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PiRV-2 stimulates sporulation in *Phytophthora infestans*

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ABSTRACT

Phytophthora infestans is the causal agent of potato and tomato late blight. This pathogen, which caused the Irish potato famine, is of profound historical significance and still poses a major threat in today's agroecosystems. Research on late blight epidemics usually focuses on pathogen virulence, host resistance, environmental factors and fungicide resistance. In this study, we examined the effect of PiRV-2, an RNA virus harbored by some *P. infestans* isolates, on its host. Comparing isogenic isolates with or without the virus demonstrated that the virus stimulated sporangia production in *P. infestans*. Transcriptome analysis suggested that it achieved sporulation stimulation likely through down-regulation of ammonium and amino acid intake in *P. infestans*. Survey of a limited *P. infestans* collection found PiRV-2 presence in most strains in the US-8 lineage, a very successful clonal lineage of *P. infestans* in North America. We suggest that PiRV-2 may affect the ecological fitness of *P. infestans* and thus could contribute to late blight epidemiology.

1. Introduction

Potato late blight caused devastation in the 1840s and led to food shortage across Europe. In Ireland, where the poor were overwhelmingly dependent on potato, it gave rise to the Irish potato famine - over one million people died and many more were forced to flee (Bourke, 1993). The causal pathogen of potato late blight is *Phytophthora infestans* (Mont.) de Bary, which also causes late blight of tomato. *P. infestans* is a member of oomycetes, a taxon which is morphologically similar to filamentous fungi and which shares similar habitats. However, these two groups are phylogenetically distinct. Oomycetes belong to the major group Stramenopila (Baldauf et al., 2000; Cavalier-Smith, 2000; Sogin and Silberman, 1998), which also includes brown algae and diatoms.

Late blight has proved to be a destructive disease that is difficult to manage and control. Even after extensive research efforts for more than one and a half centuries, it continues to devastate potatoes and tomatoes worldwide and is estimated to cause over 6.7 billion dollars annually in yield losses and control costs (Guenthner et al., 2001; Haverkort et al., 2008). Late blight can develop very rapidly, leaving very little time for growers to respond once an epidemic has started. It is a polycyclic disease: initial infection produces large number of sporangia (asexual spores), dispersal of which leads to subsequent infections. By the mid-twentieth century, late blight was managed to tolerable levels through various tools, including cultural practices, host resistance, and fungicide usage. However, by the end of twentieth century, late blight epidemics re-emerged worldwide (Fry and Goodwin, 1997b). The resurgence started in Europe in early 1980s, later in Middle East and Far East, and in North America by late 1980s and early 1990s. The resurgence was caused by multiple independent introductions of exotic and often fungicide-resistant strains from Mexico (Fry and Goodwin, 1997a), the center of genetic diversity and origin of *P. infestans*.

Prior to the late twentieth century, global populations of *P. infestans* outside Mexico were dominated by a single clonal lineage, US-1 (mating type A1) (Goodwin et al., 1994). It was soon replaced worldwide by new strains (Fry et al., 2008). These new introductions lead to the establishment of residential sexual populations in Northern Europe, though in North America, the populations remain largely clonal (Fry, 2016). In North America, US-8 has been arguably the most successful among the introduced lineages. It is extremely virulent on potato, but not as much on tomato (Fry et al., 2012). First being detected in a single county in New York in 1992, it spread to 23 states during 1994 and 1995, and was found in most potato production regions in the United States and part of Canada by 1996 (Goodwin et al., 1998). Many other clonal lineages, up to US-25, have been reported before and after US-8 (Danies et al., 2013; Hu et al., 2012). Most lineages have come and gone, but US-8 has proved to be enduring.

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Research on late blight epidemics usually focuses on pathogen virulence, host resistance, environmental factors and fungicide resistance, but any potential role of virus infection of *P. infestans* on its fitness has not been examined. We previously surveyed *P. infestans* isolates and reported four RNA viruses in this organism (Cai and Hillman, 2013; Cai et al., 2013; Cai et al., 2012, 2019; Cai et al., 2009; Hillman and Cai, 2013). Three of these viruses, PiRV-1, PiRV-3 and PiRV-4, were not found to cause measurable changes in their host that would suggest a role in enhancing or mitigating late blight.

PiRV-2 was found in two *P. infestans* isolates in the US-8 lineage, US940480 and US040009. This virus has a monopartite genome of 11,170 nt with a single large open reading frame predicted to encode an RNA-dependent RNA polymerase that is only distantly related phylogenetically to other viral RNA-dependent RNA polymerases (Cai et al., 2019). PiRV-2 appears to lack a capsid, as efforts to purify virus particles were not successful and no putative capsid protein coding region was identified in the viral genome. It is transmissible horizontally by hyphal anastomosis, and vertically by asexual reproduction through sporangia. In the study we are reporting here, we examined the effect of PiRV-2 on *P. infestans*, explored the underlying molecular mechanism likely responsible for its effect, and surveyed a small collection of *P. infestans* isolates for occurrence of PiRV-2.

2. Materials and methods

2.1. P. infestans isolates and RNA extraction

P. infestans isolates used in this study and their relevant information are listed in Supplementary Table S1. The isolates were maintained on rye agar. For mycelium production, the isolates were grown in pea broth at 18 °C in darkness. Double-stranded RNA (dsRNA) was extracted from approximately two-week old mycelium with CF-11 cellulose following the method of Morris and Dodds (Morris and Dodds, 1979) modified by Tooley et al (Tooley et al., 1989). DNaseI and S1 nuclease were used as previously described (Cai et al., 2009) to remove traces of genomic DNA and single-stranded RNA, respectively. Total RNA was extracted from mycelium using RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions.

2.2. PiRV-2 curing

Isolate US940480 was grown on a 9-cm diameter rye agar plate containing 50 μg/ml Ribavirin (SigmaAldrich) at 18 °C in the dark. When the colony diameter was approximately two-thirds of the diameter of the plate, a hyphal tip was taken from the edge with the aid of a dissecting microscope and transferred to a new plate. This process was repeated and PiRV-2 curing in the hyphal-tipping cultures was periodically examined using dsRNA extraction and RT-PCR. For this purpose, the hyphal-tipping cultures were grown in pea broth without Ribavirin to produce mycelium. DsRNA and total RNA were extracted from the mycelium as described above. DsRNA samples were run on 1% agarose gels, stained with ethidium bromide and photographed under UV light. SuperScript III one-step RT-PCR system (Invitrogen) was used in RT-PCR, with total RNA as template and primer pairs P05 (AATCC TGGTGCGACGACTAGG) / P07 (GTAGTGCGTTCAAAGTCCTGATG) or P24 (CCATACCTGGTTTTCATGAGAGCTG) / P27 (GCCTCAGGTCTTTT AGTATCTCGAC) in separate reactions and 40 amplification cycles. The RT-PCR products were separated on agarose gels, stained and photographed as described above. The hyphal-tipping cultures derived from US940480 in which PiRV-2 was cured were named US940480/PiRV-2C1 and US940480/PiRV-2C2.

2.3. PiRV-2 transmission through anastomosis

An agar plug from the edge of actively growing US940480 was placed approximately 2 mm from an agar plug from US940480/PiRV-2C1 or US940480/PiRV-2C2 on rye agar plates and grown at 18 °C in the dark. After two weeks, an agar plug was taken from the side of PiRV-2-cured cultures and placed on a fresh rye agar plate. RT-PCR was used to detect PiRV-2. The resultant cultures from US940480/PiRV-2C1 and US940480/PiRV-2C2 into which PiRV-2 was re-introduced were named US940480/PiRV-2T1 and US940480/PiRV-2T2, respectively.

2.4. Phenotype of PiRV-2

Agar plugs from US940480, US940480/PiRV-2C1, US940480/PiRV-2C2, US940480/PiRV-2T1 and US940480/PiRV-2T2 were taken from the edges of actively growing cultures with a #5 cork borer and transferred to the center of fresh 9-cm diameter rye agar plates. The plates were incubated at 18 °C in the dark. Colony diameter was measured at day 5, 7 and 9 after inoculation by averaging two measurements at 90-degree difference. Two weeks after incubation, when the plates were fully covered, they were photographed. Sporangia were harvested by flooding the colonies with 10 ml distilled water and rubbing with a sterile glass rod, and then concentrated to 2 ml by low-speed centrifugation. Sporangia concentration was determined using a hemocytometer. The experiment was conducted twice with three plates per isolate in each experiment.

2.5. Pathogenicity test

Sporangia were harvested from 2-week old cultures of US940480 and US940480/PiRV-2C1 as described above. Sporangia concentration was adjusted to 3.5×10^5 per ml with the aid of a hemocytometer. Leaves were excised from greenhouse-grown Red La Soda potato plants near flowering stage. A 50 ul aliquot of sporangia suspension (17,500 sporangia) was placed in the center of individual leaves. Control leaves were inoculated with sterile H_2O. Three leaves were used per treatment. The inoculated leaves were placed in a moisture chamber and incubated in a growth chamber at 16 °C and 12-h light. Lesion diameter was measured by averaging two measurements at 90° difference after 7 days.

2.6. Detection of PiRV-2 in P. infestans population

A panel of 54 isolates were maintained on rye agar. They were transferred to pea broth for mycelium growth. Total RNA was extracted from mycelium. Two RT-PCR reactions, with primer pairs P16 (TACG GAACAAGTCGACCTCATAGC) / P18 (GGTTTTAACTCCCATCCTACA) and p24/p27, respectively, were used to detect PiRV-2 in each isolate.

2.7. Transcriptome sequencing and analysis

Agar plugs from the edge of actively growing cultures of isolates US940480 and US940480/PiRV-2C1 were transferred to rye agar plates overlaid with cellophane, with three plates per isolate. After incubation at 18 °C in darkness for 1 week and before the onset of abundant sporangia, mycelium from each plate was harvested and total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). An Illumina TruSeq mRNA library was made for each sample according to manufacturer's instructions. The six libraries, each with a distinct barcode, were pooled and sequenced on an Illumina Miseq platform for two runs, with the first run at 1×65 bp and the second run at 1×66 bp.

Read quality was examined using FastQC (Andrews, 2010). The adapter sequences were trimmed using fastx_clipper program in the FASTX-Toolkit (http://hannonlab.cshl.edu). Reads less than 25 bp after trimming were removed from downstream analysis. The sequence reads were first mapped to PiRV-2 using TopHat v2.1.0 (Trapnell et al., 2009) and Bowtie2 v2.3.3.1 (Langmead and Salzberg, 2012) to determine if the libraries contained PiRV-2-derived sequences. The genome assembly and annotation of *P. infestans* strain T30-4 (Haas et al., 2009) were downloaded from GenBank (assembly ASM14294v1). The

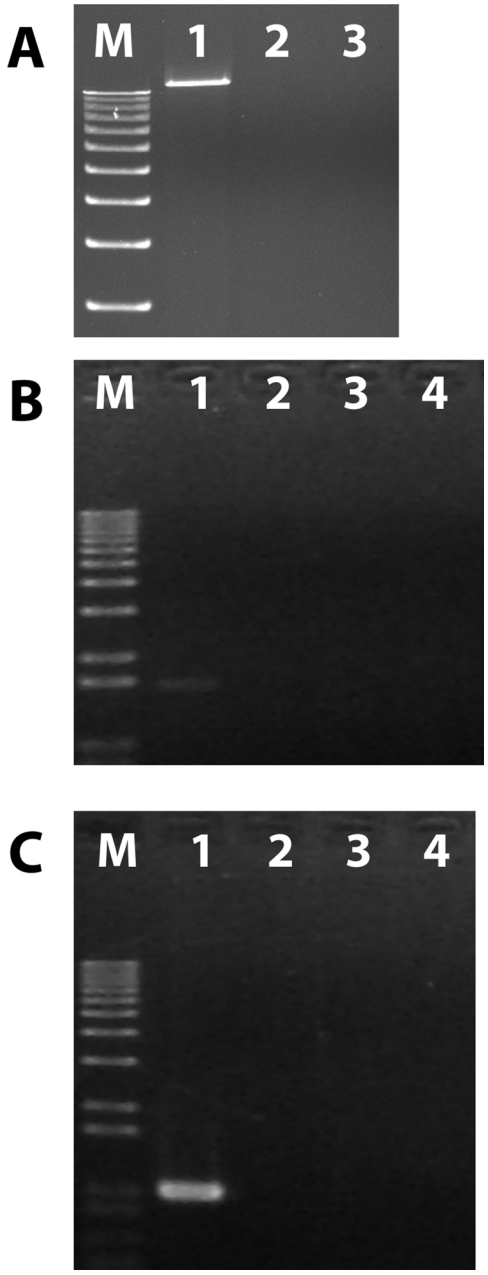

Fig. 1. Curing of PiRV-2 from isolate US940480. (**A**) dsRNA extraction; (**B**) RT-PCR with primer pair P05 and P07; and (**C**) RT-PCR with primer pair P24 and P27. Total RNA was used as template in RT-PCR. Lane designation: M, 1 kb DNA ladder; 1, US940480; 2, US940480/PiRV-2C1; 3, US940480/PiRV-2C2; and 4, negative control with sterilized H_2O as template.

sequence reads were then mapped to the genome of *P. infestans* and the read counts that mapped to individual protein-coding genes were obtained using HTSeq-count (Anders et al., 2015). Gene expression data were analyzed using DESeq2 (Love et al., 2014) to identify differentially expressed genes. Functional analysis of differentially expressed genes was conducted using DAVID version 6.8 (Huang et al., 2008) with default settings. Raw RNASeq data were submitted to GenBank (BioProject accession: PRJNA437643).

3. Results

3.1. PiRV-2 curing and re-introduction

To examine the effect of PiRV-2 on its host, we first set out to create isogenic isolates with or without this virus. To cure an isolate of PiRV-2, virus-containing isolate US940480 was grown on Ribavirin-containing rye agar and transferred by hyphal-tipping. After four iterations, the isolate was found to be cured of PiRV-2 in two cultures, as confirmed by dsRNA extraction and RT-PCR (Fig. 1). These two cultures were named US940480/PiRV-2C1 and US940480/PiRV-2C2, respectively.

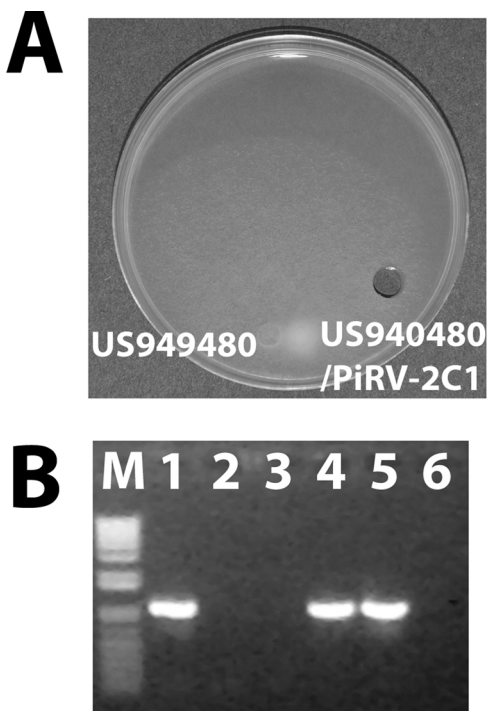

Fig. 2. PiRV-2 transmission. (**A**) Co-culturing of US940480 and US940480/PiRV-2C1 in close proximity. An agar plug was taken from the side of US940480/PiRV-2C1. (**B**) RT-PCR confirmation that PiRV-2 was transmitted into US940480/PiRV-2C1 and US940480/PiRV-2C2. The resultant cultures were named US940480/PiRV-2T1 and US940480/PiRV-2T2, respectively. Lane designation: M, 1 kb DNA ladder; 1, US940480; 2, US940480/PiRV-2C1; 3, US940480/PiRV-2C2; 4, US940480/PiRV-2T1; 5, US940480/PiRV-2T2; and 6, negative control.

To introduce PiRV-2 back into cured isolates, US940480 was co-cultured in close proximity with US940480/PiRV-2C1 on rye agar (Fig. 2A). There was no mycelium-sparse zone separating these two colonies. The virus-free isolate US940480/PiRV-2C1, starting out with dense aerial mycelium around the agar plug, soon morphed into a morphology indistinguishable from that of US940480, suggesting virus transmission. An agar plug was taken from the side of the cured isolate (Fig. 2A) and virus transmission was confirmed by RT-PCR (Fig. 2B). The same result was obtained when US940480 was co-cultured with US940480/PiRV-2C2. The resultant cultures were named US940480/PiRV-2T1 and US940480/PiRV-2T2, respectively.

3.2. PiRV-2 stimulates sporangium production in P. infestans

US940480, US940480/PiRV-2C1, US940480/PiRV-2C2, US940480/PiRV-2T1 and US940480/PiRV-2T2 were grown on rye agar to compare their growth rate, colony morphology and sporulation. Slightly faster growth rate was observed in the virus-cured isolates. The virus-cured isolates also produced denser aerial mycelium compared to US940480 based on visual observation (Fig. 3A). The most profound difference was observed in sporangia production: virus-infected isolate US940480 produced abundant sporangia, while cured isolates US940480/PiRV-2C1 and US940480/PiRV-2C2 sporulated sparsely (Fig. 3A, inset). On average, the PiRV-2-containing isolate US940480 produced 9 to 125 times as many sporangia as its isogenic, virus-free

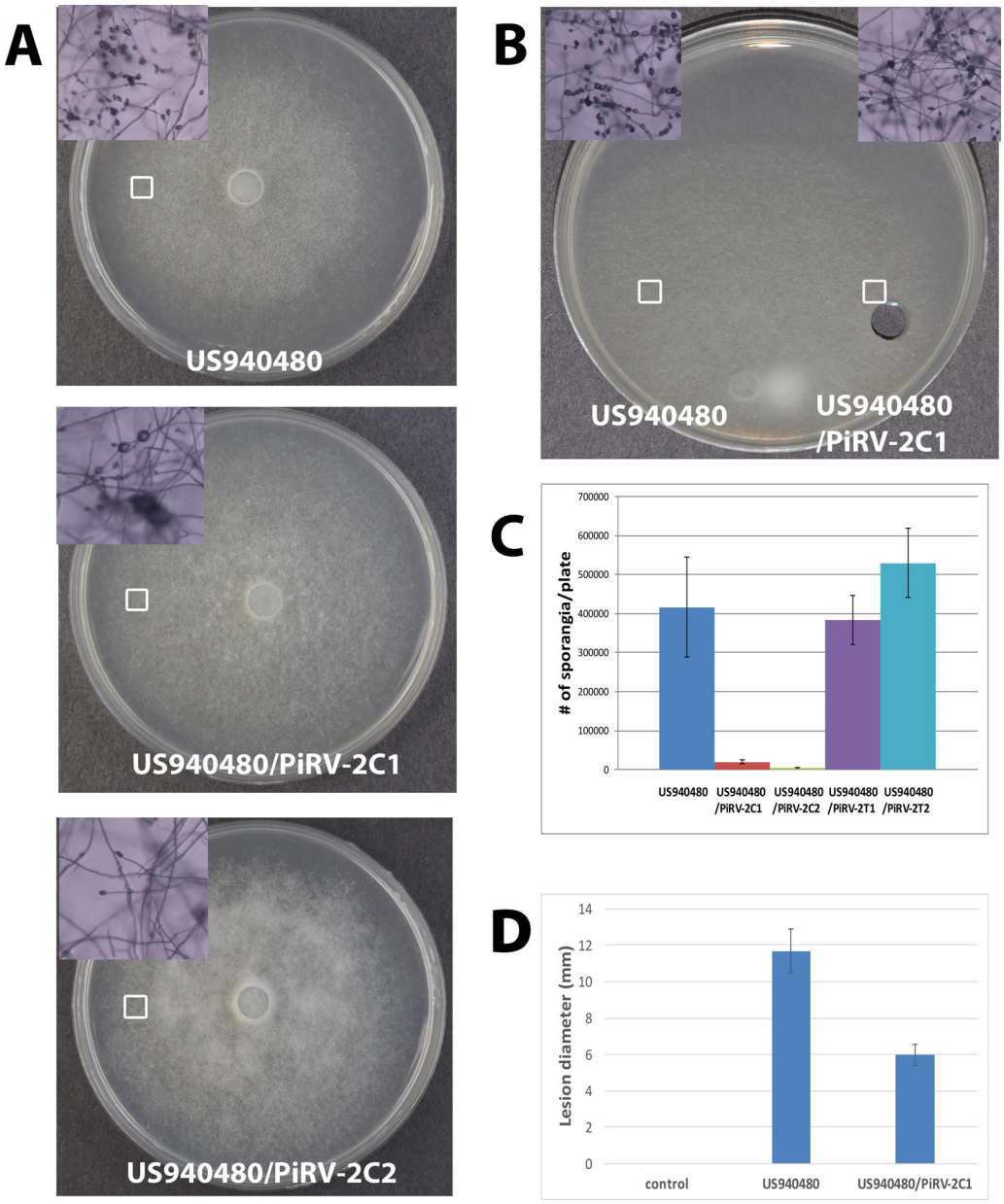

Fig. 3. PiRV-2 stimulates sporangia production in *P. infestans*. (**A**) Colony morphology and sporulation (insets) of virus-containing isolate US940480 and virus-free, cured isolates US940480/PiRV-2C1 and US940480/PiRV-2C2. (**B**) Co-culturing of US940480 and US940480/PiRV-2C1 transmitted the virus into the latter and restored its sporangium production. The white squares in **A** and **B** indicate the areas microscopically observed and photographed. (**C**) Sporangia production in isogenic PiRV-2-containing and PiRV-2-cured isolates. (**D**) Lesion size caused by isogenic PiRV-2-containing and PiRV-2-cured isolates in detached leaf assay. Vertical bars, mean; and vertical lines, standard error.

counterparts US940480/PiRV-2C1 and US940480/PiRV-2C2 did, and the difference was always statistically significant ($P = 0.05$, Fig. 3C). Co-culturing of US940480 with US940480/PiRV-2C1 or US940480/PiRV-2C2 re-introduced the virus into the virus-free isolates and restored their sporangia production (Fig. 3B-C). Sporangia production in US940480/PiRV-2T1 and US940480/PiRV-2T2 were not significantly different from sporangia production in US940480 (Fig. 3C).

To determine the impact of PiRV-2 on host virulence, US940480 and US940480/PiRV-2C1 were used to infect leaves of susceptible potato cultivar Red La Soda in detached leave assay. Lesions caused by US940480 were significantly larger than those caused by US940480/PiRV-2C1 (Fig. 3D). We were not able to obtain sufficient number of sporangia from US940480/PiRV-2C2 to compare it with US940480.

3.3. Distribution of PiRV-2 in *P. infestans* isolates

Since PiRV-2 stimulated sporulation in *P. infestans* and sporangia are an important factor in late blight epidemics, we examined its presence in a collection of *P. infestans* isolates. Using RT-PCR, PiRV-2 was found in 15 of 54 isolates examined (Fig. 4 and not shown).

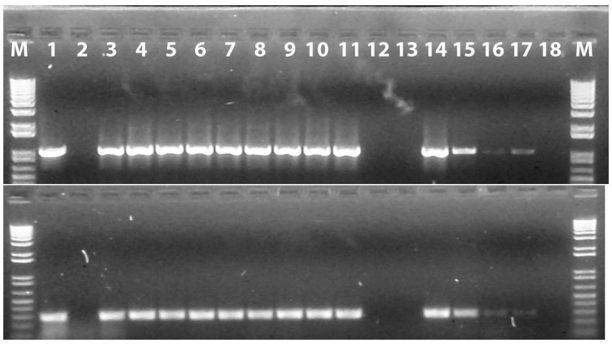

Fig. 4. Representative gel picture of RT-PCR detection of PiRV-2 in *P. infestans* collection. Upper panel, primer pair P24 and P27, lower panel, primer pair P16 and P18. Lane designation: M, 1 kb DNA ladder; 1, US940480; 2, negative control; 3, US040009; 4, P8449; 5, P9017; 6, P9051; 7, P9200; 8, P9212; 9, P9219; 10, P10107; 11, P10129; 12, US100001; 13, US100002; 14, US110001; 15, P17707; 16, P17777; 17, P17783; and 18, P17785.

Table 1
PiRV-2 distribution in *P. infestans*.

Location/lineage		# of isolates	PiRV-2 positive	PiRV-2 negative
USA	US-8	13	11	2
	US-17	1	0	1
	US-11	1	0	1
	US-22	4	3	1
	US-23	1	0	1
	ND[a]	3	0	3
South Africa		1	0	1
The Netherlands		5	0	5
Estonia		3	0	3
Mexico		41	1	40

[a] Not determined.

In a previous study, we screened 22 isolates of *P. infestans* using dsRNA extraction (Cai et al., 2009). The combined results are detailed in Supplementary Table S1 and summarized in Table 1. In North America, the virus was detected in 11 out of 13 isolates in the US-8 lineage and three out of four isolates in US-22 lineage, but not in 6 isolates belonging to US-11, US-17, US-23 or isolates whose lineages were not determined. From isolates collected outside of the USA, PiRV-2 was not detected in 9 isolates from Estonia, The Netherlands and South America. However, PiRV-2 was detected in 1 out of 41 isolates from Mexico.

3.4. PiRV-2 causes transcriptome changes in P. infestans

To explore the underlying molecular mechanism(s) through which PiRV-2 acted on its host, transcriptomes of US940480 and US940480/PiRV-2C1 were sequenced. Three independent mRNA libraries each for US940480 and US940480/PiRV-2C1 were sequenced on Illumina Miseq platform, generating 5.08 million to 7.04 million reads for each library. The reads were first mapped to the PiRV-2 genome. Two out of three US940480 mRNA libraries contained one and two reads, respectively, that were aligned to PiRV-2. No read from the three US940480/PiRV-2C1 mRNA libraries was aligned to the virus genome. This was in agreement with the fact that US940480 harbored PiRV-2 while US940480/PiRV-2C1 did not. Since PiRV-2 does not have a polyA tail (Cai et al., 2018), and mRNA was extracted from total RNA using polyT beads, it is not surprising that only trace amount of PiRV-2 RNA was introduced into the libraries.

The sequence reads were then mapped to the genome of *P. infestans* (Haas et al., 2009) to obtain read counts for individual genes. With a Benjamini-Hochberg adjusted *P* value (padj) of 0.01 and a minimum of $\log_2$ fold change (LFC) of 1 (2-fold change) requirements, 848 differentially expressed genes (DEGs) were identified, with 431 genes up-regulated by PiRV-2 and 417 genes down-regulated by this virus (Supplementary Table S2).

Four groups of genes were enriched in up-regulated DEGs. The first group (i) was histone protein genes. Of the 18 histone protein genes annotated in *P. infestans* genome, 9 were up-regulated, a 20.6-fold enrichment. Two additional histone genes were induced to a lesser extent (padj < 0.05, LFC > 0.5) and none was significantly down-regulated (Table 2). The expression of the 39 histone modifying enzyme genes in *P. infestans* was not significantly perturbed by PiRV-2. Also enriched were (ii) genes for flagellum-related proteins, (iii) proteins with epidermal growth factor (EGF)-like conserved site, and (iv) genes in the glycolytic process (Supplementary Fig. S1), with 10.0-, 9.9- and 7.4-fold enrichment, respectively.

Genes in the ribosome pathway were highly represented in the down-regulated DEGs. Of the 110 genes in *P. infestans* that mapped to the KEGG ribosome pathway, 23 were down-regulated, a 9-fold enrichment. An additional 34 genes in this pathway were down-regulated to a lesser extent (padj < 0.05, LFC < -0.5) and no gene in this pathway was significantly up-regulated (Supplementary Table S3). These down-regulated genes included both the large and small subunit ribosome proteins. The down-regulated DEGs also included a group of 98 genes with one or more transmembrane domain (Supplementary Table S4), but with only 1.5-fold enrichment. These genes included many involved in nutrient transport across the membrane, as well as several kinases and other genes. The potential molecular pathway(s) that PiRV-2 employed to act on *P. infestans* were further explored in the "Discussion" section.

Table 2
Effect of PiRV-2 on histone protein gene expression in *P. infestans*.

Locus tag	Protein name	log2FoldChange	pvalue	padj
PITG_05391	histone H1, putative	1.51	5.24E-06	0.00017
PITG_03550	histone H2B	1.58	1.91E-09	1.45E-07
PITG_03551	histone H3	1.38	6.24E-05	0.00135
PITG_03552	histone H4	1.59	3.86E-08	2.25E-06
PITG_03611	histone H2B	1.54	4.82E-07	2.10E-05
PITG_03881	histone H2A	1.64	1.32E-06	4.99E-05
PITG_03882	histone H2A type 1	1.62	1.50E-05	0.00041
PITG_05676	histone H4	1.72	1.82E-07	8.71E-06
PITG_21568	histone H1, putative	1.61	0.00031	0.00506
PITG_02255	histone H1, putative	0.54	0.00101	0.01340
PITG_05675	histone, putative	0.72	0.00163	0.01941
PITG_13828	histone H3	−0.20	0.62630	0.84345
PITG_06950	histone H3.3	−0.67	0.10672	0.35360
PITG_06953	histone H3.3	−0.58	0.01763	0.11073
PITG_06955	histone H3.3	0.06	0.92724	0.97456
PITG_04387	histone H4	NA	NA	NA
PITG_20725	histone H3.3 type 1	−0.26	0.28280	0.59615
PITG_02527	histone H2A variant 1	−0.05	0.80904	0.93215

4. Discussion

Many viruses of lower eukaryotes are asymptomatic or cause only mild symptoms in their hosts, but others cause observable symptoms (Cai and Hillman, 2013; Ghabrial et al., 2015; Pearson et al., 2009). In phytopathogenic fungi, a small number of viruses can attenuate the virulence of their hosts, resulting in a phenomenon called hypovirulence. The best examples of hypovirulence are viruses of the chestnut blight fungus, *Cryphonectria parasitica* (Hillman and Suzuki, 2004; Nuss, 2005). In addition to reducing virulence of the fungus on its chestnut host, many of these viruses also affect fitness of the fungus by reducing asexual and sexual sporulation. The L1 dsRNA of *Nectria radicicola*, the causal agent of ginseng root rot, showed the opposite effect, stimulating sporulation and increasing virulence of *N. radicicola* (Ahn and Lee, 2001). Finally, Alternaria alternata chrysovirus 1 (AaCV1), from the pathogen that causes black spot on leaves of Japanese pear, showed two-sided effects. On the one hand, a high titer of AaCV1 severely impaired the growth of *A. alternata*; on the other hand, its presence resulted in increased production of AK-toxin, a fungal virulence factor, and thus enhanced virulence of this fungus (Okada et al., 2018).

In this study, PiRV-2 shows similar effects to its host as L1 dsRNA in *N. radicicola*, in that it stimulates sporangium production and thus would be predicted to serve as a fitness factor for the host of the virus. Our conclusion is based on comparison between isogenic PiRV-2-containing and PiRV-2-cured isolates, and re-introduction of the virus into cured isolates by co-culturing US940480 with cured isolates, the classical method used to transmit fungal viruses by anastomosis. Since the virus was transmitted into unmarked isogenic cured isolates, it was not possible to molecularly differentiate the donor isolate from the recipient isolates into which PiRV-2 was re-introduced. However, when we co-cultured US940480 with other virus-free isolates of different genetic background, and agar plugs were taken from the same region on the side of virus-free isolates, PiRV-2 was not detected. Furthermore, the morphology of cured isolates adopted the morphology of the virus-infected donor, starting out with abundant aerial mycelium but acquiring a morphology with sparse aerial mycelium indistinguishable

from the PiRV-2-containing isolate US940480. Similar morphological conversions, often manifested more obviously through colony color, are observed when Cryphonectria viruses are transmitted into virus-free isolates through anastomosis (Hillman and Suzuki, 2004; Nuss, 2005; Rigling and Prospero, 2018). Our efforts to transmit the virus from US940480 into three other isolates, which likely are vegetatively incompatible with US940480, were not successful. These transmission experiments support results of the comparisons between US940480 and the isogenic, PiRV-2-cured isolates and validate our conclusion that PiRV-2 stimulates sporangium production in *P. infestans*.

The sporangium plays an important role in the late blight disease cycle (Schumann and D'Arcy, 2000). In this polycyclic disease, sporangia are dispersed via air or water to healthy tissue to initiate new infections. Within a few days after initial infection, the pathogen produces a visible lesion from which many sporangia can be produced and each is capable of initiating another cycle of pathogenesis. A virus that can stimulate sporangium production would have profound implication in late blight epidemics. In detached leave assay, the PiRV-2-containing isolate US940480 caused larger lesion than its isogenic, cured counterpart US940180/PiRV-2C1. Sporangia were readily observed from lesions infected by US940480, but not from US940480/PiRV-2C1.

Survey of a small *P. infestans* collection showed that this virus was present in most isolates of the US-8 clonal lineage of this pathogen. The US-8 lineage was first detected in the United States in 1992 and soon spread to most potato production areas in North America (Goodwin et al., 1998). From the mid-1990s until 2009, US-8 had been the dominant lineage in the USA. Recently (2012–2018), US-23 has been dominant, but US-8 still constituted a substantial portion of *P. infestans* isolated from potato in 2016–2018 (https://usablight.org, most recent data available). The fitness of *P. infestans* depends on many factors, such as inoculum production, temperature adaptation and host preference (Danies et al., 2013). Since PiRV-2 stimulates sporangium production, it thus could improve the fitness of virus-containing isolates. Based on the prevalence of PiRV-2 in US-8 isolates, we suggest that PiRV-2 may have been a contributing factor to the success of the US-8 lineage. PiRV-2 was found in one of 46 isolates from Mexico. It is assumed that the US-7 and US-8 lineages migrated to the United States in 1992 through the trade of infected plant material (Goodwin et al., 1995). Likely one of the strains carried by the infected material harbored PiRV-2 and led to the dominance of US-8 for several decades in many potato fields in North America. Only a small number of US isolates from other lineages were examined. The existence and prevalence of this virus in other lineages remain to be determined.

At the transcriptome level, the strain harboring PiRV-2 had significantly lower expression of many ribosome protein genes comparing to its isogenic, virus-free counterpart. The ribosome is the core of protein translation machinery. In Escherichia coli, *Neurospora crassa* and *Saccharomyces cerevisiae*, the expression of ribosome protein genes is proportional to growth rate and down-regulated by nutrient limitations (Kief and Warner, 1981; Nomura, 1974; Shi and Tyler, 1991). In the diploid yeast *S. cerevisiae*, nitrogen starvation induces sporulation and down-regulation of more than 40 ribosome protein genes. When nitrogen is replenished, these genes return to normal expression level and the yeast resumes vegetative growth (Chu et al., 1998; Pearson and Haber, 1980). While the sporulation in *S. cerevisiae* is a sexual process, resulting in haploid progenies from diploid parent, nitrogen starvation also induces asexual conidiation in fungi *N. crassa* and *Aspergillus nidulans* (Guignard et al., 1984; Skromne et al., 1995). Nutrient limitation has also been used to induce sporangium production in *Phytophthora* spp. (Ribeiro, 1983). There are five annotated ammonium transporter genes in *P. infestans*. Among these, the three genes with the highest expression levels were significantly down-regulated by PiRV-2 (Supplementary Table S5). In yeast, down-regulation of ribosome protein genes can also be induced through amino acid deprivation (Warner and Gorenstein, 1978). Among the 34 amino acid permease genes in *P. infestans*, eight were significantly down-regulated by PiRV-2 and none was up-regulated (Supplementary Table S5).

The main function of histone proteins in eukaryotic cells is to package and order DNA. When *S. cerevisiae* is induced into sporulation through nitrogen starvation, the amount of histone proteins per cell increases many folds in the early hours, followed by increase of DNA synthesis (Marian and Wintersberger, 1980). The four major families of histones, H2A, H2B, H3 and H4, form the nucleosome core on which DNA wraps around, while H1 links individual nucleosomes together to form higher order structure. Multiple histone protein genes were highly induced by PiRV-2, including all five major histone families.

In our study, none of the DNA polymerase genes was significantly affected by PiRV-2 and DNA content was not quantified. Genes in one of the up-regulated groups, the seven genes with EGF-like domains, have functions predicted to affect DNA synthesis (Supplementary Fig. S1B). In animal cells, binding of an EGF-like domain to a cell surface receptor is essential for activation of tyrosine kinase in the receptor cytoplasmic domain, which initiates a signal transduction and results in DNA synthesis and cell proliferation (Ng et al., 1983; Otto et al., 1981).

Previous studies have identified two genes with regulatory functions that are critical for sporangium production in *P. infestans*. A G protein β-subunit (*Pigpb1*) is induced in nutrient starved medium prior to the onset of sporangium formation, and silencing of this gene results in drastic decrease in sporangia production and dense growth of aerial mycelium (Latijnhouwers and Govers, 2003). *Picdc14* is a cell-cycle phosphatase and expressed only in sporangiophore initials, sporangiophore and sporangia (Ah Fong and Judelson, 2003). Silencing of *Picdc14* also impairs sporangium production. *Picdc14* is also induced by nutrient starvation and it is assumed to act after *Pigpb1* because silencing of the latter down-regulates its expression (Judelson and Blanco, 2005; Latijnhouwers and Govers, 2003). In our study, *Pigpb1* (PITG_06376) was not significantly affected by PiRV-2. The expression of *Picdc4* (PITG_18578) was up-regulated by 4-fold by PiRV-2 but there was high variability among the replicates (LFC = 1.97, P = 0.048, padj = 0.215).

The analysis above suggests a possible molecular mechanism for stimulation of sporulation in *P. infestans* by PiRV-2 (Fig. 5). The virus, directly or indirectly, down-regulates the expression of ammonium and amino acid transporter genes, resulting in nitrogen and amino acid deprivation. This could lead to decrease in many ribosome proteins and increase of histone proteins, prime *P. infestans* for DNA synthesis

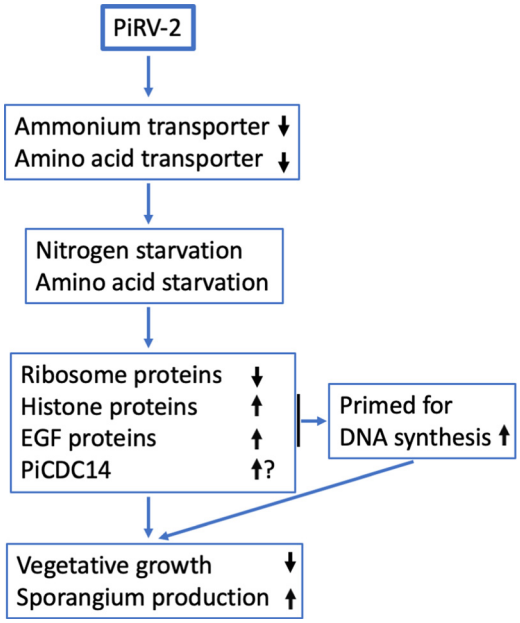

Fig. 5. Potential molecular mechanism for PiRV-2-associated stimulation of sporangium production in *P. infestans*.

through up-regulation of genes with an EGF-like domain, and possibly up-regulate the expression of *Picdc14*. These molecular processes could result in less vegetative growth in strains containing PiRV-2 (sparse aerial mycelium) and more sporulation.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197674.

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