Potato Tuber Necrosis Induced by Alfalfa Mosaic Virus Depends on Potato Cultivar Rather Than on Virus Strain

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Abstract

Alfalfa mosaic virus (AMV) was identified as the causal agent of internal tuber necrosis in the potato cultivar Innovator in New Brunswick, Canada. Further pathological characterization of the isolate (designated as isolate CaM) was performed on six potato cultivars and one breeding clone. Upon mechanical inoculation, four cultivars (Innovator, Yukon Gold, Rochdale Gold-Dorée, and Shepody) showed needle-sized necrotic spots and increasing calico symptoms on new leaves, whereas the remaining cultivars only developed calico symptoms on new leaves. All tubers of CaM-infected Innovator and Shepody plants developed sporadic internal necrotic spots, as did ca. 23 and 8% tubers of CaM-infected Yukon Gold and Rochdale Gold-Dorée, respectively. Sequence analysis of the CP gene of CaM with AMV isolates from potato, all presumed belonging to the “non-necrotic” strain and retrieved from GenBank, indicated that CaM shared >97.1% sequence identity with all but four Egyptian isolates.

At the complete genome level, phylogenetic analysis of all available sequences demonstrated that RNA 1 and RNA 3 can be grouped into three major clades each, whereas RNA 2 can be clustered into two clades. CaM and Ca175-1, an AMV isolate that was deemed non-necrotic in a previous study, had different phylogenetic clade patterns, indicating different RNA 1-RNA 2-RNA 3 haplotypes: IA-I-IB (CaM) versus Ca175-1 (IB-II-IA). Despite the difference in haplotype composition, CaM and Ca175-1 induced similar levels of internal necrosis in tubers of Innovator and its parent Shepody. The results suggest that the internal necrosis in AMV-infected tubers depends on potato cultivar rather than on AMV strain/haplotype, and CaM is just a “regular” isolate of AMV.

Keywords: alfalfa mosaic virus, potato tuber necrosis, cultivar response, phylogeny, virus haplotype

Potato, the world's third most important food crop after rice and wheat, is susceptible to numerous diseases and pests. Among them, viral diseases are particularly challenging due to the absence of a cure and the ability to pass on from one generation to another via the infected vegetative propagules (mainly potato tubers) in potato production (Slack and German 2001). Over 35 viruses and one viroid can infect potato naturally and cause a wide range of foliar symptoms ranging from latent infection to plant death, depending on virus species, strain, and potato cultivar (Salazar 1996). In addition to foliar symptoms, various tuber symptoms can also be induced by several viruses such as potato mop-top virus (PMTV, spraing symptoms), tobacco rattle virus (TRV, corky ringspot disease), potato tuber necrotic strain of potato virus Y (PVY^{NTN}, potato tuber necrotic ringspots), potato leafroll virus (PLRV, phloem net necrosis), potato tuber necrotic strain of alfalfa mosaic virus (AMV, external and internal necrosis).

Alfalfa mosaic virus is the type species of the genus *Alfavirus*, family *Bromoviridae*. The virus has a wide host range, naturally infecting more than 150 species of 22 plant families (Jaspars and Bos 1980). It can be transmitted to over 430 species of 51 dicotyledonous families. Potato is one of the natural hosts of AMV. The virus causes bright yellow blotching or mottling on leaves and was thus once called potato calico virus. AMV generally occurs at a low level in potato crops and is considered not economically important to potato production. However, a potato necrotic isolate was reported in 1950 in California, U.S.A. (Oswald 1950). The isolate induced severe foliar symptoms that include systemic necrosis on leaves and stems and premature plant death. In addition, obvious external and internal tuber necrosis was observed on all tested potato varieties. Interestingly, the occurrence of the necrotic strain of AMV appears to be rare, as there was no report for the next several decades until 2015, when AMV was determined to be the causal agent of internal tuber necrosis in potatoes of the cultivar Innovator in New Brunswick, Canada (Nie et al. 2015). However, there were significant differences between the case reported in California and the case observed in New Brunswick. The latter did not incite foliar necrotic strips or premature plant death, nor did it cause external necrosis on tubers.

AMV has been shown to exhibit a certain degree of genetic diversity (Bergua et al. 2014). The virus consists of three single-stranded sense genomic RNAs of about 3.6 kb (RNA 1), 2.6 kb (RNA 2), and 2 kb (RNA 3) (Jaspars and Bos 1980). RNA 1 and 2 encode the viral replicase subunits P1 and P2, respectively (Bol 1999, 2003). RNA 3 encodes the P3 (movement protein, MP) and coat protein (CP), which is translated via RNA 4, a subgenomic RNA derived from RNA 3 (Jaspars and Bos 1980). All RNAs have a cap structure at the 5' end and a tRNA-like structure at the 3' end (Gunn and Symons 1980; Koper-Zwarthoff et al. 1979). Phylogenetic analysis of CP genes has revealed that AMV isolates can be grouped into two

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lineages, irrespective of host origin but related to geographic origin (Parrella et al. 2000, 2011). In potato, all Canadian isolates fall into one lineage group, and were therefore considered as belonging to the same strain group (Xu and Nie 2006). Nevertheless, exceptions have been reported for Egyptian potato isolates, which appeared to form a separate clade in the phylogeny (Abdel Aleem et al. 2018). Similar to that of the CP, two phylogenetic lineages have been reported in P1 and MP genes, respectively (Bergua et al. 2014). Further analysis of the lineage type of the P1, MP, and CP genes indicate that genome reassortment have occurred among isolates in Spain, leading to different haplotypes (Bergua et al. 2014).

Despite progress in understanding genetic diversity of AMV, little is known about whether the molecular diversity is translated into pathological diversity in the virus. Keeping this in mind, we performed this research to: (1) determine whether the presumed necrotic CaM isolate of AMV identified in New Brunswick (Nie et al. 2015) is able to induce tuber necrosis in cultivars other than Innovator; (2) understand the molecular relationship between the “necrotic” isolate and other isolates, especially those deemed “non-necrotic”; and (3) investigate whether internal tuber necrosis induced by AMV is virus-strain-specific and/or potato cultivar dependent. Our results demonstrated that internal tuber necrosis can be induced by all tested isolates of AMV on Innovator and Shepody despite the fact that the isolates were different haplotypes (IA-I-IB versus IB-II-IA) based on the phylogeny lineage of RNA 1, RNA 2, and RNA 3, thus suggesting that the internal necrosis in AMV-infected tubers depends on potato cultivars rather than on the virus haplotypes. The CaM isolate that was previously deemed a necrotic strain is likely to be a regular strain in terms of pathogenicity.

Materials and Methods

Virus isolates and potato cultivars. AMV isolate CaM was obtained from potato tubers exhibiting severe internal necrosis as described previously (Nie et al. 2015) and maintained in plants/tubers of the potato clone F87084 and plants of the tobacco cultivar Samsun in the greenhouse. The isolates Ca175-1, Ca172-2, and Ca175-3 were collected in a previous study (Xu and Nie 2006) and maintained in vitro plantlets of cultivar Russet Burbank at the Charlottetown Laboratory, Canadian Food Inspection Agency, Charlottetown, Prince Edward Island, Canada, until this study. These isolates were verified to be free of other viruses and viroids by next generation sequencing as described previously (Nie et al. 2015). The in vitro plantlets containing Ca175-1, Ca172-2, or Ca175-3 were then grown in the greenhouse and used as inoculum to infect tobacco plants. The isolates were then maintained in plants/tubers of Russet Burbank and plants of the tobacco cultivar Samsun in the greenhouse.

Virus inoculation and symptom observation. For primary infection, virus-free tuber pieces of cultivars Innovator, Shepody, Chieftain, Yukon Gold, Rochdale Gold-Dorée, and Jemseg were planted in 15.2-cm pots containing Pro-Mix potting soil (Premier Horticulture, Rivière-du-Loup, Quebec, Canada), grown in the greenhouse at a temperature setup of 18/24°C (light/dark), a photoperiod of 16/8 h (light/dark), and a humidity of 75%. The ambient light was supplemented with artificial light or shading to give a light intensity of ~90 µm²/s. When the plants reached the 5-leaf stage, they were mechanically inoculated with AMV or inoculation buffer (mock) (10 mM phosphate buffer, pH 7.5, with 32 mM sodium sulfite) as described (Xu and Nie 2006). The plants were monitored for symptom expression until tuber harvest at ~80 days postinoculation (dpi). ELISA or RT-PCR was performed on the plants at 21 dpi to confirm AMV-infection. Upon harvest, tubers were washed, inspected for external symptoms, and stored at 4°C for up to 9 months. Internal necrosis was inspected at 0 (Innovator only), 3, 6, and/or 9 months postharvest after the tuber was cut open along the longitudinal axis. Each inoculation treatment contained 3 to 5 plants/cultivar, and each set of experiments was repeated once. For secondary infection experiments, tuber pieces from primary infection were planted in the greenhouse under the conditions described above. The resulting plants (3 to 5 plants/cultivar) were confirmed for AMV-infection by ELISA or RT-PCR test and monitored for symptom expression until tuber harvest. The tubers were stored at 4°C and

inspected for external and internal necrosis at 6 months postharvest as described above.

ELISA. ELISA with AMV monoclonal antibodies (Adgen Phyto-diagnostics - Neogen, Scotland, UK) was performed according to manufacturer's instructions. A sample was considered positive when absorbance at 405 nm (A_{405}) was three times the negative (healthy) control with a reading ≥ 0.050 . Samples whose A_{405} fell between the criteria (i.e., $\geq$ three times of the healthy control and < 0.050) were retested by RT-PCR (Xu and Nie 2006).

RNA extraction. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, ~0.1 g leaf tissue was added to a 2-ml microtube containing Lysis Matrix A (MP Biomedicals, Solon, OH) and homogenized with 450 µl RLT Buffer (RNeasy Plant Mini Kit) with β -mercaptoethanol using the FastPrep-24 system (MP Biomedicals) for 40 s. The mix then proceeded to the remaining steps described in the kit's manual (RNeasy Plant Mini Kit). The RNA was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Barrington, IL), adjusted to a concentration of 200 ng/µl, and stored at -80°C.

RT-PCR. For RT-PCR detection of AMV, cDNA was synthesized by using random hexanucleotide primers. Specifically, 4 µl of the above-mentioned total RNA, together with 1 µl of random hexanucleotide primers (50 µM) and 1 µl dNTPs (10 µM each of dATP, dGTP, dCTP, and dTTP) (Invitrogen), was incubated at 65°C for 5 min and chilled on ice immediately. Thereafter, 14 µl RT mix containing 4 µl 5× first-strand buffer, 2 µl DTT (0.1 M), 40 U RNaseOUT, and 200 U M-MLV reverse transcription (Invitrogen) was added to denatured RNA. The mix was then incubated at 25°C for 5 min, 37°C for 50 min, and 70°C for 15 min. PCR with the AMV-specific primers AMV-F (5'-CCATCATGAGTTCTTCA CAAAAG-3') and AMV-R (5'-TCGTACGTCATCAGTGAGAC-3') (Xu and Nie 2006) was carried out on a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The 25 µl PCR mix for each reaction contained 1× PCR buffer, 1.5 mM MgCl₂, 0.1 µM each of AMV-F and AMV-R, 0.2 mM dNTP mix, 1.25 U AmpliTaq DNA Polymerase (Applied Biosystems), and 2 µl cDNA. A 30-cycle PCR was performed, starting with a denaturation step of 95°C for 105 s, followed by 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s. Thereafter, a final elongation step of 72°C for 5 min was performed. After the reaction, 10 µl PCR products were electrophoresed on 1.5% agarose gel containing Gel Red (0.5 µg/ml) (Biotium Inc., Hayward, CA). The gel was visualized and recorded with BioSpectrum Imaging System (UVP Inc., Upland, CA).

To determine the complete genome sequence, primers for RNA 1, RNA 2, and RNA 3 were designed (Supplementary Table S1) after aligning all available sequences retrieved from GenBank. cDNAs were synthesized using the universal reverse primer (AMV universal R: 5'-GCATCCCTTAGGGGCATTCATGCA-3') for all RNA species under the conditions as described above. PCR with the common reverse primer paired with RNA 1, RNA 2, and RNA 3 corresponding specific primers (RNA 1: 5'-GTTTTTATCTTACACAC GCTTG-3'; RNA 2: 5'-GTTTTTATCTTTTCGCGATTGA-3'; RNA 3: 5'-ccgGTTTTAATACCATTTCAAAAT-3') was performed with the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) on a C1000 Touch™ Thermal Cycler for 30 cycles under conditions as described above. The PCR products were purified using MinElute Gel Extraction Kit (Qiagen) after agarose gel (1.2%) electrophoresis and cloned into Zero Blunt TOPO PCR Cloning Kit (ThermoFisher Scientific). To obtain the complete sequence of each RNA species, two clones each were sequenced at Genome Quebec (Montreal, Quebec, Canada) with the Sanger method using M13 forward and reverse sequencing primers as well as internal sequencing primers (Supplementary Table S1). The sequences were then assembled using Vector NTI bioinformatics software suite (Invitrogen). The GenBank accession numbers for the three genomic RNAs of the two AMV isolates are MK607973 to MK607978.

Sequence analysis and phylogeny. Sequence identities between virus isolates were analyzed using BLAST (<https://www.ncbi.nlm.nih.gov/BLAST>). For recombination event (RE) and phylogeny

analyses, the complete sequences of RNA 1, RNA 2, or RNA 3, including the full-length sequences reported in this study (MK607973 to MK607978), as well as the full-length sequences of the RNA species retrieved from GenBank (March 2019) were aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The aligned sequences were then subjected to phylogenetic analysis using MEGA6 software (Tamura et al. 2013) and recombination event detection using RDP4 software (version 4.71) (Martin et al. 2015). A sequence was considered “recombinant” if the recombination event was detected by at least four of the nine methods in the RDP package with P values less than 1×10^{-6} each (Gibbs et al. 2017).

Statistical analysis. Analysis of variance (ANOVA) and pairwise comparisons of internal tuber necrosis induced by different AMV isolates on the cultivars Innovator and Shepody were conducted using R software version 3.4.0 (R Core Team 2016).

Results

Pathological characterization of AMV-CaM in potato cultivars.

To test the pathogenicity of the isolate CaM, six potato cultivars (Innovator, Yukon Gold, Rochdale Gold-Dorée, Shepody, Chieftain, and Jemseg) were mechanically inoculated with the isolate. The breeding clone F87084 was included for the analysis. As shown in Figure 1A, F87084 developed calico symptoms after infection with CaM. No other symptoms were observed in the plants, and tubers obtained from the plants were free of both external and internal necrosis.

Seven days post mechanical inoculation, sporadic necrotic spots (Fig. 1B) were observed on the inoculated leaves of cultivars Innovator, Yukon Gold, Rochdale Gold-Dorée, and Shepody. However, the local lesions did not appear to enlarge or to spread on these leaves during the course of plant growth and development. Approximately 2 weeks after the inoculation, yellowish spots started to appear on the

newly emerged leaves, which may be coupled with the needle-sized necrosis, depending on cultivars (Fig. 1C to E). As time progressed, the calico symptoms became more profound in cultivars Innovator, Rochdale Gold-Dorée, F87084, Yukon Gold, and Jemseg; whereas in Chieftain, the symptoms remained largely at the same level (Fig. 2). It is also noteworthy that the distinct calico symptoms mainly occurred in leaves located 3 to 4 nodes below the uppermost leaves (Supplementary Fig. S1). As the plants grew older, the bright yellow calico turned pale yellow.

Approximately 1 in 5 tubers of AMV-infected Innovator and Shepody exhibited external small bruise-like symptoms (Fig. 3, indicated by arrow). These symptoms were typically associated with internal necrosis spanning the whole tuber (Fig. 3). Regardless of the external appearance, all tubers of AMV-infected plants of Innovator and Shepody exhibited internal necrosis (Table 1). In contrast, all tubers of AMV-negative control (mock) plants were necrosis-free. In Yukon Gold, about 23% tubers (7/31) obtained from 10 AMV-inoculated plants developed internal necrosis. In Rochdale Gold-Dorée, 3 out of 39 tubers of AMV-inoculated plants ($n = 10$) exhibited internal necrosis. Tubers of Chieftain, Jemseg, and F87084 did not exhibit internal necrosis, regardless whether they were from AMV-infected or AMV-free plants. It is noteworthy that the necrosis remained at the same level of severity during the complete storage period (data not shown).

As anticipated, calico symptoms occurred in the secondarily infected plants. Depending on cultivar, the calico symptoms ranged from slightly yellow/pale to bright yellow (Supplementary Fig. S2). Yukon Gold exhibited the most distinct calico with bright yellow color in the front leaflets and the edge of rear leaflets in leaves located 2 to 3 nodes from the top (Supplementary Fig. S2E). Overall, the symptoms in other cultivars were largely similar to those

Fig. 1. Example of symptoms expressed on leaves of potato plants after primary infection with alfalfa mosaic virus (AMV) isolate CaM. **A**, Systemic symptoms on leaves of F87084. Left, leaf of a mock-inoculated plant; right, leaf of an AMV-inoculated plant at 21 days postinoculation (dpi). **B**, Necrotic spots on an AMV-inoculated leaflet of Innovator at 7 dpi. **C** to **E**, Systemic symptoms (**C**, Chieftain; **D**, Rochdale Gold-Dorée; **E**, Yukon Gold) on leaves that emerged from AMV-inoculation at 14 dpi.

exhibited in the primary infection. No clear-cut necrotic symptoms were observed on the foliage of all tested cultivars. Similar to those observed in the primary infection, all tubers of the secondarily infected Innovator and Shepody plants (10 for Innovator; 7 for Shepody) developed internal necrosis (Table 1). In Yukon Gold and Rochdale Gold-Dorée, 20% (2/10) and 31% (8/26) tubers of secondarily infected plants (6 for Yukon Gold, 10 for Rochdale Gold-Dorée) developed internal necrosis, respectively. Tubers of Chieftain, Jemseg, and F87084 did not exhibit internal necrosis, regardless of whether they were from AMV-infected or AMV-free plants.

Molecular characterization of AMV. To investigate the difference between this isolate and other potato isolates found around the world, the CP gene, which is embedded in RNA 3, was initially compared for deciphering the necrosis-inducing ability of the isolate. The CP gene of AMV-CaM is 657 nt in length, encoding a 218 amino acid (aa)-long CP protein. Interestingly, the CP gene of other potato isolates reported in Canada is 666 nt-long encoding a 221 aa-long CP. Further comparison with isolates reported outside of Canada indicates the existence of both 666 and 657 bp types: the former includes HQ288892 (Egypt), KY700830 (5, Egypt), KY471416 (CP2, Egypt), KY549683 (RE3, Egypt), KY549684 (RE4, Egypt), AF294432 (KR1, Korea), AF294433 (KR2, Korea); the latter includes JQ685860 (Ke.Ba.Po, Iran), KX535507 (Es.Fa.Po, Iran), KX535508 (Ha.Da.Po, Iran), KX535509 (Ke.Ke.P, Iran), KX535510 (Ke.Ma.Po, Iran), KX535511 (Ke.Ma.Po2, Iran), and KX535512 (Ke.Ji.Po, Iran). Except for four isolates (CP2, RE4, 5, and RE3) from Egypt, the sequence identities between CaM and other isolates were high, ranging from 97.1 to 99.2% at the nucleotide level and 97.2 to 100% at the amino acid level (Table 2). The sequence identities between CaM and the four Egyptian isolates ranged from 93.3 to 66.3% at the nucleotide level and 89.4 to 42.8% at the amino acid level. As none of the isolates are reported as a necrotic strain regardless of the length of the CP/CP gene, sequencing of the complete genome of the isolate CaM and a representative isolate

(Ca175-1) that was determined to be nonnecrotic by Xu and Nie (2006) is thus necessary.

As shown in Table 3, RNA 1 and RNA 2 were 3,643 and 2,594 nt-long, respectively, for both isolates CaM and Ca175-1. The RNA 3, on the other hand, was 2,038 nt-long for CaM and 2,037 for Ca175-1. RNA 1 has one open reading frame (ORF) of 3,381 nt, encoding a 1,126 aa-long P1 protein/replicase; RNA 2 has one ORF of 2,373 nt, encoding a 730 aa-long P2 protein/replicase; RNA 3 has two ORFs, encoding a movement protein at 300 aa long and a CP at 218–221 aa-long CP. Sequence comparison indicated that, at the complete RNA level, isolates CaM and Ca175-1 shared 98.1,

Fig. 3. Example of necrotic symptoms on tubers of plants infected with alfalfa mosaic virus (AMV) isolate CaM. Arrow indicates the external bruise-like symptom on a tuber (cv. Shepody) that exhibited internal necrosis. Mock, tubers of mock-inoculated plants; AMV, tubers of AMV isolate CaM-infected plants.

Fig. 2. Systemic symptoms on leaves of plants of different cultivars after infection with alfalfa mosaic virus (AMV) isolate CaM. **A**, Chieftain. **B**, Yukon Gold. **C**, Innovator. **D**, Jemseg. **E**, Rochdale Gold-Dorée. **F**, Shepody. The experiments were performed independently two times, and same results were obtained. Photos were taken at 3 weeks postinoculation on one of the experiments.

95.4, and 97.2% sequence identities for RNA 1, RNA 2, and RNA 3, correspondingly. Consistent with that for the complete RNA segments, the highest sequence identities were found for P1 at 98.2% at the nucleotide level and 99.6% at the protein level, followed by CP (97.6 and 98.6% at the nucleotide and protein level, respectively), P3 (96.3 and 96.7% at the nucleotide and protein level, respectively), and P2 (95.1% at the nucleotide level and 94.1% at protein level).

Phylogenetic analysis of all available complete sequences of RNA 1, RNA 2, and RNA 3 of AMV regardless of their host of origin depicted three major clades for RNA 1 and RNA 3 and two

major clades for RNA 2 (Fig. 4). Interestingly, the isolates CaM and Ca175-1 were not always clustered within the same group. In RNA 1 and RNA 3, they were categorically placed within the major clades (clade I in RNA 1 and RNA 3) represented by the alfalfa isolate Manfredi; however, in RNA 2, CaM was grouped to the clade of Manfredi (clade I) where Ca175-1, together with its sister isolate 175 (MF990285), was placed at a major separate clade (clade II) that included the clover isolate HZ (HQ316636) (clade II-B), and the isolates Chinese (DQ139866, host unknown) and A1M4 (X01572, clover). In RNAs 1 and 3, a sequence entry from Egypt (KX579896 in RNA 1 and KY549685 in RNA 3) stood alone to

Table 1. Occurrence of tubers exhibiting internal necrosis in plants of different cultivars infected with alfalfa mosaic virus (AMV) isolate CaM

	Primary infection ^y			Secondary infection ^y		
	Repeat 1	Repeat 2	Occurrence ^z	Repeat 1	Repeat 2	Occurrence ^z
Innovator						
Mock (control)	0/2, 0/2, 0/3, 0/5	0/4, 0/5, 0/2, 0/6, 0/4	0/33 (0%)	0/3, 0/3, 0/2, 0/2, 0/2	0/2, 0/3, 0/2, 0/1, 0/2	0/23 (0%)
CaM	2/2, 3/3, 1/1, 1/1, 2/2	6/6, 4/4, 2/2, 2/2, 8/8	31/31 (100%)	2/2, 2/2, 1/1, 7/7, 2/2	2/2, 4/4, 2/2, 3/3, 5/5	30/30 (100%)
Shepody						
Mock (control)	0/1, 0/2	0/3, 0/2, 0/1	0/9 (0%)	0/1, 0/1, 0/1	0/2, 0/1, 0/3, 0/3	0/12 (0%)
CaM	2/2, 1/1, 1/1, 1/1	2/2, 1/1, 3/3, 2/2	13/13 (100%)	1/1, 1/1, 1/1	1/1, 2/2, 3/3, 2/2	11/11 (100%)
Yukon Gold						
Mock (control)	0/2, 0/2, 0/3	0/2, 0/3, 0/3, 0/4	0/19 (0%)	0/2, 0/1, 0/1	0/2, 0/1, 0/1, 0/3	0/11 (0%)
CaM	1/2, 1/4, 1/2, 0/5, 1/4	1/3, 0/2, 1/3, 0/4, 1/2	7/31 (23%)	1/1, 0/1, 0/1, 0/1	0/1, 1/5	2/10 (17%)
Rochdale						
Gold-Dorée						
Mock (control)	0/6, 0/9, 0/8, 0/5	0/4, 0/3, 0/2, 0/3	0/28 (0%)	0/4, 0/2	0/3, 0/2, 0/4, 0/1	0/16 (0%)
CaM	0/6, 1/5, 0/3, 1/4, 0/3	0/4, 1/4, 0/3, 0/4, 0/3	3/39 (8%)	1/2, 0/2, 1/2, 1/2, 2/2	1/4, 0/3, 1/4, 1/3, 0/2	8/26 (31%)
F87084						
Mock (control)	0/3, 0/4, 0/4	0/2, 0/4, 0/2	0/19 (0%)	0/4, 0/2, 0/2, 0/5	0/4, 0/3, 0/2, 0/4	0/26 (0%)
CaM	0/3, 0/2, 0/2, 0/3	0/2, 0/3, 0/2, 0/4	0/21 (0%)	0/3, 0/2, 0/2, 0/4	0/2, 0/3, 0/2, 0/5	0/23 (0%)
Jemseg						
Mock (control)	0/1, 0/2, 0/4	0/2, 0/3, 0/5, 0/2	0/19 (0%)	0/2, 0/4	0/2, 0/3, 0/4, 0/3	0/18 (0%)
CaM	0/3, 0/2, 0/2, 0/2, 0/3	0/2, 0/3, 0/2, 0/4	0/23 (0%)	0/1, 0/2, 0/2, 0/3	0/2, 0/3, 0/2, 0/4	0/19 (0%)
Chieftain						
Mock (control)	0/6, 0/4, 0/4, 0/5, 0/3	0/5, 0/4, 0/6, 0/5	0/42 (0%)	0/4, 0/2, 0/5	0/4, 0/3, 0/4, 0/5, 0/3	0/30 (0%)
CaM	0/4, 0/7, 0/6, 0/4, 0/6	0/6, 0/4, 0/4, 0/3, 0/3	0/47 (0%)	0/2, 0/3, 0/1, 0/1	0/3, 0/4, 0/2, 0/5, 0/3	0/24 (0%)

^y Number of tubers exhibiting internal necrosis/total number of tubers of each plant.

^z Sum of number of tubers exhibiting internal necrosis/sum of total number of tubers in each treatment.

Table 2. Sequence homology between coat protein (CP)/CP gene of alfalfa mosaic virus (AMV) isolate CaM and that of other AMV potato isolates

Acc. no.	Isolate	CP gene/CP length	nt sequence identity (%)	AA sequence identity (%)	Country of origin	References
KX535508	Ha.Da.Po	657/218	652/657 (99.2)	218/218 (100)	Iran	-
KX535512	Ke.Ji.Po	657/218	650/657 (98.9)	218/218 (100)	Iran	-
KX535509	Ke.Ke.Po	657/218	649/657 (98.8)	218/218 (100)	Iran	-
JQ685860	Ke.Ba.Po	657/218	648/657 (98.6)	217/218 (99.5)	Iran	-
KX535510	Ke.Ma.Po	657/218	648/657 (98.6)	218/218 (100)	Iran	-
DQ314749	Ca375	666/221	645/654 (98.6)	216/218 (99.1)	Canada	Xu and Nie 2006
KX535507	Es.Fa.Po	657/218	646/657 (98.3)	218/218 (100)	Iran	-
DQ314752	Ca400	666/221	643/654 (98.3)	216/218 (99.1)	Canada	Xu and Nie 2006
DQ314751	Ca399	666/221	641/654 (98.0)	214/218 (98.2)	Canada	Xu and Nie 2006
DQ314753	Ca401	666/221	641/654 (98.0)	214/218 (98.2)	Canada	Xu and Nie 2006
HQ288892	n/a	666/221	641/654 (98.0)	212/218 (97.2)	Egypt	El-Helaly et al. 2012
KX535511	Ke.Ma.Po2	657/218	642/657 (97.7)	216/218 (99.1)	Iran	-
DQ314754	Ca508	666/221	639/654 (97.7)	214/218 (98.2)	Canada	Xu and Nie 2006
DQ314755	Ca518	666/221	639/654 (97.7)	214/218 (98.2)	Canada	Xu and Nie 2006
DQ314756	Ca616	666/221	639/654 (97.7)	214/218 (98.2)	Canada	Xu and Nie 2006
AF294433	KR2	666/221	638/654 (97.6)	215/218 (98.6)	Korea	-
DQ314750	Ca175	666/221	636/654 (97.2)	213/218 (97.7)	Canada	Xu and Nie 2006
MF990286	175	666/221	638/654 (97.6)	213/218 (97.7)	Canada	-
AF294432	KR1	666/221	635/654 (97.1)	212/218 (97.2)	Korea	-
KY471416	CP2	666/221	612/656 (93.3)	195/218 (89.4)	Egypt	Abdel Aleem et al. 2018
KY549684	RE4	666/221	544/662 (82.2)	155/219 (70.8)	Egypt	Abdel Aleem et al. 2018
KY700830	5	666/221	500/671 (74.5)	126/224 (56.3)	Egypt	Abdel Aleem et al. 2018
KY549683	RE3	666/221	435/656 (66.3)	96/224 (42.9)	Egypt	Abdel Aleem et al. 2018

form its own clade (clade III in RNAs 1 and 3) (Fig. 4). Further examination revealed that isolates CaM and Ca175-1 grouped separately in subclades of clade I in both RNA 1 and RNA 3. In RNA 1, CaM clustered more closely to the subclade (I-A) represented by Manfredi (KC881008), whereas Ca175-1 and its sister isolate 175 (MF990284) formed a separate subclade (I-B) with the clover isolate 425 Leiden (L10063). In RNA 3, Ca175-1 and its sister isolate 175 (MF990286) clustered with the subclade (I-A) represented by the alfalfa isolate Manfredi (KC881010), whereas CaM grouped with the clover isolates HZ (HQ316637), the Lucerne isolate S (X00819), *Lavandula stoechas* isolate Lst (FN667967), the *Trifolium pratense* isolate AMV-PV1 (MG600289), and the Lucerne isolate YSMV (M59241) to form the subclade I-B. Based on the phylogeny lineage of RNA 1, RNA 2, and RNA 3, CaM and Ca175-1 belonged to two different haplotypes, i.e., IA-I-IB for CaM and IB-II-IA for Ca175-1.

Recombination detection analysis of the RNA species detected one recombination event in RNA 1 of ER1 (KX579896, major parent, unknown; minor parent, 425 Leiden; putative breakpoint, nt 2665; supported by seven algorithms in the RDP package, all with $P < 1 \times 10^{-7}$), an Egyptian potato isolate that formed its own clade (clade III) in the phylogenetic tree (Fig. 4). It is noteworthy that several insignificantly/insufficiently supported recombination events

(3 in RNA 1, 1 in RNA 2, and 3 in RNA 3) were also observed in the analysis (Supplementary Table S2).

Internal necrosis induction in Innovator and Shepody by Ca175-1 as well as its sister isolates Ca175-2 and Ca175-3. To test internal tuber necrosis development in AMV-infected Innovator and Shepody cultivars by the CaM isolate, virus-free plants of each cultivar were inoculated with CaM, Ca175-1, Ca175-2, and Ca175-3. Isolates Ca175-1, Ca175-2, and Ca175-3 were obtained from the same field in a survey carried out by Xu and Nie (2006), and did not incite internal necrosis in Chieftain and Jemseg (data not shown). Newly emerged leaves developed pale mosaic symptoms and needle-sized necrotic spots 2 weeks postinoculation in plants infected with any of the tested AMV isolates including CaM. The infection was confirmed by ELISA. Examination of tubers from the plants demonstrated that all tested AMV isolates induced internal necrosis in tubers of both cultivars Innovator and Shepody (Fig. 5) at a mean frequency from three repeated experiments (two primary and one secondary infection experiments) equal to or higher than 81%. Tubers of the mock-inoculated plants were completely internal necrosis-free (Table 4). For Innovator, the mean frequencies of tuber necrosis induced by different AMV isolates were: CaM, 96%; Ca175-1, 85%; Ca175-2, 97%; and Ca175-3, 89%. For Shepody, the tuber necrosis frequencies were: CaM, 100%; Ca175-1, 88%;

Table 3. Sequence homology between alfalfa mosaic virus isolates CaM and Ca175-1^z

Isolate	RNA 1		RNA 2		RNA 3		
	Full length (nt)	P1 gene (nt) / P1 protein (aa)	Full length (nt)	P2 gene (nt) / P2 protein (aa)	Full length (nt)	P3 gene (nt) / P3 protein (aa)	P4 gene (nt) / P4 protein (aa)
CaM	3,643	3,381 / 1,126	2,594	2,373 / 790	2,038	903 / 300	657 / 218
Ca175-1	3,643	3,381 / 1,126	2,954	2,373 / 790	2,037	903 / 300	666 / 221
Identity (CaM versus Ca175-1)							
	3,575 / 3,643 (98.1%)	3,320 / 3,381 (98.2%)	2,475 / 2,594 (95.4%)	2,257 / 2,373 (95.1%)	1,981 / 2,038 (97.2%)	870 / 903 (96.3%)	638 / 654 (97.6%)
	-	1,122 / 1,126 (99.6%)	-	744 / 790 (94.1%)	-	290 / 300 (96.7%)	215 / 218 (98.6%)

^z The GenBank accession numbers are: MK607973 (CaM RNA 1), MK607974 (Ca175-1 RNA 1), MK607975 (CaM RNA 2), MK607976 (Ca175-1 RNA 2), MK607977 (CaM RNA 3), and MK607978 (Ca175-1 RNA 3).

Fig. 4. Phylogenetic trees of genomic RNA species of alfalfa mosaic virus (AMV). Phylogenetic analyses were performed with MEGA6 (Tamura et al. 2013) using the Maximum Likelihood method. Numbers at nodes are percent support values based on 1,000 bootstrap iterations; and values greater than 70% are indicated. The tree for each RNA species is drawn to scale, with branch lengths measured in the number of substitutions per site. Each isolate is labeled by its accession number and name. Host and country of origin of the isolate are shown in brackets. The isolates reported in this study are indicated by ^z. Accession numbers of RNA 1 to 3 for isolate CaM are MK607973, MK607975, and MK607977, correspondingly; and for Ca175-1 are MK607974, MK607976, and MK607978, correspondingly.

Ca175-2, 95%; and Ca175-3, 95%. Statistical analysis indicated that the tuber necrosis occurring frequencies in different treatments were not significantly different at $P = 0.05$.

Discussion

Several viruses include PVY strain NTN (PVY^{NTN}), PMTV, TRV, PLRV, and AMV necrotic strain, together with the bacterium '*Candidatus Liberibacter solanacearum*', have been known to cause various forms of necrosis on potato tubers (Karasev and Gray 2013; Loebenstein 2001; Oswald 1950; Salazar 1996; Secor et al. 2009; Yellareddygar et al. 2018a, b). The affected tubers lose their market value, leading to significant economic losses to both the producers and the processors. The CaM isolate of AMV identified in New Brunswick (Nie et al. 2015) was presumed to be a necrotic strain similar to the one reported in California (Oswald 1950). However, comparison of the symptoms of CaM infection in seven different potato cultivars/clones with the described symptoms of the AMV necrotic strain infection indicates considerable differences: the latter included severe foliar necrosis and plant death in all 10 tested cultivars/seedlings (White Rose, Netted Gem, Pontiac, Mohawk, Chippewa, Teton, Houma, Sequoia, Red Warba, and USDA seedling 41956)

and severe external tuber necrosis in eight of the 10 cultivars (all but Sequoia and Red Warba). In contrast, CaM induced mainly the characteristic calico symptoms on leaves of the tested cultivars and internal necrosis in tubers of four cultivars. Although needle-sized necrotic spots occurred on the uninoculated leaves of cultivars including Innovator, Shepody, and Yukon Gold, the necrosis remained at the same level. Moreover, no premature leaf drop or plant death was observed in any of the tested cultivars upon CaM infection. These results demonstrate clearly that the two isolates possess different pathogenicity on potato, with the California isolate being unarguably a necrotic strain and CaM being a "regular" strain. The internal tuber necrosis of CaM-infected Innovator and Shepody were likely due to these cultivars' sensitivity to AMV in general rather than due to the isolate's pathogenicity. This hypothesis was further supported by the occurrence of internal necrosis in tubers of Innovator and Shepody plants infected with any of three AMV isolates that had been deemed nonnecrotic previously (Xu and Nie 2006).

Symptom expression in plants upon virus infection is determined by both virus or virus strain and host or host genotype (Salazar 1996). The characteristic potato tuber necrotic ringspots caused by PVY only occur in certain potato cultivars (e.g., Yukon Gold, Yukon

Fig. 5. Induction of internal tuber necrosis by different isolates of alfalfa mosaic virus (AMV) in cultivars Innovator and Shepody.

Table 4. Frequency of tubers exhibiting internal necrosis from Shepody and Innovator plants infected with different isolates of alfalfa mosaic virus (AMV)

	Primary infection ^y		Secondary infection ^y	Occurrence ^z
	Repeat 1	Repeat 2		
Innovator				
Mock (control)	0/2, 0/3, 0/1, 0/3	0/4, 0/1, 0/1, 0/1	0/8, 0/2, 0/5, 0/5	0/36 (0%) a
CaM	1/1, 3/3, 1/1, 1/1	1/1, 2/2, 2/2, 1/1, 2/2	3/3, 3/3, 1/2, 3/3	24/25 (96%) b
Ca175-1	2/2, 1/1, 2/3	1/1, 1/1, 1/1, 3/3	1/2, 3/5, 2/2	17/21 (81%) b
Ca175-2	1/1, 1/1, 2/2, 2/3	3/3, 2/2, 2/2, 3/3, 1/1	5/5, 3/3, 2/2, 2/2	29/30 (97%) b
Ca175-3	1/1, 1/1, 1/1, 1/1	2/2, 2/2, 1/1, 2/2	2/2, 2/2, 6/6, 3/6	24/27 (89%) b
Shepody				
Mock (control)	0/3, 0/2, 0/4	0/2, 0/1, 0/3, 0/1	0/3, 0/3, 0/3, 0/2	0/27 (0%) a
CaM	2/2, 3/3, 1/1, 2/2, 3/3	2/2, 1/1, 1/1, 1/1	2/2, 1/1, 2/2, 2/2	23/23 (100%) b
Ca175-1	3/3, 2/2, 2/2	1/1	2/2, 1/3, 2/2, 1/1	14/16 (88%) b
Ca175-2	2/2, 1/1, 3/3, 1/1	2/2	3/3, 4/4, 2/3, 2/2	18/19 (95%) b
Ca175-3	2/2, 3/3, 1/1, 2/2, 2/2	1/1, 2/2, 2/2	3/3, 1/2, 2/2	21/22 (95%) b

^y Number of tubers exhibiting internal necrosis/total number of tubers of each plant.

^z Sum of number of tubers exhibiting internal necrosis/sum of total number of tubers in each treatment. Values with the same letter are not significantly different at $P = 0.01$.

Gem, AC Chaleur) upon infection by an isolate belonging to PVY^{NTN} strain group (Karasev and Gray 2013; Nie et al. 2013; MacKenzie et al. 2019). Nevertheless, most, if not all, potato cultivars/clones tested to date exhibit a certain degree of susceptibility to PMTV-induced spraing disease and TRV-induced corky ringspot disease (Yellareddygar et al. 2018a, b), suggesting that the PMTV-induced tuber necrosis is potato genotype independent and likely to be virus strain independent. On the other hand, cases of potato cultivar/genotype-dependent and virus strain-independent tuber necrosis, in addition to the apparent cultivar-dependent responses manifested in internal tuber necrosis upon AMV infection, are shown by the potato tuber net-necrosis induced by PLRV in cultivars such as Russet Burbank (sensitive to net-necrosis), Premier Russet (moderate sensitive to net-necrosis), and Bannock Russet (very resistant to net-necrosis) (Douglas and Pavek 1972; Novy et al. 2002, 2008). It is noteworthy that Innovator is a progeny of Shepody, and the same level of sensitivity to AMV-induced internal tuber necrosis between the two suggests that the trait is heritable. More research is needed to unveil the genetics controlling the trait as well as the gene(s) involved in the process. From the practical perspective, selection for cultivars that are insensitive to AMV-induced tuber necrosis is necessary in order to minimize the disease-caused economic losses.

AMV diversity is manifested at the individual genomic RNA level as well as the haplotype level (Bergua et al. 2014). Indeed, at least two major phylogeny clades were found for each RNA species when all available complete sequences, including CaM and Ca175-1, were analyzed. The clade diversity in each RNA could thus lead to different haplotype combinations in AMV isolates, as evidenced by the apparent haplotypes IA-I-B (RNAs 1-2-3) for CaM and IB-II-IA (RNAs 1-2-3) for Ca175-1. Bergua et al. (2014) reported a total of seven haplotypes in AMV populations in Spain. It is noteworthy that for the AMV isolates studied in Spain, the P2 coding region, which is embedded in RNA 2, demonstrated a single cluster; whereas in this study, not only were two major clusters (clades) observed for all RNA 2 of AMV isolates worldwide, but also the two Canadian potato AMV isolates CaM and Ca175-1 were found to be grouped into two separate clades. Considering this difference between AMV isolates from Spain and Canada, it is reasonable to speculate that the number of AMV haplotypes in Canada may be at the same level of that in Spain, if not higher. Clearly, more research is needed in order to unveil the population structure of AMV in Canada. What interests us most is that the haplotype difference between CaM and Ca175-1 as well as its sister isolates Ca175-2 and Ca175-3 did not lead to different pathogenicity in potato, further strengthening the notion that the internal tuber necrosis induced by AMV is virus strain/haplotype independent but potato cultivar dependent, and the CaM is just a “regular” isolate.

Alfalfa has been implicated in influencing AMV occurrence in neighboring cultivated plants such as snap bean (Mueller et al. 2012), as well as potato (Oswald 1950). However, the virus does not appear to be easily transmitted to potato plants (McDonald and Suzuki 1983); and the calico disease of potato, an indicator of AMV infection, is rarely encountered in potato crops in both Canada (McDonald and Suzuki 1983; Xu and Nie 2006; M. Singh, *unpublished data*) and the United States (Zeng et al. 2019). This may explain why the AMV-internal tuber necrosis in sensitive cultivars was not uncovered until now.

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