter were extracted from leaves of symptomatic plants and visualized using transmission electron microscopy; and (iii) coat protein gene sequence analysis revealed 94 to 99% nucleotide similarity with reference TSV sequences. In newer cultivars, 99% of uprights with scarred berries

tested positive for TSV. In older cultivars, 31% of uprights with scarred berries tested positive for TSV and the remaining 69% of uprights with scarred berries tested positive for *Blueberry shock virus*. TSV overwintered in cranberry plants, and leaves, pollen, and fruit tested positive for TSV the year following symptom occurrence. Attempts to inoculate cranberry using infected pollen or sap as inoculum failed, but several herbaceous hosts tested TSV positive following mechanical inoculation. Phylogenetic analysis of the coat protein gene of 26 TSV isolates from various cultivars of cranberry in Wisconsin, New Jersey, and Massachusetts revealed diversity. This work provides information that will be useful in understanding the epidemiology of TSV in cranberry and in the development of management strategies.

Cranberry (*Vaccinium macrocarpon* Aiton) is the most important fruit crop in Wisconsin in terms of both area planted and economic value. With over 8,250 ha currently in production and a farm gate value of approximately \$145 million in 2014 (USDA-NASS 2014), the Wisconsin cranberry crop accounts for almost 60% of the nation's supply (Colquhoun and Johnson 2010). Cranberry is a vegetatively propagated perennial plant for which the cost of establishing and nurturing beds to full production is estimated to be approximately \$74,000/ha (Roper 2008). This makes the potential cost of diseases, especially viral diseases for which there are no treatments or cures, particularly high. To date, three viruses (*Red ringspot virus*, *Tobacco streak virus* [TSV], and *Blueberry scorch virus*) have been described in cranberry and, in some cases, infections of cranberry with these viruses are latent (Caruso and Ramsdell 1995; Jones et al. 2001; Wegener et al. 2004).

TSV was first reported in asymptomatic cranberry plants during a routine screen of plant material imported into Scotland from New Jersey (Jones et al. 2001). The TSV-positive plants in New Jersey had been transported there from Wisconsin as part of a germplasm collection (Martin et al. 2012). TSV was later confirmed in asymptomatic plants in the plots in New Jersey from which the infected material originated (Martin et al. 2009). In July 2012, cranberry plants bearing disfigured, scarred berries were first observed at three commercial cranberry farms in west-central Wisconsin. The scars appeared on berries as necrotic, irregularly shaped, indented lesions ranging in length from <2 to >10 mm (Fig. 1). Affected berries turned red in color prematurely (Fig. 2). Some affected berries aborted while others matured but were distorted with lesions causing deep indentations and/or cracks. All other organs appeared normal on plants

bearing affected fruit. In almost all cases where symptoms occurred, every berry on a vertically oriented stem (hereafter referred to as an "upright") was symptomatic, and every upright on an affected stolon produced symptomatic fruit, suggesting a systemic infection. Incidence of symptomatic uprights was highly variable among farms, ranging from very difficult to locate to nearly 100% symptomatic uprights in small (approximately 1-m²) patches (Fig. 2). Because TSV had been reported previously in cranberry (Jones et al. 2001; Martin et al. 2009, 2012), we investigated TSV as a possible cause of berry scarring and disfigurement and reported preliminary results (Wells et al. 2014).

TSV is the type member of the genus *Ilarvirus* (family *Bromoviridae*) and has a host range of 140 genera (Fulton 1948; Rabedaux et al. 2005). TSV occurs worldwide, and has been reported from North America, South America, Europe, India, Japan, Australia, New Zealand, and South Africa (Scott 2001). TSV infection can lead to significant yield loss (Reddy and Zehr 2004) and, in many crops studied, TSV is transmitted by pollen, seed, and vegetatively propagated materials (Ghanekar and Schwenk 1974; Greber et al. 1991). Mechanical transmission is possible as well but, in nature, this is believed to occur more frequently in annual hosts than in woody, perennial hosts (Eastwell 2010). The ability to be transmitted both vertically and horizontally can lead to rapid spread of the virus, particularly in vegetatively propagated perennial crops such as cranberry. The widespread distribution and host range of TSV make it difficult to identify exactly when, where, and how TSV enters new plant hosts. Nevertheless, gathering information regarding the relatedness of TSV strains in cranberry to each other as well as to previously reported strains of the virus has the potential to provide insight into how long TSV has been in cranberry, as well as its possible origins.

The specific objectives of this research were to: (i) determine the relationship of TSV in cranberry with berry-scarring symptoms; (ii) determine whether TSV overwinters in cranberry plants; (iii) determine whether TSV is pollenborne or seedborne in cranberry; and (iv) investigate genetic diversity and the possible relationships among TSV strains using molecular phylogenetic analysis of the coat protein (CP) gene.

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Materials and Methods

Detection and identification of TSV in cranberry. *Enzyme-linked immunosorbent assay.* A kit was used for double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) detection and identification of TSV in cranberry plant samples, herbaceous plant samples, or weed samples, according to the manufacturer's instructions (Agdia, Inc., Elkhart, IN). Plant samples were stored at approximately 4°C until they were processed. Leaves from known TSV-negative cranberry plants were included in every plate as negative controls, in addition to the positive control provided with the kit and a buffer-only negative control. For all DAS-ELISA tests, samples were considered positive when the absorbance at 405 nm was greater than three times the average values obtained for the healthy controls, as read by a Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA).

Detection and identification of TSV virions by immunosorbent electron microscopy. In 2012, partially-purified extracts were prepared from 2 to 3 g of infected current-season leaf samples from up-rights bearing scarred fruit ('Mullica Queen') by differential centrifugation, as described previously (Ahlawat et al. 1996). Virions were trapped using rabbit antibodies to TSV (American Type Culture Collection PVAS 49) and observed by transmission electron microscopy in preparations negatively stained with 2% (wt/vol) sodium phosphotungstate, pH 7.0.

Reverse-transcription polymerase chain reaction and CP nucleotide sequence analysis. Leaf samples were stored at 4°C for up to 4 weeks until RNA was extracted. Total RNA was extracted from leaves of infected up-rights using the Qiagen RNeasy Mini Plant Kit (Qiagen, Valencia, CA). For cDNA synthesis, total RNA was incubated according to the recommended protocol after iScript (5×) reverse-transcription supermix (Bio-Rad Laboratories) was added. Following reverse transcription, forward and reverse primers (TSV CP F and TSV CP R, respectively; Tzanetakis et al. 2004) that

Fig. 1. Irregularly shaped, indented lesions observed on cranberry fruit.

Fig. 2. Scarring and premature reddening observed on cranberry fruit at the early fruit set stage of development. Area photographed is approximately 1 m².

amplify an 867-bp fragment of RNA 3, encompassing the CP gene, were used in the polymerase chain reaction (PCR). PCR amplifications were carried out using GoTaq Green Master mix (Promega Corp., Madison, WI) in an Eppendorf master cycler gradient thermal cycler (Eppendorf, Westbury, NY). The thermal cycling profile consisted of an initial denaturation at 95°C for 5 min; followed by 40 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.5% Tris-acetate-EDTA agarose gel. Gels were stained with SYBR Safe (Life Technologies, Grand Island, NY) and viewed on a UV-transilluminator. A 100-bp ladder (Promega Corp.) was used to estimate sizes of PCR products.

The MinElute DNA gel extraction kit (Qiagen) and pGEM-T Easy Vector (Promega Corp.) were used for PCR product purification and cloning, respectively. Sequencing of cloned inserts was performed using the GenomeLab DTCS Quick-Start sequencing kit (Beckman-Coulter, Brea, CA) and the universal M13 forward and reverse primers on the CEQ8000 Genetic Analysis System (Beckman-Coulter). Sequences were first edited using the SeqBuilder module from the Lasergene software package (DNASTAR, Madison, WI) and then aligned using MegAlign Pro (DNASTAR). Sequences were compared with RNA3 (CP) sequences for TSV strains cataloged in the National Center for Biotechnology Information (NCBI) GenBank.

Association of TSV with berry scarring. In 2013 and 2014, up-rights bearing scarred fruit from hybrid cultivars released after 2002 ('Crimson Queen', 'Demoranville', 'Grygleski Hybrid 1', 'HyRed', and Mullica Queen) as well as older cultivars ('Norman LeMunyon', 'Pilgrim', and 'Stevens') (Table 1) of cranberry were collected from several commercial farms throughout the cranberry-growing region of central Wisconsin. Uprights of Crimson Queen, HyRed, Mullica Queen, Pilgrim, and Stevens bearing non-scarred fruit were collected from farms on which berry-scarring symptoms had not been reported to avoid sampling asymptomatic plants that had developed tolerance to TSV (Wells et al. 2014). Initially in 2013, only scarred berries were tested for TSV. If a scarred berry tested negative for TSV, a mixture of current-season and previous-season leaves from the affected upright was tested for TSV. In 2014, a sample consisted of a single upright from which current-season leaves, previous-season leaves, and fruit were tested separately for TSV using DAS-ELISA. If any one of these parts tested positive, then the sample was recorded as positive. This sampling method helped avoid false negatives due to uneven distribution of the virus within plants. In total, 681 and 210 up-rights bearing scarred fruit or nonscarred fruit, respectively, were tested for TSV.

Table 1. Association of berry scarring with *Tobacco streak virus* (TSV) in cranberry

Cultivar	Number of TSV-positive up-rights/total number of up-rights with scarred berries tested ^a	Uprights with scarred berries that tested TSV positive (%)
Crimson Queen	25/25	100
HyRed	14/14	100
Mullica Queen	481/485 ^b	99
Demoranville	11/12	92
Grygleski Hybrid 1	6/7	86
Pilgrim	9/10 ^c	90
Stevens	34/117 ^c	29
LeMunyon	0/11 ^c	0

^a Leaves and fruit of up-rights bearing scarred berries were tested for TSV using double-antibody sandwich enzyme-linked immunosorbent assay. Samples were considered positive when the absorbance at 405 nm was greater than three times the average value of the negative control as read by a Bio-Rad plate reader.

^b For 227 Mullica Queen samples, current-season leaves, previous-season leaves, and symptomatic fruit were tested separately. TSV was detected in 173/227 (76%) of current-season leaves, 210/227 (93%) of previous-season leaves, and 75/227 (33%) of symptomatic berries.

^c Scarred fruit that tested TSV negative tested positive for *Blueberry shock virus*.

Overwintering of TSV in cranberry plants. In 2012, 65 TSV-positive uprights (Mullica Queen) bearing scarred fruit were tagged with twist ties at one farm in central Wisconsin. In 2013, 30 and 22 TSV-positive uprights (Mullica Queen) bearing scarred fruit were tagged with twist ties on the same farm as in 2012 and an additional farm, respectively. All tagging was arbitrarily performed as uprights with scarred berries were observed in the field, and plant parts were tested for TSV as they became available throughout the growing season. In November 2012, plants bearing 28 of the tagged uprights were dug from the field and planted in a 1:2 sand-peat mixture in pots measuring 15 cm in diameter. The potted plants were overwintered in a cold room in Madison, WI for 37 days at approximately 4°C until being transferred to a greenhouse in January (approximately 24°C, 15-h day length provided by supplemental fluorescent lighting). The 37 remaining tagged plants were left to overwinter in the field in 2012. All uprights tagged in 2013 were left to overwinter in the field. In 2013 and 2014, leaves, pollen, and fruit of plants tagged in the field in 2012 and 2013, respectively, were tested for TSV using DAS-ELISA and reverse-transcription (RT)-PCR. Likewise, leaves, pollen, and fruit of plants dug from the field and overwintered in a cold room were tested for TSV.

Detection of TSV in cranberry pollen. In 2013, 2014, and 2015, pollen was collected from flower-bearing uprights (Mullica Queen) that had tested positive for TSV the previous year as well as from uprights in close proximity to the known TSV-positive uprights. Pollen from six to eight flowers was collected into one sterile 1.5-ml microcentrifuge tube to constitute one 0.5- to 2.0-mg sample. Thirty-two samples were collected in total in all 3 years. Tubes containing pollen were stored at 4°C for up to 3 days until tested for TSV. Pollen was washed in 1 ml of ELISA general extraction buffer (GEB; Agdia, Inc.) in a 1.5-ml microcentrifuge tube. The suspension was centrifuged at 13,000 × g for 5 min. The resulting supernatant served as the pollen wash. In 2014 and 2015, these steps were repeated until two to five successive washes were obtained for each pollen sample. The pollen pellet was then crushed using a minipestle, resuspended in 1 ml of GEB, and centrifuged at 13,000 × g for 5 min. This supernatant served as the pollen extract. The pollen washes and pollen extracts were then assayed for TSV by DAS-ELISA (Table 2). Pollen from known healthy, TSV-negative plants was included in every assay plate as a negative control in addition to a buffer-only negative control. Positive controls provided with the kit were also included in every assay plate.

Seed transmission of TSV. In September 2013, ripe berries were collected from TSV-positive uprights and TSV-negative uprights (Mullica Queen). Both scarred and nonscarred berries were collected from TSV-positive uprights. Whole berries were surface disinfested for 30 s in a 70% ethanol solution followed by 10 min in a 10% household bleach solution (final concentration: 0.6% sodium hypochlorite) containing one drop (approximately 3 µl) of Tween 80 per 100 ml of solution. Berries were then rinsed in sterile distilled water and sliced in half transversely. Seeds were extracted using sterile tweezers and stored at 4°C. Seeds were sown in 36-cm² tissue culture boxes (45 seeds/box; Magenta, Chicago) containing 75 ml of water agar (8 g of granulated agar [Fisher Scientific, Fair Lawn, NJ] per

1 liter of deionized H₂O). After approximately 2 months of growth on a lab bench exposed to sunlight, seedlings were tested for TSV using DAS-ELISA. A series of 14 assays were conducted over a period of 12 weeks, running 90 seedlings in any given assay. Seedlings grown from seeds harvested from fruit of known TSV-negative plants were included in every plate as negative controls.

Transmission of TSV to cranberry and other potential hosts. Cranberry plants (Mullica Queen) at a farm where berry-scarring symptoms had not been observed were dug prior to bloom in June 2013 and 2014, planted in a 1:2 sand-peat mixture in pots measuring 15 cm in diameter, and grown in a greenhouse at approximately 24°C, with a 15-h day length provided by supplemental fluorescent lighting. Clumps of cranberry plants in 25 pots in 2013 and 51 pots in 2014 were inoculated with TSV-infected pollen or sap from infected leaves by various methods (Table 3). For pollination inoculations, all flowers that were receptive at the time of inoculation were pollinated, and at least four uprights per pot were inoculated for all other inoculation methods. Additionally, nine 2-year-old rooted cranberry cuttings (Crimson Queen) were inoculated with TSV-infected sap in 2013, and three 2-year-old rooted cranberry cuttings were inoculated with TSV-infected pollen in 2014 (Table 3).

Inoculations were carried out in a greenhouse on 24 and 28 June 2013 and 25 and 26 June as well as 1 July 2014, when the majority of flowers on all plants were receptive to pollen. Pollen was collected from TSV-infected flower-bearing uprights (Mullica Queen), transported to the greenhouse on ice, and used as inoculum on the same day as collection. The following day, pollen was confirmed as TSV positive by DAS-ELISA. In pollination inoculations, pollen was applied to the stigmata of flowers. Cranberry plants which were determined free of TSV were self-pollinated and served as controls.

Table 3. Methods by which transmission of *Tobacco streak virus* (TSV) to cranberry plants was attempted^a

Year of inoculation	TSV inoculum preparation ^b	Inoculation procedure ^c	Number of plants inoculated
2013	TSV (+) pollen	Pollination	13
2013	TSV (+) pollen	Leaf rubbing	6
2013	TSV (+) sap	Leaf rubbing	6
2013	TSV (+) sap	Leaf wounding	9
2013	TSV (–) pollen	Pollination	8
2013	TSV (–) pollen	Leaf rubbing	4
2013	TSV (–) sap	Leaf wounding	3
2013	Buffer	Leaf rubbing	5
2013	No treatment	No treatment	2
2014	TSV (+) pollen	Pollination	21
2014	TSV (+) pollen	Leaf rubbing	10 ^d
2014	TSV (+) sap	Leaf rubbing	20 ^e
2014	TSV (+) pollen	Ovary wounding	10 ^d
2014	TSV (+) sap	Ovary wounding	20 ^e
2014	TSV (+) pollen	Flower wounding	3
2014	TSV (–) pollen	Pollination	3
2014	TSV (–) sap	Leaf rubbing	3
2014	Buffer	Leaf rubbing	2
2014	Buffer	Ovary wounding	5
2014	TSV (–) pollen	Flower wounding	3
2014	No treatment	No treatment	7

^a All but one of the inoculated plants tested negative for TSV in both years.

^b In 2013, pollen was collected from cranberry flowers from uprights on which scarred fruit had been observed in the 2012 growing season. In 2014, pollen was collected from flowers from uprights on which fruit had not been monitored for scarring in the 2012 or 2013 growing season.

^c Rub inoculations (leaf and berry) were done with a sterile cotton swab or with gloved fingers after dusting with diatomaceous earth and surface wounding with a needle. Ovary-wounding inoculations were done with a needle dipped in inoculum. Leaf- and flower-wounding inoculations were done by pressing inoculum into leaves or flowers using forceps with a serrated tip after dusting with diatomaceous earth and rubbing with inoculum.

^d Leaf rubbing and ovary wounding with TSV-positive pollen were performed on the same plants.

^e Leaf rubbing and ovary wounding with TV-positive sap were performed on the same plants.

Table 2. Pollen-wash samples tested for *Tobacco streak virus* (TSV) in infected cranberry

Wash	Number of TSV-positive pollen washes/number of TSV-positive pollen samples		
	2013	2014	2015
1	1/8 ^a	10/15	6/9
2	...	5/14 ^b	4/9
3	...	3/10	0/9
4	...	0/10	...
5	...	0/10	...

^a In 2013, only one wash was completed for each pollen sample.

^b For one sample, wash number 2 tested TSV positive when wash number 1 tested TSV negative.

In leaf-rub inoculations, current-season leaves and previous-season leaves were dusted with diatomaceous earth and scratched with a fine needle, and then inoculum (TSV-positive sap or pollen) was rubbed onto leaves with a sterile cotton swab or with gloved fingers. Approximately 2 h after rub inoculations, all inoculated plant parts were rinsed with distilled water. In leaf-wounding inoculations, current-season leaves and previous-season leaves were dusted with diatomaceous earth, TSV-positive sap was rubbed onto leaves with a sterile cotton swab, and then the inoculum was pressed into leaves using sterile forceps with a serrated tip. Sap was obtained from cranberry plants by grinding infected leaves (1:10, wt/vol) in 0.013 M Tris-HCl, 0.067 M K₂HPO₄, pH 8, containing 0.02 M 2-mercaptoethanol and 2% polyvinyl pyrrolidone (PVP, MW 40,000) (Spiegel and Cohen 1985). In ovary-wounding inoculations, ovaries were pierced with a fine needle that had been dipped in either TSV-positive sap or pollen. In flower-wounding inoculations, flowers (Crimson Queen) were gently wounded within a couple of days prior to opening using spiked forceps dipped in TSV-infected sap inoculum. Any viable flowers were self pollinated. Control plants were inoculated with sap or pollen from known TSV-negative cranberry plants. Plants were observed at least three times per week for symptoms and, after approximately 3 months, leaves and berries were tested for TSV using DAS-ELISA. To determine whether symptom development or detection of TSV required time, the inoculated and control potted plants were overwintered in a cold frame in Madison, WI for approximately 4 months until being transferred to a greenhouse in March (approximately 24°C, with a 15-h day length provided by supplemental fluorescent lighting). Any viable flowers were self pollinated. Plants were again observed at least three times per week for symptoms and, after approximately 1 month, new and previous-season leaves were tested for TSV using DAS-ELISA.

Table 4. Mechanical transmission of *Tobacco streak virus* to herbaceous plants

Plant species inoculated	Inoculum type ^a	Number of TSV-positive plants/number of inoculated plants
<i>Amaranthus caudatus</i>	Pollen	0/3
	Sap	0/10
<i>Capsicum annuum</i> 'Carmen'	Sap	0/9
<i>Chenopodium</i> sp.	Sap	0/6
<i>Glycine max</i> 'Corsoy 79'	Sap	1/5
<i>Gomphrena globosa</i> 'Audray Bicolor Rose'	Pollen	0/3
	Sap	9/9
<i>G. globosa</i> 'Globe Amaranth'	Sap	16/25
<i>Helianthus annuus</i> 'Sunspot'	Pollen	0/3
	Sap	2/9 ^b
<i>H. annuus</i> 'Large Gray Stripe'	Sap	0/4
<i>H. annuus</i> 'Irish Eyes'	Sap	0/8
<i>Nicotiana affinis</i> 'Crimson King'	Pollen	0/3
	Sap	0/9
<i>N. benthamiana</i>	Sap	1/11
<i>N. tabacum</i> 'W38'	Pollen	0/3
	Sap	1/9
<i>N. tabacum</i> 'Xanthi'	Pollen	1/2
	Sap	3/23
<i>Phaseolus vulgaris</i> 'Nickel'	Pollen	0/3
	Sap	0/9
<i>P. vulgaris</i> 'Burpee's Stringless'	Sap	2/10
<i>P. vulgaris</i> 'Bountiful'	Sap	0/7
<i>Vicia faba</i> 'Broad Windsor Long Pod'	Sap	0/9

^a Pollen was collected from cranberry flowers from uprights on which fruit had not been monitored for scarring in previous growing seasons. Rub inoculations (pollen and sap) were done with gloved fingers after dusting with diatomaceous earth.

^b All infected plants were symptomless, except *H. annuus* 'Sunspot', which developed necrotic leaf spots.

In 2014, seeds of 11 herbaceous plant species (Table 4) were grown either in a growth chamber at approximately 24°C, with 14 h of supplemental fluorescent lighting, or in a greenhouse at approximately 21°C, under natural light. For some species, multiple cultivars were tested. In total, 192 plants were mechanically inoculated with either TSV-positive sap or pollen. Rub inoculations of leaves were performed using buffers and methods described above approximately 1 month after sowing. Three control plants for each plant species were inoculated with sap or pollen from known TSV-negative cranberry plants.

Phylogenetic analysis of TSV CP coding region. Leaf samples were collected in 2013 and 2014 from cranberry uprights that showed berry scarring symptoms and that tested positive for TSV by DAS-ELISA. Leaf samples were collected from 2, 3, and 19 uprights from commercial cranberry beds in Massachusetts, New Jersey, and Wisconsin, respectively. Sequences 'NJ88-25' and 'NJ88-93' were obtained from cranberry leaf samples collected from the plots in New Jersey, from which the infected material shipped to Scotland (Jones et al. 2001) originated. Nucleotide sequences of the CP gene were cloned and sequenced as described above. Sequences of three known TSV strains, JX463347, X00435, and JX46336, from *Ajuga* sp., the type isolate of TSV from *Trifolium* sp., and a more divergent isolate from *Helianthus annuus*, respectively, were retrieved from the NCBI GenBank for comparison with TSV strains extracted from cranberry. Nucleotide sequences were aligned using MUSCLE (Edgar 2004) implemented in MEGA, version 6 (Tamura et al. 2013). Phylogenetic trees were constructed based on the maximum-likelihood method and a bootstrap test consisting of 1,000 pseudo-replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein 1985). The distances were computed using the maximum-composite-likelihood method (Tamura et al. 2004). The resulting tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Results

Detection and identification of TSV in cranberry. Three independent, complementary methods were used to detect and identify TSV in cranberry plants. First, leaves, pollen, and berries tested positive by DAS-ELISA (details below). Second, transmission electron microscopy of leaf sap from uprights with scarred berries showed isometric particles approximately 33 nm in diameter (Fig. 3), which

Fig. 3. Transmission electron micrograph of isometric virus particles from cranberry sap preparation.

is characteristic of TSV (Lister et al. 1972). Although these two methods are widely used to detect TSV, TSV antiserum is known to cross react with certain other ilarviruses (Tzanetakis et al. 2004). However, a third method, RT-PCR, yielded the expected 867-bp product from leaves and scarred berries of symptomatic uprights, while no products were obtained from healthy cranberry uprights. Results from sequencing of the RT-PCR product, which corresponds to the CP coding region, revealed 94 to 99% nucleotide similarity with reference TSV sequences. All samples that tested TSV positive by RT-PCR tested TSV positive by DAS-ELISA, and all samples that tested TSV negative by DAS-ELISA also tested TSV negative by RT-PCR. High-quality RNA is sometimes difficult to extract from cranberry fruit using the methods described above, and some berry samples that tested TSV positive by ELISA likely tested TSV negative by RT-PCR due to poor-quality RNA.

Association of TSV with berry scarring. Of 543 uprights of the newer hybrid cranberry cultivars (Crimson Queen, Demoranville, Grygleski Hybrid 1, HyRed, and Mullica Queen) bearing scarred fruit that were tested for TSV, 537 uprights (approximately 99%) tested positive (Table 1). However, of the 138 uprights of older cultivars bearing scarred fruit that were tested for TSV, 43 (approximately 31%) tested positive for TSV (Table 1). Upon further investigation, uprights of the older cultivars (Norman LeMunyon, Pilgrim, and Stevens) bearing scarred fruit that tested negative for TSV tested positive for *Blueberry shock virus* (BShV) by ELISA by a commercial lab (Agdia, Inc.), or by us using a kit similar to the TSV ELISA kit, used according to the manufacturer's instructions (Agdia, Inc.). Of the 227 uprights (Mullica Queen) bearing scarred fruit, for which current-season leaves, previous-season leaves, and a symptomatic berry were tested separately, all tested positive for TSV. Of the 227 current-season and previous-season leaf samples tested, 173 (76%) and 210 (93%) were TSV positive, respectively; and, of the 227 symptomatic berries tested, 75 (33%) tested TSV positive according to DAS-ELISA. All uprights bearing asymptomatic fruit collected from farms where berry-scarring symptoms had not been reported tested negative for TSV.

Overwintering of TSV in cranberry plants. In 2013 and 2014, current-season leaves, previous-season leaves, pollen, and fruit of all plants tagged in the field during the previous year tested positive for TSV by DAS-ELISA and RT-PCR. TSV was first detected in the previous-season leaves and new vegetative growth of these tagged uprights in the field on 22 May 2013 and 6 June 2014, which corresponded to the very early stages of bud break. Pollen and fruit were tested for TSV as soon as they became available in the growing season. Leaves, pollen, and fruit of all plants determined to be TSV positive the previous year that were dug from the field and overwintered in a cold room also tested positive for TSV.

Detection of TSV in cranberry pollen. In 2013, of eight samples tested, one pollen wash and eight pollen pellets were positive for TSV. In 2014, of 15 samples tested, 11 pollen-wash samples and 15 pollen pellets were positive for the virus. Of the 11 TSV-positive pollen-wash samples, 10 of the first washes were positive, 5 of the second washes were positive, 3 of the third washes were positive, and none of the fourth or fifth washes were positive. In 2015, of nine samples tested, six pollen-wash samples and nine pollen pellets were positive for TSV. Of these six TSV-positive pollen-wash samples, all of the first washes were positive, four of the second washes were positive, and none of the third washes were positive. All pollen washes and pellets from healthy, TSV-negative cranberry uprights tested negative for TSV.

Seed transmission of TSV. All seedlings grew normally. Of the 1,251 seedlings grown from seeds harvested from fruit from TSV-positive uprights, none tested positive for TSV. All of the seedlings grown from seeds originating from healthy, TSV-negative cranberry uprights also tested negative for TSV.

Transmission of TSV to cranberry and other hosts. In 2013 and 2014, all inoculated cranberry plants grew normally and no symptoms developed on fruit or leaves. Leaves but not berries from one of the plants inoculated by pollination with TSV-

positive pollen in 2014 tested positive for TSV by DAS-ELISA. All plant parts tested on the remaining 75 inoculated plants were negative for TSV, and all control plants inoculated with TSV-negative pollen, TSV-negative sap, or buffer tested negative for the virus as well.

Of the 192 herbaceous host plants inoculated with TSV-positive sap or pollen, 36 (approximately 19%) tested positive for the virus after approximately 25 days of growth (Table 4). The highest incidence of detection of TSV was in *Gomphrena globosa* inoculated with sap. All plants that tested positive for TSV had been inoculated with sap, except for one plant of *Nicotiana tabacum* 'Xanthi' that tested positive after inoculation with infected pollen. All infected plants were symptomless except *H. annuus*, which developed necrotic leaf spots. Plants inoculated with TSV-negative cranberry sap or pollen grew normally and tested negative for TSV.

Phylogenetic analysis of TSV isolates based on CP coding sequence. Nucleotide sequencing of the CP encoding gene of 26 TSV isolates from cranberry uprights revealed a degree of divergence from previously published sequences of TSV from plants other than cranberry, as well as variation among strains collected from cranberry (Fig. 4). Although sample numbers were limited, there was no evidence of grouping by state or cultivar.

Discussion

TSV has been reported in cranberry previously (Jones et al. 2001) but the current study represents the first report of symptoms associated with TSV infection in this crop. Additionally, the results demonstrate that TSV persists in cranberry plants overwinter, TSV is pollenborne in cranberry, several of the industry's most popular cultivars are susceptible to TSV, and TSV isolates collected from various locations in Wisconsin, New Jersey, and Massachusetts are variable in CP gene sequence.

This work investigated several methods of experimentally transmitting TSV to cranberry plants, in an attempt to reproduce symptoms. In both years, all inoculated cranberry plants grew normally and produced asymptomatic fruit, and all plant parts tested were negative for TSV, with the exception of leaves from one plant that had been pollinated with TSV-positive pollen in 2014. Plants used for inoculations were dug from a commercial cranberry marsh where berry-scarring symptoms had not been observed, and all plant parts tested prior to inoculation tested negative for TSV by DAS-ELISA. However, viruses are sometimes unevenly distributed within plants (Kritzman et al. 2001; Latvala et al. 1997; Smith et al. 2006), and we cannot rule out the possibility that this plant was TSV positive prior to being inoculated. In fact, testing of multiple plant parts of TSV-infected cranberry uprights revealed uneven distribution of the virus within cranberry uprights. Further investigation into the distribution of TSV within affected cranberry plants is necessary to elucidate the disease cycle of TSV in cranberry. Additionally, due to space limitations, inoculated plants were grown in the same greenhouse as known TSV-positive plants and, therefore, it is possible that TSV was vectored to this plant in another manner (e.g., by an arthropod) while in the greenhouse. Mechanical transmission of viruses, especially ilarviruses, to woody plant hosts is difficult, and the infection rate is usually low and inconsistent (Fulton 1966). Because of these limitations, Koch's postulates have not been fulfilled for many of the diseases associated with viruses in woody hosts (Goheen 1988; Martin et al. 2011; Mink 1995; Thekke-Veetil et al. 2014). Despite the unsuccessful attempts at completing Koch's postulates, the association of TSV with berry scarring in cranberry is strong (Table 1). In cultivars released after 2002, 99% of uprights with scarred berries tested positive for TSV. In older cultivars, 31% of uprights with scarred berries tested positive for TSV, and the remaining 69% of uprights with scarred berries tested positive for BShV, another ilarvirus. The difference in TSV infection observed between newer and older cultivars is likely a result of closer examination and more intensive testing of newer cultivars compared with older cultivars because of the value of the newer cultivars to the current and future industry. The origins of BShV and the significance of this virus in Wisconsin and other cranberry-growing areas await investigation.

Many viruses that infect perennial plants have the ability to persist from year to year within their hosts (Boland et al. 2004; Bristow et al. 2002; Duffus 1971; Martin et al. 2012; Pscheidt and Harper 2014). Consequently, it is not surprising that TSV overwinters in cranberry; however, this suggests that, once infected, this virus will persist in a cranberry planting. With proper maintenance, cranberry beds can remain in production for several decades; some beds in Massachusetts and New Jersey were established over 100 years ago (Martin et al. 2012; Oudemans et al. 1998). As such, it will be imperative to conduct further research to investigate the long-term effects that TSV may have in this perennial crop.

TSV is pollenborne in hosts other than cranberry and, in that regard, is similar to other ilarviruses such as BISHV (Bristow and Martin 1999), *Prunus necrotic ringspot virus* (PNRSV) (Hamilton et al. 1984), and *Prune dwarf virus* (Hamilton et al. 1984). In assays designed to gain insight into the presence and location of the virus in or on pollen grains from cranberry, TSV was detected in a subset of pollen washes and all pollen extracts tested. These data suggest that TSV is located both inside and on the outside of pollen grains of cranberry, although we cannot rule out the possibility that detection in pollen washes was the result of infected pollen grains rupturing during centrifugation. Detection of TSV both in and on pollen was demonstrated previously for pollen of TSV-infected tomato, tobacco, and sow thistle plants (Sdoodee and Teakle 1993). The sources of primary inoculum are unknown for the outbreaks of TSV in cranberry beds in recent years, and it is not yet known whether TSV is transmitted by pollen in cranberry.

In crops such as tomato, cucumber, peanut, and cowpea, the involvement of thrips in transmission of TSV has been demonstrated (Prasada Rao et al. 2003; Sdoodee and Teakle 1993). The authors of those studies proposed that thrips obtain TSV-infected plant material (e.g., pollen) on their bodies while feeding on infected plant parts. When these thrips move to uninfected plants, they mechanically inoculate the healthy plants with TSV as the abrasion caused by their feeding creates an entry point for the virus (Rabedeaux et al. 2005). Flower-feeding thrips are routinely detected in cranberry beds in Wisconsin but their involvement in the transmission of TSV in cranberry is unknown.

In blueberry and cherry, the ilarviruses BISHV and PNRSV, respectively, are transmitted horizontally by pollination, making honeybees and native pollinators abundant vectors of these viruses (Bristow and Martin 1999; George and Davidson 1963). Although, in most crops, TSV is not reported to be spread by pollination (Johnson et al. 1984; Sdoodee and Teakle 1988), the detection of the virus in or on cranberry pollen is concerning because it implies that pollinators might be involved in spreading TSV in cranberry. Cranberry growers rely on native bee populations as well as commercial honeybee hives and bumblebee colonies for pollination. During routine flower visits, pollinators might move TSV-contaminated pollen from flowers of infected plants to flowers of healthy plants, depositing pollen for inoculation by thrips or other feeding insects (Bristow and Martin 1999; Mink 1992). It is also possible that pollinators could simultaneously move contaminated pollen and insects such as thrips from infected plants to healthy plants, further

Fig. 4. Inferred phylogenetic tree of 26 *Tobacco streak virus* strains from cranberry based on coat protein nucleic acid sequences. Three reference strains from GenBank (preceded by GB) from *Ajuga* spp., *Trifolium* spp., and *Helianthus annuus* were used for comparison. Bootstrap values based on 1,000 iterations are given for nodes with branch support greater than 50. Those preceded by WI, NJ, and MA were collected in Wisconsin, New Jersey, and Massachusetts, respectively. Abbreviations for cranberry cultivars: CQ = Crimson Queen, MQ = Mullica Queen, ST = Stevens, PI = Pilgrim, DM = Demoranville, and HO = Howes.

exacerbating the spread of the virus (Bristow and Martin 1999; Mink 1992). Therefore, even if TSV is not transmitted through pollination, pollinators could greatly increase the rate and distance over which the virus is spread, increasing the number of plants infected (Mink 1993).

About 20% of plant virus diseases are believed to be seed transmissible (Amari et al. 2009; Mink 1993). TSV is seedborne in several crops, and this method of transmission is key to the perpetuation of this virus (Ghanekar and Schwenk 1974; Kaiser, et al. 1991; Rabedeaux et al. 2005; Sdoodee and Teakle 1988; Sharman et al. 2009; Walter et al. 1992). Cranberry plants for commercial plantings are vegetatively propagated by rooted cuttings, making seed transmission of relatively little significance to the industry. However, volunteer seedlings sometimes do occur if unharvested fruit remain in a bed, or if seeds are discarded by birds or other animals. The data collected from our seed transmission experiments indicate that none of the 1,251 seedlings tested were TSV positive by DAS-ELISA. We cannot dismiss the possibility that use of a different testing method could reveal TSV-positive seedlings but our data suggest that, if seed transmission of TSV does occur in cranberry, it occurs at a very low rate. Nevertheless, if the virus is seed transmitted, even at a low rate, volunteer seedlings would have the potential to serve as reservoirs of TSV in commercial plantings or surrounding natural areas which are a habitat for native and feral cranberry plants.

TSV occurs on numerous farms in the major cranberry-growing regions of Wisconsin and at a few locations in New Jersey and Massachusetts. However, testing in New Jersey and Massachusetts has not been extensive, and the virus may be more widespread in those states. When CP encoding nucleic acid sequences from 26 TSV isolates from cranberry were analyzed, variation among strains was revealed (Fig. 4). However, except for the two isolates from Massachusetts, isolates did not consistently cluster based on location or cultivar of origin. The CP gene was chosen because GenBank accessions representing this gene are readily available. When TSV was first reported in cranberry, three distinct isolates of TSV were detected in a relatively small number of infected plants from New Jersey (Jones et al. 2001). Therefore, the sequence variability we observed in our analyses was not unexpected. This variation suggests that either TSV was introduced into cranberry some time ago and, over time, has been spreading and mutating, or the introduction of variable isolates of TSV into cranberry has been more recent, and is possibly the result of multiple introductions or sources. Because TSV has been reported in diverse hosts, including some families and genera that are weeds on cranberry farms, it is possible that weeds are a reservoir for TSV. However, we failed to detect TSV in 23 samples of *Polygonum*, *Rubus*, *Rumex*, *Taraxacum*, and *Trifolium* spp. collected from cranberry farms in New Jersey and Wisconsin (L. D. Wells-Hansen, unpublished data). The possibility of infection by multiple strains of TSV in a single plant and the effect on symptom development has not yet been determined for cranberry but is an area that should be explored.

In summary, our research confirms that recently observed berry-scarring symptoms on cranberry are associated with TSV infection. The agronomic importance of TSV on a commercial scale in cranberry is not yet known but continued research needs to be conducted in an effort to better understand the impact and epidemiology of TSV in cranberry. This information will ultimately aid in the development of management recommendations in the future. As with all vegetatively propagated crops, caution should be taken to plant virus-free material when establishing new cranberry plantings.

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