Internalization and Dissemination of Human Norovirus and Animal Caliciviruses in Hydroponically Grown Romaine Lettuce

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Fresh produce is a major vehicle for the transmission of human norovirus (NoV) because it is easily contaminated during both pre- and postharvest stages. However, the ecology of human NoV in fresh produce is poorly understood. In this study, we determined whether human NoV and its surrogates can be internalized via roots and disseminated to edible portions of the plant. The roots of romaine lettuce growing in hydroponic feed water were inoculated with 1×10^6 RNA copies/ml of a human NoV genotype II strain (GII.4) or 1×10^6 to 2×10^6 PFU/ml of animal caliciviruses (Tulane virus [TV] and murine norovirus [MNV-1]), and plants were allowed to grow for 2 weeks. Leaves, shoots, and roots were homogenized, and viral titers and/or RNA copies were determined by plaque assay and/or real-time reverse transcription (RT)-PCR. For human NoV, high levels of viral-genome RNA (10^5 to 10^6 RNA copies/g) were detected in leaves, shoots, and roots at day 1 postinoculation and remained stable over the 14-day study period. For MNV-1 and TV, relatively low levels of infectious virus particles (10^1 to 10^3 PFU/g) were detected in leaves and shoots at days 1 and 2 postinoculation, but virus reached a peak titer (10^5 to 10^6 PFU/g) at day 3 or 7 postinoculation. In addition, human NoV had a rate of internalization comparable with that of TV as determined by real-time RT-PCR, whereas TV was more efficiently internalized than MNV-1 as determined by plaque assay. Taken together, these results demonstrated that human NoV and animal caliciviruses became internalized via roots and efficiently disseminated to the shoots and leaves of the lettuce.

The *Caliciviridae* family includes a number of significant enteric viruses that cause gastroenteritis in humans and animals. Examples of these viruses include human norovirus (NoV), human sapovirus, and the newly discovered monkey calicivirus (Tulane virus [TV]) (15, 16). Human NoV is the leading cause of nonbacterial gastroenteritis worldwide, contributing to over 95% of all nonbacterial acute gastroenteritis each year and more than 60% of all food-borne illnesses reported annually (43). The virus is highly contagious, resistant to commercially used disinfectants, and has a low infectious dose (2, 29, 49). However, human NoV remains difficult to study because it cannot be grown in cell culture and it lacks a small-animal model (14, 44). Furthermore, there is no vaccine or antiviral drug against this virus (33, 47). Currently, human NoV is classified as a category B biodefense agent by the National Institute of Allergy and Infectious Diseases (NIAID).

To date, most of our understanding of the stability and persistence of human NoV in foods comes from the study of surrogate viruses. Three cultivable animal caliciviruses, feline calicivirus (FCV), canine calicivirus (CaCV), and murine norovirus (MNV), have been used extensively as human NoV surrogates (2, 7, 30, 40, 44, 56). Although these animal caliciviruses share various degrees of genetic relatedness with human NoV, they differ from human NoV in clinical manifestations, host receptors, susceptible cell types, pathogenesis, and immunity (23, 26, 44, 47, 48). Therefore, whether these surrogates truly represent human NoV remains unknown (42). Recently, a new primate calicivirus, TV, was discovered in the stools of captive rhesus macaques in the Tulane National Primate Research Center (18). TV replicates *in vitro* in rhesus monkey kidney cells and causes typical cytopathic effect (CPE) (17, 18). Importantly, TV also recognizes the histo-blood group antigens (HBGAs) similarly to human NoV. The complete genome of TV has been sequenced, and TV is genetically closely related

to human NoV when compared with other caliciviruses (17, 18). Thus, TV could serve as a useful surrogate for human NoV.

In recent years, the consumption of fresh produce has increased as individuals strive to maintain a healthy diet. However, disease surveillance has shown that vegetables and fruits are major vehicles for the transmission of human NoV, since they normally undergo little or no processing and are easily contaminated pre- and postharvest through irrigation, fertilizers, soil, wildlife, domestic animals, packaging, and food handlers (1, 13, 28, 32, 41). It has been reported that norovirus accounts for more than 40% of outbreaks caused by fresh produce in the United States annually (11, 45). Fresh produce-related outbreaks caused by noroviruses have been reported for lettuce, salad, fruit salad, tomato, carrot, melon, strawberry, raspberry, orange juice, fresh cut fruit, coleslaw, spring onion, and other vegetables (11, 13, 21, 32, 38). In another survey, it was found that salads, lettuce, and fruits contributed 67%, 47%, and 67%, respectively, to human NoV gastroenteritis in the United States from 1990 to 2005 (11). The increasing outbreaks of viruses in fresh produce give considerable urgency to understanding the ecology of enteric viruses in vegetables and fruits and the mechanism of viral contamination and persistence in fresh produce.

Internalization of pathogens is considered one of the potential routes for contamination of fresh produce. It has been well established that food-borne bacterial pathogens, such as *Escherichia coli*

Received 2 April 2012 Accepted 18 June 2012

Published ahead of print 22 June 2012

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doi:10.1128/AEM.01081-12

O157:H7 and *Salmonella*, become internalized and disseminated in plant crops, including lettuce, spinach, tomato, and mung bean shoots, via the plant root systems, through wounds in the cuticle, or through stomata in laboratory settings (4, 22, 25). The efficiency of internalization of bacterial pathogens in plants can be affected by many factors, such as the type of plant, plant stress, bacterial species and strain, bacterial dose, environmental humidity, and temperature (3, 13, 25, 45). In contrast, Sharma et al. failed to detect any internalization of *E. coli* O157:H7 in spinach grown in soil, suggesting that uptake of *E. coli* O157:H7 from soil to internal plant tissue is a rare event (46). To date, whether pathogen internalization occurs in fresh produce during field production is still poorly understood. Recently, Erickson et al. found that internalization of *E. coli* O157:H7 via plant roots in the field is a rare phenomenon and that when it does occur, *E. coli* O157:H7 does not persist 7 days later (15). Hence, internalization of pathogens in fresh produce during field production remains poorly understood and is highly debated. The feasibility of internalization of human enteric viruses by plants is supported by the ability of plants to internalize their own viral pathogens, which can be taken up from soil and water (22, 36, 39). Many plant viruses are transported with water, minerals, and photosynthetic products throughout the plant tissues (36). In addition, many plant viruses encode the viral movement (M) protein that assists in their movement within the plant system (36). As the size of a virus is approximately 1,000 times smaller than that of a bacterium, in theory, the efficiency of a smaller pathogen to enter and disseminate in plants would be elevated. Since human enteric viruses may be present in sewage-contaminated soil or water, they may potentially be taken into the plant through the roots and/or leaves. The dissemination of the viruses via the vascular system of the plant could also facilitate movement of the virus from the inedible portions of the plant (roots) to the edible portions of the plant (leaves).

To date, only two studies have examined whether human NoV and its surrogates can be internalized and disseminated in plants (52, 55; reviewed in reference 22). Urbanucci et al. found that canine calicivirus RNA could be detected in the aerial tissues of lettuce grown both hydroponically and in soil, though not all samples in the treatment groups tested positive (52). In contrast, when a human NoV genogroup II (GII) strain was used under the same experimental conditions, no viral RNA in the lettuce was detected even after challenge with a high level of human NoV (10^6 to 10^7 RNA copies/ml) (52). Most recently, Wei et al. found that less than 2 log of infectious MNV-1 could be detected in leaf samples from days 1 to 5 when the roots were challenged with a high level of MNV-1 (5×10^8 PFU/ml) (55). However, no infectious virus was detected when the roots were challenged with a low level of MNV-1 (5×10^5 PFU/ml). Furthermore, infectious MNV-1 was undetectable when lettuce was grown in soil inoculated with even a high level of MNV-1 (5×10^8 PFU/ml) (55). These two studies demonstrated that low levels of virus internalization of human NoV surrogates, such as MNV-1 and CaCV, can occur in growing lettuce. However, based on Urbanucci's study (52), it seems that human NoV cannot be internalized via roots and disseminated to leaves of lettuce. The basis for the differences seen in the rates of internalization by human NoV and its surrogates has not been elucidated.

The objective of this study was to evaluate the internalization and dissemination of human NoV in hydroponically growing lettuce using a GII genotype 4 (GII.4) human NoV strain, which is

currently the prevalent strain circulating in many countries. In addition, we compared the efficiency of viral internalization and dissemination of different caliciviruses (MNV-1, TV, and human NoV) in lettuce.

MATERIALS AND METHODS

Viruses and cell culture. Murine norovirus strain MNV-1 was generously provided by Herbert W. Virgin IV, Washington University School of Medicine. Tulane virus was a generous gift from Xi Jiang at Cincinnati Children's Hospital Medical Center. MNV-1 and TV were propagated in confluent monolayers of the murine macrophage cell line RAW 264.7 and the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA), respectively. RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C in a 5% CO₂ atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.1. After 1 h of incubation at 37°C, 15 ml of DMEM with 2% FBS was added. The virus was harvested 2 days postinoculation by three freeze-thaw cycles and low-speed centrifugation at $1,000 \times g$ for 30 min. MK2-LLC cells were cultured at 37°C in a 5% CO₂ atmosphere in low-serum Eagle's minimum essential medium (Opti-MEM) supplemented with 2% FBS. For growing TV stock, MK2-LLC cells were washed with Hanks' balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 0.1. After 1 h of incubation at 37°C, 15 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days postinoculation and subjected to three freeze-thaw cycles, followed by centrifugation at $1,000 \times g$ for 30 min.

Characterization of a human norovirus GII.4 strain. Human NoV clinical isolate 5 M was originally isolated from an outbreak of acute gastroenteritis in Ohio. The stool samples were diluted 1:10 in phosphate-buffered saline (PBS), shaken vigorously for 10 min at 4°C, and centrifuged for 10 min at $5,000 \times g$. The sample was filtered through a 0.45- μ m filter, aliquoted, and stored at -80°C until use. The entire genomic cDNA of the human NoV strain 5 M was amplified by reverse transcription (RT)-PCR using five to six overlapping fragments. The PCR products were then purified, cloned into a pGEM-T easy vector (Promega), and sequenced at the Plant Microbe Genetics Facility at The Ohio State University. The full-length genome of the viral isolate was 7,558 nucleotides and has been deposited in GenBank with accession number JQ798158. Sequence comparison found that the strain belongs to the norovirus genotype GII.4. The genomic RNA was then quantified by real-time RT-PCR, and the GII.4 isolate 5 M was found to have 6.7×10^6 genomic RNA copies/ml.

Plant cultivation for hydroponic growth. Seeds of romaine lettuce (*Lactuca sativa*) were planted in 2-in. plug trays and grown under greenhouse conditions. Twenty days after germination, plants were removed from the soil and inserted in the hydroponic growth system. The hydroponic feed water was supplemented with a nutrient solution containing nitrogen, phosphorus, and potassium. The feed water was aerated using a pump system. This aeration allowed for movement of the water within the growth tank. The water was not replaced over the study period. The feed water was also supplemented with 1% penicillin, kanamycin, and streptomycin to inhibit microbial growth. After viral inoculation, the plants were grown in the laboratory under a fluorescent light cycle of 12 h light and 12 h darkness. The temperature and relative humidity were maintained at 20°C and 40%, respectively.

Viral inoculation and sample collection. The hydroponic feed water was inoculated with either MNV-1 or TV. The total volume of the hydroponic feed water reservoir was 800 ml, which was inoculated with 50 ml of viruses to have a starting titer of 1×10^6 PFU/ml.

The roots of lettuce were maintained in virus-inoculated feed water for 14 days. No fresh water or virus inoculum was added after the initial virus inoculation. The viral titer in the water was monitored throughout the experiment. Controls received no viral inoculation in feed water. At days 0 (before viral inoculation), 1, 2, 3, 7, and 14, the leaves, shoots, and roots

FIG 1 Schematic of harvesting procedure of romaine lettuce. Leaf tissue represents the aerial tissues of the lettuce starting 2 in. above the root juncture and was harvested first. Shoot tissue represents the 2-in. portion of the aerial tissue connected to the root juncture and was not in contact with the feed water, which was harvested second. Root tissue consists of all lettuce roots and was in direct contact with the feed water and was harvested third.

were harvested and weighed. Three plants were harvested each day for each treatment group. To prevent any possible contamination, leaves were harvested separately from shoots and roots for each plant (Fig. 1). A physical barrier was installed to separate shoots from roots. The shoot is the 2-inch-high portion connected to the roots and directly above the physical barrier. The samples were homogenized by freezing with liquid nitrogen and grinding with a mortar and pestle. Homogenized tissue was resuspended in 5 ml PBS, pH 7.0. Sample homogenates were centrifuged at $1,000 \times g$ to remove cellular debris, and the virus-containing supernatant was transferred to a new collection tube for viral enumeration by plaque assay. At days 0, 1, 2, 3, 7, and 14, 500- μ l samples of feed water were collected for determination of viral titer by plaque assay. Where indicated, the harvested plant tissues were treated with 1,000 ppm of sodium hypochlorite (pH 10.0), which was prepared from chlorine bleach containing 6% sodium hypochlorite. The purpose of this treatment is to inactivate any virus that may be present on the surface of plant tissues. For chlorine-treated samples, following harvest, each tissue was submerged in a 50-ml conical tube containing 1,000 ppm chlorine and incubated at room temperature for 5 min. After the chlorine wash, samples were placed in a new 50-ml tube containing tap water and submerged for 5 min with gentle agitation. Following the tap water wash, samples were placed in a 50-ml tube containing 0.25 M sodium thiosulfate to neutralize residual chlorine. All solutions were changed between samples to maintain the oxidation potential of the chlorine solution. Samples were then homogenized and processed as described above. For human NoV, the feed water was inoculated to a starting concentration of 1×10^6 RNA copies/ml, while controls received no inoculums. Sample collection methods were the same as described above. Quantification of viral genomic RNA was executed using RT-quantitative PCR (qPCR). Each experiment was run once, with three plants for each data point. Each tissue sample was processed individually and the PFU count or RNA copy number per gram was calculated using the weight of the sample. Each data point represents the average of 3 samples.

Virus enumeration by plaque assay. MNV-1 and TV were quantified by plaque assay in RAW 264.7 and LLC-MK2 cells, respectively (15, 23). Briefly, cells were seeded into 6-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h of incubation, RAW 264.7 and MK2-LLC cell monolayers were infected with 400 μ l of a

10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37°C with gentle agitation every 10 min. The cells were overlaid with 3 ml of Eagle's minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg kanamycin/ml, 0.05 mg gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37°C and 5% CO₂ for 2 days, the plates were fixed in 10% formaldehyde. The plaques were visualized by staining with 0.05% (wt/vol) crystal violet. Viral titer was expressed as the mean log₁₀ PFU/ml $\pm$ standard deviation.

Quantification of viral RNA by real-time RT-PCR. Since human NoV cannot be grown in cell culture, real-time RT-PCR was used to quantify viral genomic RNA copies. Briefly, total RNA was extracted from samples using an RNeasy kit (Qiagen), followed by real-time PCR. First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using the primer VP1-P1 (5'-TTATAATACACGTCTGCGC CC-3'), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom TaqMan primers and probes (Forward primer, 5'-CACCGCCGGGAAAATCA-3', reverse primer, 5'-GCCTTCAGTTGGGAAATTTGG-3', and reporter, 5'-FAM-ATTTCAGCAGTCCC-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). PCR and cycling parameters followed the manufacturer's protocol (Invitrogen). Briefly, TaqMan Fast Universal Master Mix was used for all reactions. For cycling parameters, a holding stage at 95°C was maintained for 20 s prior to cycling, followed by 50 cycles of 95°C for 1 s for annealing and 60°C for 20 s for extension. Standard curves and StepOne software version 2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as the mean log₁₀ genomic RNA copies/ml $\pm$ standard deviation.

To compare the internalization rate between human NoV and Tulane virus, Tulane virus RNA was also quantified by RT-qPCR. First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using the primer TVRT (5'-AATTCCACCTTCAACCCAAGTG-3'), which targets the VP1 gene of Tulane virus. The VP1 gene was then quantified by real-time PCR using custom TaqMan primers and probes (forward primer, 5'-TTGCAGGAGGGTTTCAAGATG-3', reverse primer, 5'-CACGGTTTCATTGTCCCCATA-3', and probe, 5'-FAM-TGATGCA CACATGTGGA-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems). PCR, cycling parameters, and quantification method were identical to those used with human NoV.

RNAse treatment of lettuce tissues. Following harvest, processed romaine lettuce samples were stored at -80°C. Samples were then thawed, and 100- μ l aliquots were incubated with 10 μ l (0.5 μ g/ μ l) of RNase (Invitrogen) at 37°C for 1 h. Samples were subjected to RNA extraction using an RNeasy kit (Qiagen), followed by real-time RT-PCR using the procedures described above.

Statistical analysis. Statistical analysis by one-way multiple comparisons was performed by using Minitab 16 statistical analysis software (Minitab, Inc., State College, PA). A *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession number. The full-length genomic sequence of human NoV strain 5M described in the present study has GenBank accession number [JQ798158](https://www.ncbi.nlm.nih.gov/nuclot/JQ798158).

RESULTS

TV was efficiently internalized and disseminated in romaine lettuce grown hydroponically. The TV feed water in the reservoir for romaine lettuce hydroponic growth had a starting titer of 1.25×10^6 PFU/ml. To prevent contamination, the leaves, shoots, and roots of the lettuce were harvested separately at days 0, 1, 2, 3, 7, and 14 after viral inoculation (Fig. 1). The kinetics of the internalization and dissemination of TV were monitored. TV was detected in leaves as soon as day 1 postinoculation with an average titer of 5.7 PFU/g. The viral titer in the leaves gradually increased through day 14 (Fig. 2). At day 7 postinoculation, the viral titer reached 9.8×10^5 PFU/g, which was significantly higher than on

FIG 2 Internalization of TV in romaine lettuce grown hydroponically. Feed water was inoculated with TV stock to a starting titer on day 0 of 1.25×10^6 PFU/ml. The titer of the feed water was monitored throughout the 14-day study period and is reported as PFU/ml. At days 1, 2, 3, 7, and 14, roots, shoots, and leaves of romaine lettuce were harvested, homogenized with liquid nitrogen and mortar and pestle, and resuspended in 5 ml of sterile PBS. Sample homogenate was then subjected to centrifugation at $1,000 \times g$ for 30 min, and the supernatant was tested for infectious viral particles by plaque assay. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent ± 1 standard deviation.

days 1, 2, and 3 (Fig. 2). The TV titer in the leaves on day 14 was 6.3×10^5 , which was comparable to the titer on day 7 (Fig. 2). Similarly, infectious TV was also detected in the shoots on all days tested, with a viral titer in the shoots of 7.8×10^3 PFU/g on day 1 (Fig. 2). The viral titer gradually increased and reached a peak titer of 2.4×10^6 PFU/g on day 7. The TV titer in the shoots on day 14 was 1.3×10^6 , which was a slight decrease compared to the titer on day 7 ($P > 0.05$). During the experimental period, the viral titer in shoots was significantly higher than that in leaves ($P < 0.05$). As expected, TV was detected in lettuce roots since they were in direct contact with virus-contaminated feed water. On day 1, the titer in roots was 1.5×10^5 PFU/g, and the root titer increased until day 14 (Fig. 2). The TV titers in the roots on days 7 and 14 were 1.2×10^6 PFU/g and 1.0×10^6 PFU/g, respectively, and the viral titer in shoots at day 7 was higher than that found in roots ($P < 0.05$). These results suggest that TV efficiently attached to roots, internalized in roots, and disseminated into shoots and leaves of the lettuce. Concurrently, the titer of the feed water was also monitored each day until the plants were harvested. Consistent with the increasing viral titer in lettuce, the titer of the TV in the feed water gradually decreased during the experimental period. On days 1, 2, 3, 7, and 14, the titers in the feed water were 3.75×10^5 , 7.5×10^5 , 3.5×10^4 , 7.5×10^4 , and 5.0×10^3 PFU/ml, respectively. To further confirm that the decreasing titer in feed water was due to internalization via roots and not to the instability of TV in feed water, TV was diluted in feed water (without lettuce) and the viral titer was monitored until day 14. TV was found to be highly stable in the feed water alone over the 14-day period, with no significant reduction in viral titer (data not shown). Taken together, these results suggest that TV was internalized via roots and disseminated to the shoot and leaf portions of the plants.

As the plants were grown hydroponically, it is possible that the

FIG 3 Chlorine treatment of lettuce tissue after TV internalization and dissemination. Feed water was inoculated with TV stock to a starting titer on day 0 of 1.25×10^6 PFU/ml. At days 1, 2, 3, 7, and 14, roots, shoots, and leaves of romaine lettuce were harvested and submerged in 50 ml of chlorine solution (1,000 ppm) for 5 min. Plant tissues were washed with 50 ml of tap water for 5 min by gentle agitation. Following tap water wash, samples were homogenized with liquid nitrogen and mortar and pestle and resuspended in 5 ml of sterile PBS. The residual chlorine was neutralized by 0.25 M sodium thiosulfate. Sample homogenate was then subjected to centrifugation at $1,000 \times g$ for 30 min, and the supernatant was tested for infectious viral particles by plaque assay. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent ± 1 standard deviation.

shoots and leaves of lettuce may have been contaminated by virus moving on the external surface of the plant through capillary action. To exclude this possibility, we performed an identical experiment with the exception that the harvested plant tissues were submerged in 50 ml of 1,000 ppm chlorine for 5 min. It was found that TV was completely inactivated when incubated with 1,000 ppm of chlorine for 1 min (data not shown). Treatment of lettuce with 1,000 ppm of chlorine for 5 min is sufficient to inactivate TV that may be present on the surface of the plant tissues (data not shown). As shown in Fig. 3, there were no significant differences observed in TV internalization in chlorine-treated shoots and leaves on any of the study days compared to that in the untreated samples ($P > 0.05$) during the experimental period. However, there was a significant difference in the detection of TV between untreated roots and chlorine-treated roots on day 1 ($P < 0.05$). Presumably, this is due to the inactivation of surface virus by chlorine, because roots directly contacted the virus-contaminated feed water. However, there were no differences in TV internalization in the chlorine-treated roots on day 2, 3, 7, or 14 compared to that in the roots receiving no treatment ($P > 0.05$). Taken together, the experiment confirmed that TV was indeed absorbed by roots and disseminated to shoots and leaves of lettuce.

Internalization and dissemination of MNV-1 in romaine lettuce grown hydroponically. The kinetics of MNV-1 internalization in lettuce was also determined. The starting titer (day 0) of MNV-1 in feed water in the reservoir for hydroponically grown romaine lettuce was 2.5×10^6 PFU/ml. The experimental design was identical to that described above. Figure 4 shows the dynamics of MNV-1 titers in leaves, shoots, roots, and feed water. In leaf tissues, MNV-1 was detected on days 1, 2, 3, 7, and 14 using plaque

FIG 4 Internalization of MNV-1 in romaine lettuce grown hydroponically. Feed water was inoculated with MNV-1 stock to a starting titer on day 0 of 2.5×10^6 PFU/ml. The titer of the feed water was monitored throughout the 14-day study period and is reported as PFU/ml. At each time point, roots, shoots, and leaves of romaine lettuce were harvested, homogenized with liquid nitrogen and mortar and pestle, and resuspended in 5 ml of sterile PBS. Sample homogenate was then subjected to centrifugation at $1,000 \times g$ for 30 min, and the supernatant was tested for infectious viral particles. Data shown in the internalization kinetics plot were determined by plaque assay, and results are reported as PFU/g. Data points are the averages of three replicates. Error bars represent ± 1 standard deviation.

assays. On day 1, the viral titer detected in the leaves was 5.9×10^1 PFU/g, and it increased to 3.3×10^5 PFU/g on day 3 and remained at this level for the duration of the study (Fig. 4). Similarly, all shoots harvested from days 1 to 14 were positive for infectious MNV-1. On day 1, 5.9×10^1 PFU/g of MNV-1 was detected in the shoots. The titer increased until day 3 to 3.3×10^5 PFU/g, and again the level of virus detected in the shoots remained stable until day 14 (Fig. 4). All plaque assay results for roots were positive. MNV-1 was detected in the roots on day 1 at 6.5×10^3 PFU/g; the titer increased until day 3 to reach 2.5×10^5 PFU/g, and it was maintained in the roots until day 14 (Fig. 4). The MNV-1 titer in the feed water gradually decreased. The initial titer (day 0) in feed water was 2.5×10^6 PFU/ml. On day 1, the titer decreased to 2.5×10^5 PFU/ml, on day 2, the titer was further decreased to 2.5×10^4 PFU/ml, and a similar titer was maintained until day 14 (Fig. 4). In feed water without lettuce as a control, the MNV-1 titer was not significantly decreased (data not shown). This result indicates that the decreasing titer in feed water of growing lettuce was due to the internalization of MNV-1 via roots of lettuce and not to the instability of MNV-1 in feed water. The starting titers of the feed water of both TV and MNV-1 were comparable. However, the titers of TV detected in the roots on days 1, 7, and 14 were significantly higher than those of MNV-1. The TV titers detected in the shoots on days 7 and 14 were also significantly higher than the MNV-1 titers detected in shoots. However, the TV titer in the leaves was only significantly higher than the MNV-1 titer on day 7. These results indicate that TV was more efficient than MNV-1 in attachment, internalization, and dissemination in lettuce.

Internalization and dissemination of human NoV in romaine lettuce grown hydroponically. To determine the rate of human NoV internalization, romaine lettuce was grown hydro-

FIG 5 Detection of internalized human NoV GII.4 RNA in romaine lettuce grown hydroponically. Feed water was inoculated with human NoV GII.4 stock to a starting titer on day 0 of 2.9×10^6 RNA copies/ml. The titer of the feed water was monitored throughout the 14-day study period and is reported as RNA copies/ml. At each time point, roots, shoots, and leaves of romaine lettuce were harvested, homogenized with liquid nitrogen and mortar and pestle, and resuspended in 5 ml of sterile PBS. Sample homogenate was then subjected to centrifugation at $1,000 \times g$ for 30 min, and the supernatant was tested for the presence of human NoV RNA. Data shown in the internalization kinetics plot were determined by RT-qPCR, and results are reported as RNA copies/g. Data points are the averages of three replicates. Error bars represent ± 1 standard deviation.

ponically and the feed water source was inoculated with human NoV GII.4 isolate 5 M at a starting titer of 2.9×10^6 RNA copies/ml. The experimental design and procedures were identical to those described above. The kinetics of viral RNA in leaf, shoot, root, and feed water were quantified by real-time RT-PCR. A high level of human NoV RNA (6.9×10^5 RNA copies/g) was detected in the leaf tissue of the lettuce on day 1 postinoculation, and the human NoV RNA detected in the leaves remained stable over the 14-day study period (Fig. 5). Human NoV RNA was also detected in the shoots of lettuce on day 1 postinoculation at a titer of 2.1×10^6 RNA copies/g (Fig. 5), which was significantly higher than that in leaves ($P < 0.05$). Similarly, the RNA copies detected in the shoots remained stable over the study period to a final titer of 4.4×10^5 RNA copies/g on day 14 (Fig. 5). Root samples were also positive for human NoV RNA on day 1 postinoculation, with a titer of 3.9×10^5 RNA copies/g (Fig. 5). The human NoV RNA detected in the roots reached a peak titer (3.15×10^6 RNA copies/g) at day 3 postinoculation and decreased to 1.95×10^4 RNA copies/g on day 14. The human NoV RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/ml on day 14 (Fig. 5). These results demonstrated that human NoV was efficiently internalized and disseminated in lettuce grown hydroponically.

Subsequently, we compared the internalization rates of human NoV and Tulane virus. Tulane virus was quantified by both plaque assay and real-time RT-PCR. As shown in Fig. 6, Tulane virus RNA was detected at a high titer in the leaves (1.9×10^6 RNA copies/g) on day 1 postinoculation and remained stable over the 14-day study period. Similarly, the RNA detected in the shoots was also detected at day 1 postinoculation at a titer of 1.2×10^6 RNA copies/g (Fig. 6). The TV RNA detected in the shoots also re-

FIG 6 Detection of internalized TV RNA in romaine lettuce grown hydroponically. Feed water was inoculated with TV stock to a starting titer on day 0 of 1.4×10^6 RNA copies/ml. The titer of the feed water was monitored throughout the 14-day study period and is reported as RNA copies/ml. At each time point, roots, shoots, and leaves of romaine lettuce were harvested, homogenized with liquid nitrogen and mortar and pestle, and resuspended in 5 ml of sterile PBS. Sample homogenate was then subjected to centrifugation at $1,000 \times g$ for 30 min, and the supernatant was tested for the presence of TV RNA. Data shown in the internalization kinetics plot were determined by RT-qPCR, and results are reported as RNA copies/g. Data points are the averages of three replicates. Error bars represent ± 1 standard deviation.

maintained stable over the 14-day study period, with no significant change in the amount of RNA detected throughout the study ($P > 0.05$). TV RNA was also detected in the roots of lettuce on day 1 postinoculation at a titer of 3.2×10^6 RNA copies/g (Fig. 6). The RNA titer found in the roots remained stable over the 14-day study period and was similar to the results obtained for RNA copies in the leaf and shoot tissue. The Tulane virus RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/ml on day 14 (Fig. 6).

Upon comparison, it was realized that there was a significant difference in the kinetics of Tulane virus internalization determined by the two detection methods, real-time RT-PCR and plaque assay. A higher level of Tulane virus RNA (2.5×10^4 to 1.4×10^5 RNA copies/g) was detected in leaves and shoots at days 1 and 2 postinoculation using real-time RT-PCR (Fig. 6), compared to a relatively low level of infectious viral particles (1 to 3 log PFU/g) in leaves and shoots at days 1 and 2 using the plaque assay (Fig. 2). It was hypothesized that there might be noninfectious viral particles or naked RNA present in leaves and shoots at days 1 and 2. To address this possibility, we treated all of the samples with 5 μ g of RNase A in order to degrade any exogenous RNA before RNA extraction, and viral RNA was quantified by real-time RT-PCR. In all day 1 samples tested, there was an approximately 2.5-log reduction in the amount of Tulane virus RNA detected in the roots after RNase treatment compared to the amount in samples which were not treated with RNase (Fig. 7A). Also, day 1 shoots treated with RNase had an approximately 1.3-log reduction in viral RNA compared to the amount in untreated samples (Fig. 7B). In plant tissues on other days, there was a 1-log reduction or less in RNA detection due to treatment with RNase (Fig. 7A to C). This indicates that some naked viral RNA was present in plant tissues which was degraded by RNase treatment. It is likely that the

FIG 7 Detection of internalized TV RNA in romaine lettuce treated with RNase. Amounts of 100 μ l of processed lettuce samples from the experiment whose results are shown in Fig. 6 were treated with 5 μ g of RNase at 37°C for 1 h. The RNA was extracted using an RNeasy kit (Qiagen), followed by real-time RT-PCR. Results are reported as RNA copies/g. Data points are the averages of three replicates. Error bars represent ± 1 standard deviation. Statistical differences ($P < 0.05$) are indicated by an asterisk.

naked viral RNA originated from virus particles damaged within the plant tissues.

To determine whether the amount of human NoV RNA detectable in lettuce samples would be affected by pretreatment with RNase, the same RNase treatment used on Tulane virus in lettuce samples was applied to human NoV samples. In contrast to TV, pretreatment with RNase did not have a significant effect on the amount of human NoV RNA that was detected in day 1 lettuce root samples (Fig. 8A). There was an approximately 1-log reduction in the viral RNA detected in shoots after treatment with RNase (Fig. 8B). For both TV and human NoV, there was not a reduction in the RNA detected in the leaf tissue on day 1 due to

FIG 8 Detection of internalized human NoV RNA in romaine lettuce treated with RNase. Amounts of 100 μ l of processed lettuce samples from the experiment whose results are shown in Fig. 5 were treated with 5 μ g of RNase at 37°C for 1 h. The RNA was extracted using an RNeasy kit (Qiagen), followed by real-time RT-PCR. Results are reported as RNA copies/g. Data points are the averages of three replicates. Error bars represent ± 1 standard deviation. Statistical differences ($P < 0.05$) are indicated by an asterisk.

treatment with RNase (Fig. 7C and 8C). The RNase treatment reduced the amount of viral RNA detected in plant tissues by approximately 0.5 to 1.5 log on all other study days (Fig. 8A to C). Finally, we increased the RNase level to 25 μ g per sample, followed by RNA extraction and real-time RT-PCR. The viral RNA levels detected using 25 μ g of RNase treatment were similar to those detected after treatment with 5 μ g of RNase (data not shown). These results suggest that (i) the levels of human NoV RNA detected are from the mixture of intact virus particles and naked viral

RNA in plant tissues; (ii) RNase treatment degraded the naked RNA; and (iii) intact virus particles persisted in plant tissues for at least 14 days.

DISCUSSION

Human NoV is the leading causative agent of fresh produce-associated outbreaks. However, the interaction of human NoV with fresh produce is poorly understood. In this study, using hydroponically grown romaine lettuce as a model, we experimentally demonstrated that human NoV and its surrogates became internalized via roots and was efficiently disseminated to the shoots and leaves of the plants. Although it has been documented that a low level of internalization and dissemination of MNV-1 and CaCV occurs in lettuce (52, 55), this is the first report of the successful detection of internalization and dissemination of human NoV in plants.

Fresh produce is one of the major high-risk foods for human NoV contamination because it can become contaminated at any point during processing, including both preharvest and postharvest stages. To date, most surveillance is focused on outbreak data and the associated foods (8, 9, 35, 43). There is no standard procedure or method that is routinely used for monitoring the presence of viruses in fresh produce at any level from farm to table. A study was conducted on leafy greens, red fruits, and other produce purchased from markets in Canada, France, and Belgium (3). It was found that human NoV was detected in 28.2%, 33.3%, and 50% of the leafy greens tested in Canada, Belgium, and France, respectively, by using real-time RT-PCR (3). This demonstrates that human NoV is present in leafy greens purchased at local markets (3). Previously, it was shown that hepatitis A virus RNA could be detected inside green onions which were grown hydroponically in feed water inoculated with this virus (9). Poliovirus was found in leaves of tomato plants after growth in soil irrigated with poliovirus-contaminated water at levels of 10^3 to 10^4 PFU/ml (37). Bacteriophage f2 was also detected in beans (*Phaseolus vulgaris* L.) grown hydroponically when challenged with 10^{10} PFU/ml of the virus (53). These results indicate that viral internalization during hydroponic growth of crops does occur, although the level of virus detected varies among experiments. Since human NoV may be present in sewage-contaminated soil or water, it may also be taken into the plant through the roots. Once viruses are internalized, it would be significantly more challenging to eliminate them, since traditional sanitation measures usually target the pathogens on the surface of fresh produce (13, 29, 54). Of further concern is that these internalized viruses can potentially survive for long periods (weeks to months) in fresh produce since human NoV is highly stable in this environment (2, 11, 13). The development of food processing technologies such as irradiation and high-pressure processing may be necessary to eliminate viral hazards from produce (13, 19, 31, 45).

During either the preharvest or field-growing stage of produce production, the use of irrigation water contaminated with norovirus poses the most significant risk for disseminating disease. Agriculture is responsible for the largest usage of freshwater worldwide, and about 70% of this usage is for irrigation. Nearly 17% of all cropland is irrigated, which equates to one-third of the world-wide food supply being exposed to irrigation water (6, 27). The use of feces or fecally contaminated irrigation water has been shown to play a role in spreading enteric microorganisms (1, 6, 10, 54). For this reason, the use of night soil or irrigation with un-

treated human wastewater is illegal in the United States and is not recommended by the World Health Organization. However, nearly 70% of all the irrigated crop land is found in developing countries where irrigation water regulations may not exist (10). Groundwater is generally regarded as being free of microbial contamination and is considered a safe source of irrigation water. However, recent studies in the United States indicate that 8 to 31% of ground water is contaminated with viruses (1, 5). While irrigation water is commonly screened for fecal coliforms, it is rarely tested for the presence of viruses. All these factors contribute to irrigation water posing a significant risk for distributing viral pathogens to fresh produce. Plants may also be contaminated through fertilizers (human waste, feces, and sewage), soils (contaminated by human feces), wildlife (birds, deer, wild pigs, and flies carrying human and animal waste), and domestic animals (spreading contaminants while foraging through fields) (3, 11, 13, 21, 27, 45).

Previously, Urbanucci et al. investigated the internalization of human NoV in lettuce (52). However, no viral RNA was detected in leaves when lettuce was grown hydroponically or in soil after challenge with a high level (10^6 to 10^7 RNA copies/ml) of human NoV. In contrast, we found that a high level of human NoV RNA was detected at day 1 and was persistent in roots, shoots, and leaves for at least 14 days when the roots were challenged with 10^6 RNA copies/ml of human NoV. Several factors may be responsible for this apparent discrepancy. One possibility is variations in the experimental conditions between studies, such as the environmental growth conditions, the type of lettuce tested, the viral strain, and the amount of viral inoculum. In our study, we used romaine lettuce, whereas Rapid lettuce *L. sativa* var. *cripsa* was used in Urbanucci's study (52). It is known that environmental factors (such as temperature and relative humidity conditions) affect the transpiration rate of lettuce, which may have a significant effect on viral internalization and dissemination. In our experiments, the plants were grown at 20°C and a relative humidity of 40%, but the growth conditions were not reported in Urbanucci's study (52). Thus, we cannot directly compare whether these environmental factors contributed to the different results. A plant's transpiration rate increases as the relative humidity of the air decreases, and this increase in transpiration seems to correlate to an increase in viral internalization and dissemination. For example, Wei et al. showed a significant increase in MNV-1 internalization in lettuce when the relative humidity was 70% compared to its internalization at 99% humidity (55). In our study, we decreased the relative humidity to 40% and the dissemination of MNV-1 to leaves was 4 to 5 log PFU/g (Fig. 3), compared to the results reported by Wei et al., where the titer of internalized MNV-1 was 2.6 RT-qPCR units/50 mg with 70% relative humidity (55).

Similar to bacterial internalization, it is also possible that different viral strains may have differing rates of internalization and dissemination. In our study, we used a genogroup II genotype 4 (GII.4) strain of human NoV. Although Urbanucci et al. also used a GII virus, the specific genotype was not reported in their study (52). Within genogroup II, at least 33 human NoV genotypes have been identified (57). It is well known that different human NoV genotypes have different binding affinities to the virus's functional receptors, the histo-blood group antigens (HBGAs) (23, 24, 47). HBGAs are carbohydrate complexes that are present on the surface of erythrocytes, as well as the intestinal, genitourinary, and

respiratory epithelia. There are three major families of HBGAs, Lewis, ABO, and secretor, and each is specifically recognized by different human NoV strains. Interestingly, recent studies have shown that human NoV binds to HBGA-like molecules which exist in fresh produce (such as lettuce, blueberries, and strawberries) (20, 51). In fact, some carbohydrate moieties are the analogues of human NoV receptors, such as glucose and glycan, and are highly abundant in vegetables and fruits. It is possible that these HBGA-like molecules may play a role in viral attachment, internalization, and dissemination. A recent study by Esseili et al. demonstrated that human NoV GII.4 virus-like particles (VLPs) bound to the cell wall material of young and old leaves, the green leaf lamina, and also, the principle vein of Romaine lettuce (16). This binding was found to be strongest in the cell wall material of old leaves and the green leaf lamina compared to other plant tissues tested. This was believed to be due to the fact that the cell walls of older leaves are more complex and contain a higher carbohydrate concentration (16). To further demonstrate that the human NoV VLPs were binding to carbohydrates, sodium periodate treatment was used to oxidize carbohydrates in the cell wall extract, and this treatment significantly reduced the binding efficiency of the human NoV VLPs (16).

The fact that human NoV GII.4 VLPs have been shown to attach specifically to carbohydrates found in romaine lettuce may explain the large amount of bioaccumulation of human NoV GII.4 RNA observed in our study. This possibility is further supported by the fact that HBGA-like receptors exist in the gastrointestinal epithelial cells of oysters, mussels, and clams, which are also a high-risk food for human NoV contamination (34, 50). These HBGA-like receptors were shown to play an essential role in bioaccumulation of human NoV in oysters, mussels, and clams (28, 34, 50). Furthermore, different human NoV strains are known to have different binding affinities to shellfish because of their differences in receptor usage (28, 34). In our study, we also demonstrated that human NoV and TV have similar efficiencies in internalization and dissemination in lettuce (Fig. 5) under the same experimental conditions, whereas TV appears to have a much higher internalization rate than MNV-1 (Fig. 2 and 4). The differences in internalization kinetics may also be related to the properties of each virus, such as surface structure, receptor binding affinity, and charge. A recent study has shown that TV also binds to HBGAs, the functional receptor of human NoV (17), but further studies are required to identify whether receptor binding contributes to the bioaccumulation of human NoV in fresh produce.

Since human NoV is not cultivable in cell culture, real-time RT-PCR is frequently used for the detection of human NoV. The major disadvantage of real-time RT-PCR is that it cannot discriminate infectious and noninfectious viral particles. Thus, one may argue that the high level of RNA copies detected in lettuce may be due to the presence of naked human NoV RNA, rather than infectious viral particles. To rule out that possibility, all samples were treated with RNase to degrade naked viral RNA, followed by RNA extraction and real-time RT-PCR. RNase treatment led to a decrease of 0.5 to 1.5 log in human NoV RNA copies in lettuce tissue from days 2 to 14, suggesting that naked human NoV RNA, which may come from damaged human NoV particles, is present in these samples. Interestingly, high levels of TV RNA were detected in leaves and shoots at days 1 and 2 by real-time RT-PCR (Fig. 6), whereas low levels of infectious viral particles were isolated from

leaves and shoots at days 1 and 2 using the plaque assay (Fig. 2). After RNase treatment, there was an approximately 2.5-log reduction in the amount of TV RNA in shoots (Fig. 7B). In leaves harvested on day 1, 2.5×10^4 RNA copies/g were detected by real-time RT-PCR (Fig. 6), whereas less than 1 log virus was detected by plaque assay (Fig. 2). Interestingly, RNase treatment did not decrease the level of viral RNA copies ($P > 0.05$), suggesting that some noninfectious particles but not naked viral RNA may be present in leaves. A similar phenomenon was observed by Wei et al. (55). They found that a high level of MNV-1 RNA copies in lettuce was detected by real-time RT-PCR, whereas no infectious MNV-1 was detected by plaque assay. It is likely that these viral particles were damaged and noninfectious, whereas viral genome RNA persisted in the lettuce tissues. Although plants lack an immune system comparable to that of animals, they have developed an array of structural, chemical, enzyme, and protein-based defenses designed to detect and inactivate invading organisms (12, 39). It is possible that different viruses may have different degrees of stability against these plant defense systems. Therefore, the data generated from human NoV may be more useful than data generated from the use of surrogate viruses.

In summary, our study elucidates a major gap in our understanding of the ecology of human NoV in fresh produce, specifically, our understanding of the fate of human NoV after attaching to the roots of growing lettuce. Dissection of the mechanism of virus-plant interaction will facilitate the development of novel interventions to prevent viral attachment and internalization in plants.

ACKNOWLEDGMENTS

This study was supported by a special emphasis grant (grant 2010-01498) from the USDA National Integrated Food Safety Initiative (NIFSI), a food safety challenge grant (grant 2011-68003-30005), and the NoroCORE project (grant 2011-68003-30395) from the USDA Agriculture and Food Research Initiative (AFRI) to J.L.

We thank Xi Jiang for Tulane virus and Herbert W. Virgin IV for murine norovirus.

REFERENCES

- Abbaszadegan M, LeChevallier M, Gerba CP. 2003. Occurrence of viruses in US groundwater. *J. Am. Water Works Assoc.* 95:107–120.
- Bae J, Schwab KJ. 2008. Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Appl. Environ. Microbiol.* 74:477–484.
- Baert L, et al. 2011. Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health. *Int. J. Food Microbiol.* 151:261–269.
- Bernstein N, Sela S, Pinto R, Ioffe M. 2007. Evidence for internalization of *Escherichia coli* into the aerial parts of maize via the root system. *J. Food Prot.* 70:471–475.
- Borchardt MA, Bertz PD, Spencer SK, Battagelli DA. 2003. Incidence of enteric viruses in groundwater from household wells in Wisconsin. *Appl. Environ. Microbiol.* 69:1172–1180.
- Bosch JC. 1983. Viruses in environmental waters, p 148–177. In Berg G (ed), *Viral pollution of the environment*. CRC Press, Inc., Boca Raton, FL.
- Cannon JL, et al. 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *J. Food Prot.* 69:2761–2765.
- Centers for Disease Control and Prevention. 2010. CDC reports 1 in 6 get sick from foodborne illnesses each year. CDC, Atlanta, GA. www.cdc.gov/media/pressrel/2010/r101215.html.
- Chancellor DD, et al. 2006. Green onions: potential mechanism for hepatitis contamination. *J. Food Prot.* 69:1468–1472.
- Choi C, Song I, Stine S, Pimentel J, Gerba CP. 2004. Role of irrigation and wastewater: comparison of subsurface irrigation and furrow irrigation. *Water Sci. Technol.* 50:61–68.
- DeWaal CS, Bhuiya F. 2007. Outbreaks by the numbers: fruits and vegetables 1990–2005. Presented at the 2007 Annual Meeting of the International Association for Food Protection, Lake Buena Vista, Florida. <http://www.cspinet.org/foodsafety/IAFPPoster.pdf>.
- de Wit PJ. 2007. How plants recognize pathogens and defend themselves. *Cell. Mol. Life Sci.* 64:2726–2732.
- Doyle MP, Erickson MC. 2008. Summer meeting 2007—the problems with fresh produce: an overview. *J. Appl. Microbiol.* 105:317–330.
- Duizer E, et al. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* 85:79–87.
- Erickson MC, et al. 2010. Infrequent internalization of *Escherichia coli* O157:H7 into field-grown leafy greens. *J. Food Prot.* 73:500–506.
- Esseili MA, Wang Q, Saif LJ. 2012. Human GII.4 norovirus-like particles (VLPs) bind to carbohydrates of Romaine lettuce leaf cell wall materials. *Appl. Environ. Microbiol.* 78:786–794.
- Farkas T, et al. 2010. Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *J. Virol.* 84:8617–8625.
- Farkas T, Sestak K, Wei C, Jiang X. 2008. Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *J. Virol.* 82:5408–5416.
- Feng K, Divers E, Ma Y, Li J. 2011. Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. *Appl. Environ. Microbiol.* 77:3507–3517.
- Gandhi KM, Mandrell RE, Tian P. 2010. Binding of Norwalk virus viral-like particles to veins of romaine lettuce. *Appl. Environ. Microbiol.* 76:7997–8003.
- Heaton JC, Jones K. 2008. Microbial contamination of fruit and vegetables and the behavior of enteropathogens in the phyllosphere: a review. *J. Appl. Microbiol.* 104:613–626.
- Hirneisen KA, Sharma M, Kniel KE. 2012. Human enteric pathogen internalization by root uptake into food crops. *Foodborne Pathog. Dis.* 9:396–405.
- Huang P, et al. 2005. Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. *J. Virol.* 79:6714–6722.
- Hutson AM, Atmar RL, Graham DY, Estes MK. 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J. Infect. Dis.* 185:1335–1337.
- Jablason J, Warriner K, Griffiths M. 2005. Interactions of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. *Int. J. Food Microbiol.* 99:7–18.
- Karst SM, Wobus CE, Lay M, Davidson J, Virgin HW IV. 2003. STAT 1-dependent innate immunity to a Norwalk-like virus. *Science* 299:1575–1578.
- Koopmans M, Duizer E. 2004. Foodborne viruses: an emerging problem. *Int. J. Food Microbiol.* 90:23–41.
- Le Guyader FS, et al. 2006. Norwalk virus-specific binding to oyster digestive tissues. *Emerg. Infect. Dis.* 12:931–936.
- Li J, Predmore A, Divers E, Lou F. 2012. New interventions against human norovirus: progress, opportunities, and challenges. *Annu. Rev. Food Sci. Technol.* 3:331–352.
- Lou F, Neetoo H, Chen H, Li J. 2011. Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. *Appl. Environ. Microbiol.* 77:1862–1871.
- Lou F, Neetoo H, Li J, Chen H, Li J. 2011. Lack of correlation between virus barosensitivity and the presence of a viral envelope during inactivation of human rotavirus, vesicular stomatitis virus, and avian metapneumovirus by high-pressure processing. *Appl. Environ. Microbiol.* 77:8538–8547.
- Lynch MF, Tauxe RV, Hedberg CW. 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiol. Infect.* 137:307–315.
- Ma Y, Li J. 2011. Vesicular stomatitis virus as a vector to deliver human norovirus virus-like particles: a new vaccine candidate against an important non-cultivable virus. *J. Virol.* 85:2942–2952.
- Maalouf H, et al. 2011. Strain-dependent norovirus bioaccumulation in oysters. *Appl. Environ. Microbiol.* 77:3189–3196.
- Mead PS, et al. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.

36. Moreno IM, Thompson JR, García-Arenal F. 2004. Analysis of the systemic colonization of cucumber plants by cucumber green mottle mosaic virus. *J. Gen. Virol.* 85:749–759.
37. Oron G, Goemans M, Manor Y, Feyen J. 1995. Poliovirus distribution in the soil-plant system under reuse of secondary wastewater. *Water Res.* 29:1069–1078.
38. Ponka A, Maunula L, Von Bonsdorff CH, Lyytikäinen O. 1999. An outbreak of calicivirus associated with the consumption of frozen raspberries. *Epidemiol. Infect.* 123:469–474.
39. Postel S, Kemmerling B. 2009. Plant systems for recognition of pathogen-associated molecular patterns. *Semin. Cell Dev. Biol.* 20:1025–1031.
40. Predmore A, Li J. 2011. Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers. *Appl. Environ. Microbiol.* 77:4829–4838.
41. Rawsthorne H, Phister TG, Jaykus LA. 2009. Development of a fluorescent in situ method for visualization of enteric viruses. *Appl. Environ. Microbiol.* 75:7822–7827.
42. Richards GP. 2012. Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. *Food Environ. Virol.* 4:6–13.
43. Scallan E, et al. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.
44. Scipioni A, Mauroy A, Vinje J, Thiry E. 2008. Animal noroviruses. *Vet. J.* 178:32–45.
45. Seymour IJ, Appleton H. 2001. Foodborne viruses and fresh produce. *J. Appl. Microbiol.* 91:759–773.
46. Sharma M, et al. 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. *J. Food Prot.* 72:1513–1520.
47. Tan M, Jiang X. 2005. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. *Trends Microbiol.* 13:285–293.
48. Taube S, et al. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. *J. Virol.* 83:4092–4101.
49. Teunis PFM, et al. 2008. Norwalk virus: How infectious is it? *J. Med. Virol.* 80:1468–1476.
50. Tian P, Engelbrektson AL, Jiang X, Zhong W, Mandrell RE. 2007. Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. *J. Food Prot.* 70:2140–2147.
51. Tian P, Yang D, Mandrell R. 2011. Differences in the binding of human norovirus to and from romaine lettuce and raspberries by water and electrolyzed waters. *J. Food Prot.* 74:1364–1369.
52. Urbanucci A, Myrmel M, Berg I, von Bonsdorff CH, Maunula L. 2009. Potential internalization of caliciviruses in lettuce. *Int. J. Food Microbiol.* 135:175–178.
53. Ward RL, Mahler RJ. 1982. Uptake of bacteriophage f2 through plant roots. *Appl. Environ. Microbiol.* 43:1098–1103.
54. Wei J, Jin Y, Sims T, Kniel KE. 2010. Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by Romaine lettuce. *Appl. Environ. Microbiol.* 76:578–583.
55. Wei J, Jin Y, Sims T, Kniel KE. 2011. Murine norovirus-1 internalization into *Lactuca sativa* during irrigation. *Appl. Environ. Microbiol.* 77:2508–2512.
56. Wobus CE, Thackray LB, Virgin HW IV. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. *J. Virol.* 80:5104–5112.
57. Zheng DP, et al. 2006. Norovirus classification and proposed strain nomenclature. *Virology* 346:312–323.