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Effects of the deletion and over-expression of *Fusarium graminearum* gene *FgHal2* on host response to mycovirus *Fusarium graminearum virus 1*

JISUK YU, KYUNG-MI LEE, MOONIL SON AND KOOK-HYUNG KIM*

Department of Agricultural Biotechnology, Center for Fungal Pathogenesis and Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, South Korea

SUMMARY

The mycovirus *Fusarium graminearum virus 1* (FgV1) is associated with reduced virulence (hypovirulence) of *Fusarium graminearum*. Transcriptomic and proteomic expression profiling have shown that many *F. graminearum* genes are differentially expressed as a consequence of FgV1 infection. Several of these genes may be related to the maintenance of the virus life cycle. The host gene, *FgHal2*, which has a highly conserved 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain or inositol monophosphatase (IMPase) superfamily domain, shows reduced expression in response to FgV1 infection. We generated targeted gene deletion and over-expression mutants to clarify the possible function(s) of *FgHal2* and its relationship to FgV1. The gene deletion mutant showed retarded growth, reduced aerial mycelia formation and reduced pigmentation, whereas over-expression mutants were morphologically similar to the wild-type (WT). Furthermore, compared with the WT, the gene deletion mutant produced fewer conidia and these showed abnormal morphology. The *FgHal2* expression level was decreased by FgV1 infection at 120 h post-inoculation (hpi), whereas the levels were nine-fold greater for both the virus-free and virus-infected over-expression mutant than for the WT. FgV1 RNA accumulation was decreased in the deletion mutant at 48, 72 and 120 hpi. FgV1 RNA accumulation in the over-expression mutant was reduced relative to that of the WT at 48 and 120 hpi, but was similar to that of the WT at 72 hpi. The vertical transmission rate of FgV1 in the gene deletion mutant was low, suggesting that *FgHal2* may be required for the maintenance of FgV1 in the host cell. Together, these results indicate that the putative 3'(2'),5'-bisphosphate nucleotidase gene, *FgHal2*, has diverse biological functions in the host fungus and may affect the viral RNA accumulation and transmission of FgV1.

Keywords: 3'(2'),5'-bisphosphate nucleotidase, *FgHal2*, *Fusarium graminearum*, *Fusarium graminearum virus 1*, secondary metabolism, virus–host interaction.

**Correspondence*: Email: kookkim@snu.ac.kr

INTRODUCTION

Mycoviruses are common in plant-pathogenic and other fungi and have genomes of double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) or DNA (Xie and Jiang, 2014). Although many mycoviruses infect their fungal hosts asymptomatically, some viruses confer hypovirulence, i.e. they reduce the virulence of the pathogenic fungus (Pearson *et al*., 2009). From an agricultural perspective, mycovirus-induced hypovirulence could contribute to the development of sustainable biological control (Ghabrial and Suzuki, 2009; Cho *et al*., 2013). Furthermore, the study of the interactions between a hypovirulence-associated virus and its host provides an opportunity to characterize viral determinants for the virus disease cycle and to identify the fungal host genes involved in the interaction (Ghabrial and Suzuki, 2009).

Previous research has demonstrated that mycovirus infections alter the expression of a broad range of host genes and cause hypovirulence or phenotypic alterations in the fungal host (Allen and Nuss, 2004; Lee *et al*., 2014; McBride *et al*., 2013). Several host genes regulated by *Cryphonectria hypovirus 1* strain EP713 (CHV1) infection have been identified in the plant-pathogenic fungus *Cryphonectria parasitica*. For example, the expression of PRO1, which is required for female fertility, conidiation and the stable inheritance of CHV1 infection, is down-regulated (Sun *et al*., 2009). CpBir, which is involved in fungal conidiation and virulence, is also significantly down-regulated by CHV1 infection and affects the transmission of the hypovirus in *C. parasitica* (Gao *et al*., 2013). CHV1 infection also down-regulates the expression of the tannic acid-inducible, small heat shock protein Hsp24, and is associated with the fungal stress response and virulence (Baek *et al*., 2014).

Fusarium graminearum Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch] is a homothallic ascomycetous fungus which causes Fusarium head blight (FHB) in small grain cereals and ear rot in maize (Desjardins and Proctor, 2007). In addition to reducing the yield of economically important crops, this plant-pathogenic fungus produces mycotoxins, such as trichothecenes and zearalenone, in cereals, which are very harmful to humans and other animals (Goswami and Kistler, 2004). Recently, several mycoviruses have been detected and identified in *Fusarium*

species, including *Fusarium graminearum* (Cho *et al*., 2013). Among *Fusarium graminearum* viruses, *Fusarium graminearum virus 1* strain DK21 (currently named FgV1) has been associated with reduced host virulence, a reduced rate of mycelial growth, increased pigmentation and inhibition of mycotoxin production (Chu *et al*., 2002). FgV1 mainly occurs as the dsRNA form, but expresses its genome in a manner similar to that of positive-strand potex-like RNA viruses, and is phylogenetically similar to members of the genus *Hypovirus* based on the alignment of proteins containing RNA-dependent RNA polymerase domains (Kwon *et al*., 2009b).

With the availability of well-developed forward and reverse genetic tools, including an efficient DNA-mediated transformation system and the availability of a completely sequenced genome, *F. graminearum* can be used as a model system to understand virus–host interactions (Güldener *et al*., 2006; Son *et al*., 2013). By using transcriptomic and proteomic approaches, we have determined previously that many fungal host genes are differentially expressed in response to FgV1 infection. These host genes are involved in cell signalling pathways, the stress response, post-transcriptional gene silencing, differentiation and other processes (Cho *et al*., 2012; Kwon *et al*., 2009a; Lee *et al*., 2014). Researchers have proposed that some of these genes might function as host factors to maintain the life cycle of FgV1 (Kwon *et al*., 2009a; Son *et al*., 2013). For example, Hex1, a major protein of the Woronin body, plays a crucial role in maintaining the cellular integrity and pathogenicity of *F. graminearum* (Son *et al*., 2013). Moreover, the accumulation of FgV1 viral RNA depends on the *HEX1* gene expression level, although the molecular mechanisms underlying Hex1 protein–FgV1 interactions are not clearly understood (Son *et al*., 2013). Other host genes in *F. graminearum* which may directly and/or indirectly affect the life cycle of FgVs, however, have not been identified.

This article concerns the characterization of one *F. graminearum* gene, *FgHal2*, which shows a reduced gene expression level (transcript and protein expression profiles) in response to FgV1 infection (Kwon *et al*., 2009a; Lee *et al*., 2014). *FgHal2* is an orthologue of the yeast gene *Hal2* (*MET22*) and encodes a hypothetical protein that has a conserved domain of 3'-phosphoadenosine-5'-phosphate (pAp) phosphatase. *Hal2* encodes 3'(2'),5'-bisphosphate nucleotidase, which is involved in methionine biosynthesis in the sulfate assimilation pathway and which also affects salt tolerance in yeast (Gil-Mascarell *et al*., 1999). Met22p removes the 3' phosphate from pAp, thus producing adenosine monophosphate (AMP), and also hydrolyses 3'-phosphoadenosine-5'-phosphosulfate (pApS) (Hudson and York, 2012). Met22p can suppress viral RNA recombination of *Tomato bushy stunt virus* (TBSV) in yeast and is related to the Xrn1p 5'–3' ribonuclease, a known suppressor of viral RNA recombination (Jaag and Nagy, 2010). *SAL1*, one of the *Arabidopsis* orthologues of yeast *Hal2*, also has multiple functions. It catabolizes inositol 1,4,5-trisphosphate (IP3) and pAp (Estavillo *et al*., 2011; Hudson and York, 2012), represses post-transcriptional gene silencing by degrading the exoribonuclease inhibitor pAp, and functions in stress signalling and developmental processes in plants (Estavillo *et al*., 2011; Gy *et al*., 2007).

In this study, we provide evidence that the putative 3'(2'),5'-bisphosphate nucleotidase gene, *FgHal2*, in *F. graminearum* is down-regulated following FgV1 infection. We also investigate the possible function(s) of *FgHal2* in *F. graminearum* using gene deletion and gene over-expression mutants. We found that deletion of *FgHal2* reduced conidiation, mycelial growth and the production of secondary metabolites. Moreover, deletion of *FgHal2* decreased viral RNA accumulation and the vertical transmission of FgV1 via conidia. Together, these results indicate that *F. graminearum* can down-regulate one of its major multifunctional genes, *FgHal2*, in response to FgV1 infection.

RESULTS

Sequence analysis of *FgHAL2*

The genomic sequence of *FGSG_09532*, corresponding to the *FgHAL2* gene, was determined at the Munich Information Centre of Protein Sequences (MIPS) and the Fusarium comparative database [Fusarium Comparative Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology (MIT) (http://www.broadinstitute.org)]. We aligned amino acid sequences of *FgHal2* and *FgHal2* orthologues using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/). The result of multiple amino acid sequence alignment indicated that the 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) or inositol monophosphatase (IMPase) superfamily domain is highly conserved among all orthologues and *FgHal2* (Fig. 1). The deduced amino acid sequence of *FgHal2* in *F. graminearum* shares 45% sequence identity with the *Saccharomyces cerevisiae* Met22 protein. *FgHal2* also shows high sequence identity with proteins in *F. oxysporum* (95%), *F. verticillioides* (95%), *Neurospora crassa* (76%) and *Magnaporthe oryzae* (78%). The *F. graminearum* chromosome has two other putative genes that contain a PAP phosphatase-like domain: *Fg07103* and *Fg01708*. The comparison of the deduced amino acid sequence of *FgHal2* with those of *Fg07103* and *Fg01708* showed 42% and 35% sequence identity, respectively. When we conducted real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify the alteration in the expression level of *Fg07103*, *Fg01708* and *FgHal2* after FgV1 infection, only the *FgHal2* gene was down-regulated after virus infection; the transcription levels of *Fg07103* and *Fg01708* genes did not change significantly (Fig. S1, see Supporting Information).

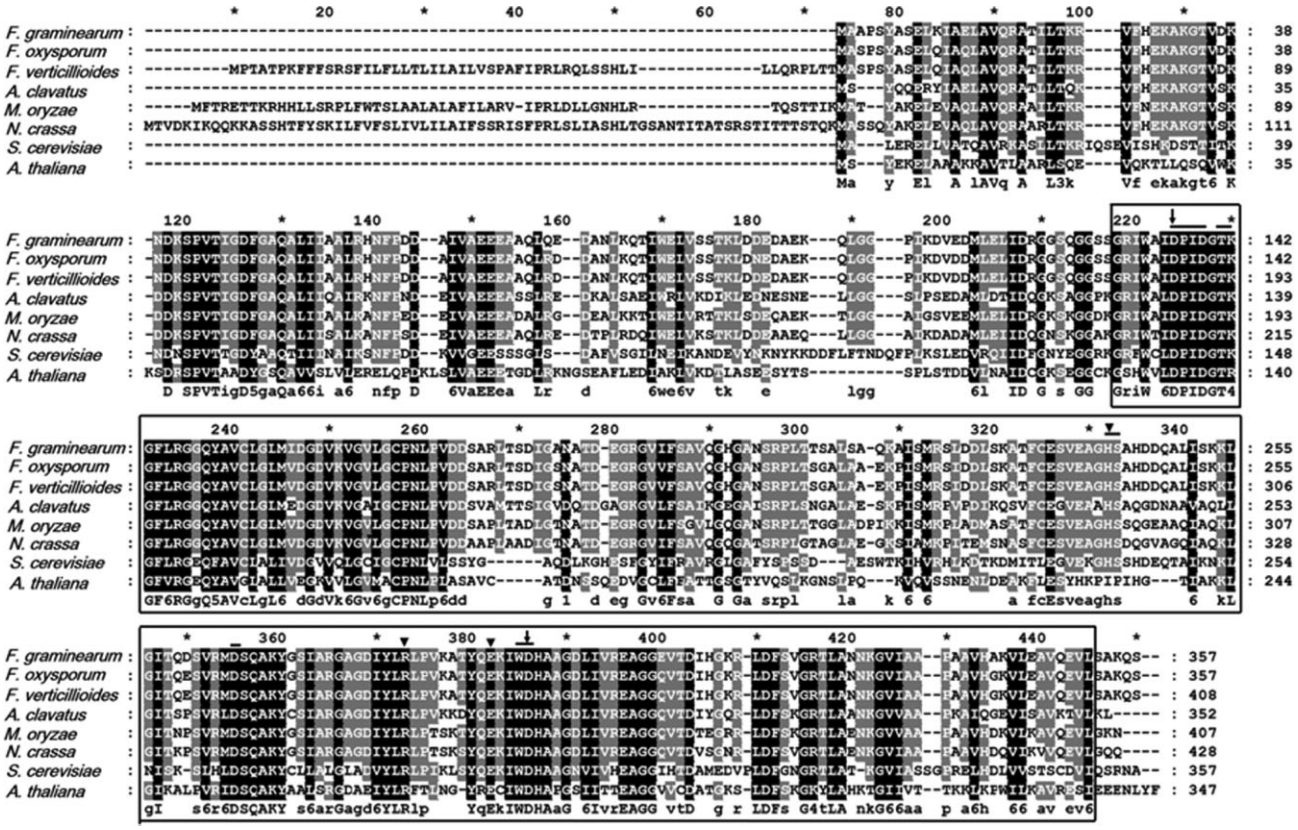

Fig. 1 Amino acid sequence alignment of FgHal2 corresponding to the conserved 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain of the predicted FgHal2 protein and orthologues. The positions of the amino acid sequences are indicated on the right. Species and GenBank accession numbers are as follows: *Arabidopsis thaliana* (NP201205.1); *Aspergillus clavatus* (XP001268064.1); *Magnaporthe oryzae* (XP003719499.1); *Fusarium graminearum* (XP389708.1); *Fusarium oxysporum* (EGU82355.1); *Fusarium verticillioides* (EWG42162); *Neurospora crassa* (EAA28448.2); *Saccharomyces cerevisiae* (NP014577.1) The conserved amino acids are shaded. The alignment was generated with GeneDoc software. The PAP phosphatase-like domain [cd01517] is boxed; the conserved active site residues are indicated by a bar above the sequence; the substrate binding site residues are indicated by arrowheads; and the putative Li^+/Na^+ binding sites are indicated by black arrows.

Generation of mutants

To investigate the function of *FgHal2* in *F. graminearum*, we generated gene deletion and gene over-expression mutants using a homologous recombination strategy (Fig. 2A). For the construction of the gene deletion mutant, the 5'- and 3'-flanking regions were amplified from wild-type (WT) *G. zeae* PH-1 genomic DNA by PCR and fused to the geneticin (gen) resistance cassette. The fused DNA construct was used to transform fungal protoplast, and gen-resistant transformants were confirmed by Southern blot hybridization. For the construction of the complementation strain of *FgHal2*, the open reading frame (ORF) of *FgHal2* and the green fluorescent protein (GFP) fusion construct under the control of the *FgHal2* native promoter were inserted into the genome of the *FgHal2* deletion mutant. We also generated an *FgHal2* over-expression strain, which contained the elongation factor 1α (EF1α) promoter of *F. verticillioides* upstream of the *FgHal2* ORF. All transformed fungal mutants were confirmed by Southern blot analysis (Fig. 2B). qRT-PCR of the virus-free strains confirmed that the transcript level of *FgHal2* was not detected at all in the deletion mutant, greatly increased in the over-expression mutant, and similar in the WT and the complementation mutant (Fig. 2C). All fungal strains used in this study are listed in Table 1.

Effect of FgV1 infection on *FgHal2* gene expression level

To determine whether the expression level of *FgHal2* mRNA differed among the *F. graminearum* strains, we introduced FgV1 into the virus-free *FgHal2* gene deletion strain, complementation strain and over-expression strain through hyphal anastomosis. As indicated by qRT-PCR, the expression level of *FgHal2* mRNAs was significantly lower in the FgV1-infected WT than in the virus-free WT at 120 h post-inoculation (hpi) (Fig. 2C). The *FgHal2* transcript level was also significantly lower in the virus-infected WT and the complemented strain than in the corresponding virus-free strains

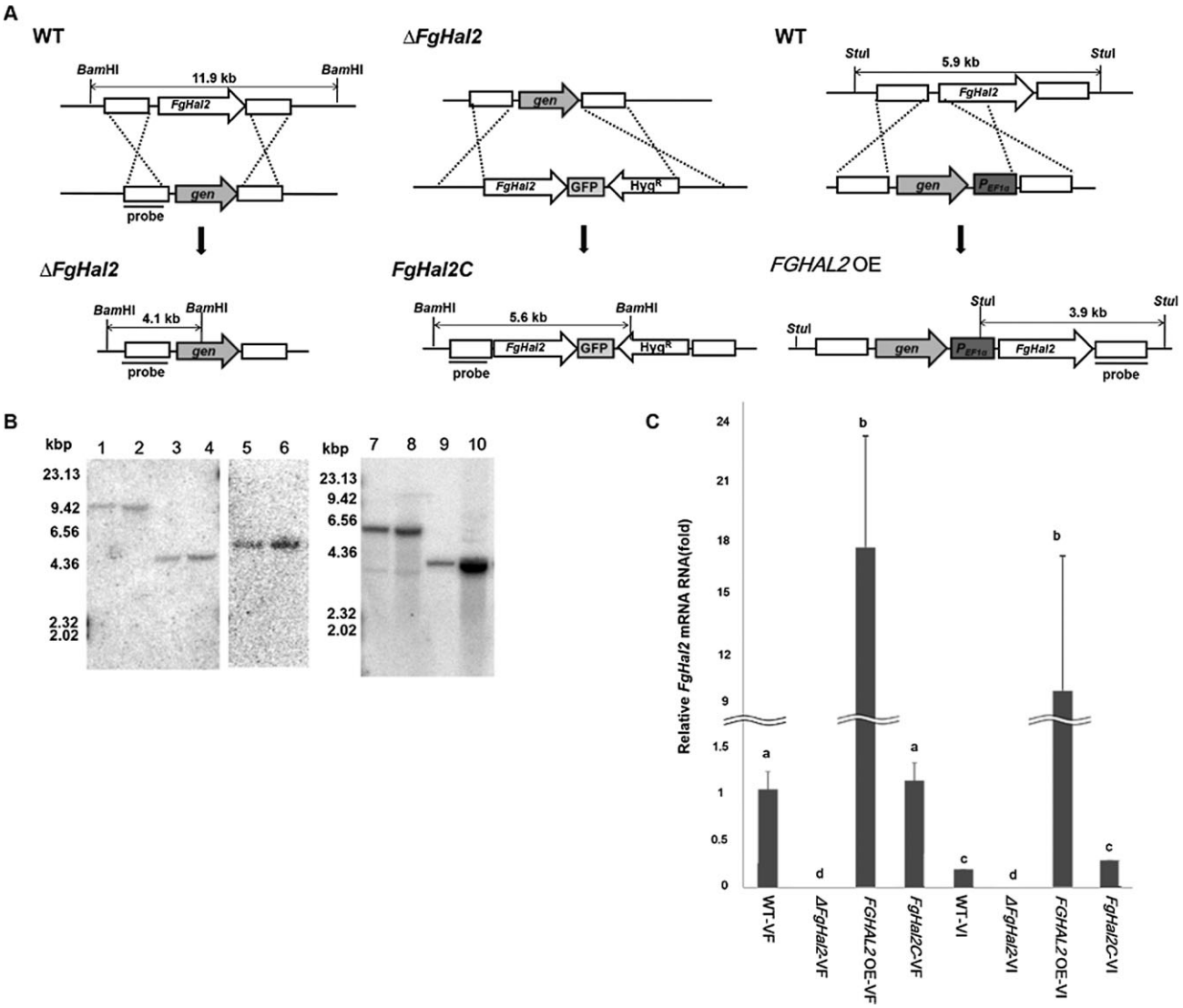

Fig. 2 Generation of *FgHal2* gene deletion, complementation and over-expression mutants of *Fusarium graminearum*. (A) Schematic representation of the homologous gene recombination strategy used to generate the *FgHal2* deletion mutants (left), *FgHal2* complementation mutant (middle) and *FgHal2* over-expression mutant (right). The *FgHal2* complementation mutant strain was fused with green fluorescent protein (GFP). The promoter was replaced with the *elongation factor 1*α (*EF1*α) promoter in the *FGHAL2* OE strain. WT, *F. graminearum* wild-type strain PH-1; Δ*FgHal2*, *FgHal2* gene deletion mutant; *FgHal2C*, *FgHal2* complementation and GFP-fused mutant; *FGHAL2* OE, *FgHal2* over-expression mutant. (B) Southern blot hybridization of the *F. graminearum* mutant strains. The sizes of the DNA standards are indicated to the left of the blot. Lanes 1–6 indicate Southern blot images of *Bam*HI-digested genomic DNA of each strain. Lanes 1 and 2 represent the WT, lanes 3 and 4 represent Δ*FgHal2*, and lanes 5 and 6 represent the complementary strain (*FgHal2C*). A ^{32}P-labelled DNA fragment of the 5' flanking region of the *FgHal2* gene was used as a probe for Southern blot hybridization (lanes 1–6). Lanes 7–10 show Southern blot images of *Stu*I-digested genomic DNA of each strain. Lanes 7 and 8 represent the WT, and lanes 9 and 10 represent *FGHAL2* OE. A ^{32}P-labelled DNA fragment of the 3' flanking region of the *FgHal2* gene was used as a probe (lanes 7–10). In lanes 1–10, odd-numbered lanes indicate virus-free strains and even-numbered lanes indicate virus-infected strains. (C) Relative *FgHal2* mRNA transcript levels in the WT and mutant strains. Relative transcript levels were normalized to elongation factor 1α and ubiquitin C-terminal hydrolase. cDNAs were generated from total RNA extracts from mycelia harvested after 120 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on Tukey's test. VF, virus-free; VI, *Fusarium graminearum virus 1* (FgV1)-infected strains.

when the strains were incubated for 48 and 72 h in complete medium (CM) liquid culture (data not shown). Previously, *FgHal2* was found to be down-regulated by FgV1 infection when two-dimensional electrophoresis was used to determine the protein expression level of virus-infected and virus-free *F. graminearum* (Kwon *et al*., 2009a). This down-regulation was consistently observed at the transcription level when qRT-PCR was performed in the current study. These results demonstrate that FgV1 infection reduces the expression of *FgHal2* in *F. graminearum*. *FgHal2* transcript levels in over-expression strains, however, were similar

regardless of virus infection. The latter finding might be explained by the strong constitutive *EF1α* promoter that was inserted into the over-expression strain.

Effects of *FgHal2* deletion and over-expression on vegetative growth and pigmentation

WT-VF, *FGHAL2* OE-VF and *FgHal2C*-VF showed similar morphologies on CM at 120 hpi, but the deletion of *FgHal2* (Δ*FgHal2*-VF) led to abnormal colony morphology, a slow growth rate, reduced aerial mycelium formation and reduced red pigmentation (Fig. 3). Δ*FgHal2*-VF formed a hyphal mass that was pale yellow or white, whereas the WT, complementation strain and over-expression strain formed a pink or red hyphal mass. Relative to the virus-free strains, the virus-infected strains had an irregular morphology, increased pigmentation and reduced aerial mycelia at 120 hpi on CM, as reported previously (Lee *et al*., 2014). Radial growth on CM was reduced for the deletion mutant whether it was virus-free (Δ*FgHal2*-VF) or virus-infected (Δ*FgHal2*-VI). The radial growth of *FGHAL2* OE-VF did not differ from that of WT-VF, and the same was true for *FGHAL2* OE-VI and WT-VI.

As shown in Fig. 3, both the deletion of *FgHal2* and FgV1 infection reduced the production of aerial mycelia. Because the aerial hyphae of many fungi have hydrophobic cell surfaces (Jiang *et al*., 2011), we hypothesized that deletion of *FgHal2* or infection by FgV1 might reduce the hydrophobicity of the aerial hyphae. To measure the hydrophobicity of the hyphal surface, we applied 15 μL of water containing bromophenol blue to 3-day-old colonies of all virus-free and virus-infected strains (Fig. S2, see Supporting Information). The water formed spherical droplets on all virus-free strains that produced abundant mycelia, including the WT, over-expression strain and complementation strain. In contrast, water droplets were absorbed by the mycelia of the knockout mutant and virus-infected WT and mutant strains. These results indicate that *FgHal2*, in addition to being involved in mycelial growth, is involved in the hydrophobicity of *F. graminearum* aerial hyphae. FgV1 infection also affected the hydrophobicity of the aerial mycelia, perhaps because FgV1 infection reduces the expression of *FgHal2* (Fig. 2C).

Table 1 *Fusarium graminearum* strains used in this study.

Strain	Description	References
WT-VF	Wild-type *F. graminearum* (lineage 7), *Gibberella zeae* PH-1	Lee *et al*. (2014)
WT-VI	Wild-type PH-1 infected with FgV1	Lee *et al*. (2014)
Δ*FgHal2*-VF	*FgHal2* gene deletion mutant in WT-VF genetic background	This study
Δ*FgHal2*-VI	FgV1 introduced into Δ*FgHal2*-VF by hyphal anastomosis	This study
FGHAL2 OE-VF	*FgHal2* gene over-expression mutant in WT-VF genetic background	This study
FGHAL2 OE-VI	FgV1 introduced into *FGHAL2* OE-VF by hyphal anastomosis	This study
FgHal2C-VF	*FgHal2* gene complementation mutant in WT-VF genetic background	This study
FgHal2C-VI	FgV1 introduced into *FgHal2C*-VF by hyphal anastomosis	This study

Effects of *FgHal2* deletion and over-expression on conidiation and conidial morphology

To determine the effects of *FgHal2* on conidiation and conidial morphology, we quantified the production of conidia by virus-free

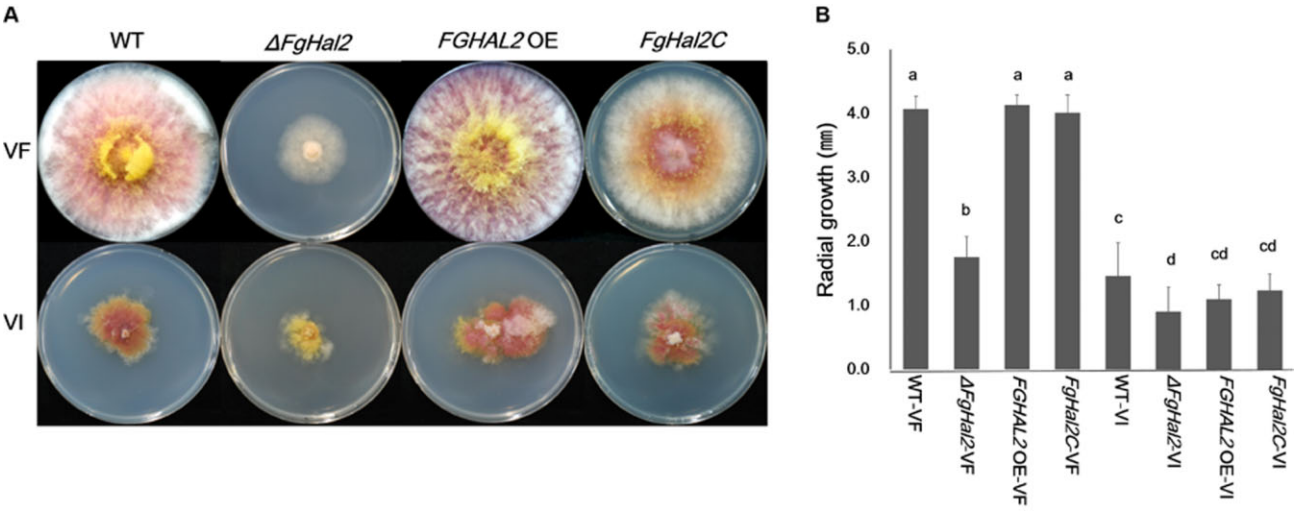

Fig. 3 Colony morphology and mycelial growth of virus-free and *Fusarium graminearum virus 1* (FgV1)-infected strains [wild-type (WT) and *FgHal2* gene deletion, complementation and over-expression mutants] of *F. graminearum*. (A) Colony morphology on complete medium (CM) agar. Colonies were photographed after 5 days of incubation. (B) Radial growth after 5 days of incubation on CM agar. Means and standard deviations were calculated from three repeated experiments. Bars with different letters are significantly different ($P < 0.05$) based on Tukey's test, which was conducted using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA).

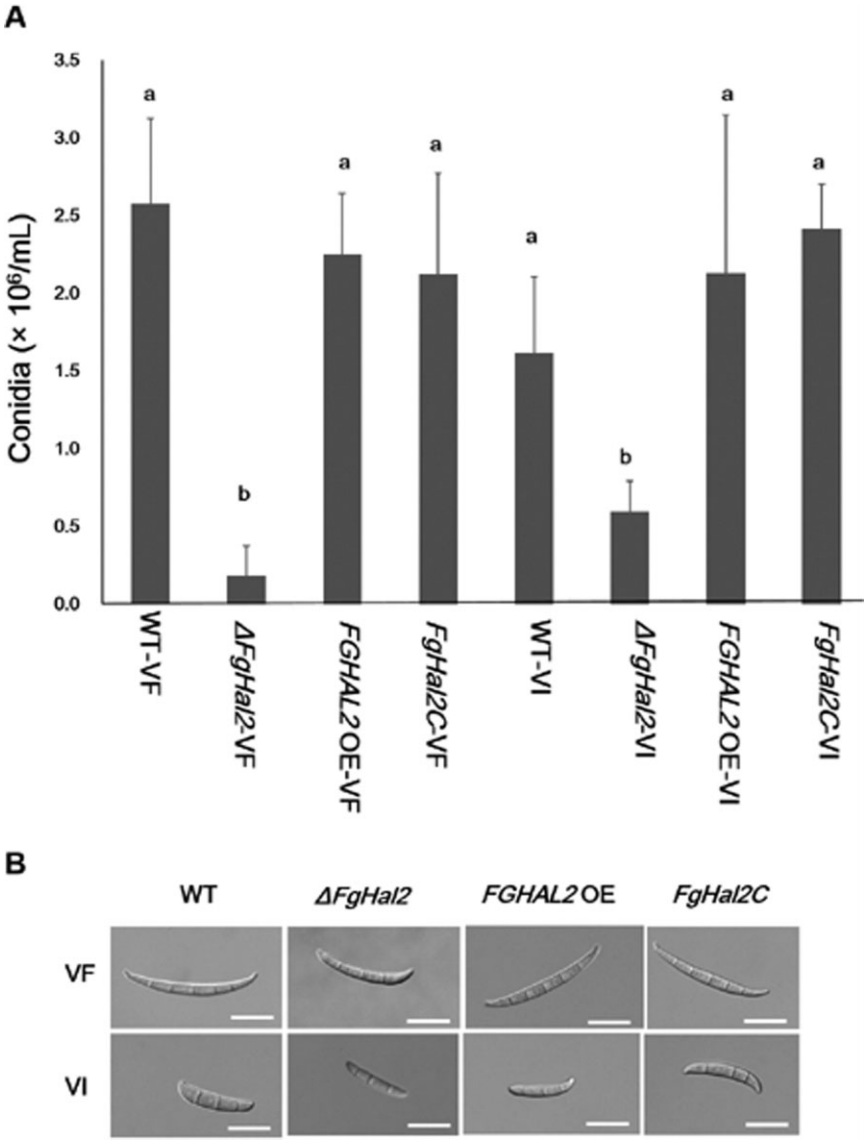

Fig. 4 Conidial production by virus-free and *Fusarium graminearum virus 1* (FgV1)-infected strains [wild-type (WT) and *FgHal2* gene deletion, complementation and over-expression mutants] of *F. graminearum*. (A) Conidia production after 5 days on carboxymethyl cellulose (CMC). Values are means and standard deviations of three repeated experiments. Means with different letters are significantly different ($P < 0.05$) based on Tukey's test. (B) Conidial morphology. Representative conidia of virus-free (VF) and virus-infected (VI) strains were photographed with differential interference contrast optics. Bar, 20 μm.

and virus-infected strains on carboxymethyl cellulose (CMC) medium (Fig. 4A). Deletion of *FgHal2* dramatically reduced conidial production on CMC medium regardless of FgV1 infection. Conidial production did not differ significantly among the WT, complementation strain or over-expression strain regardless of FgV1 infection (Fig. 4A). Our previous study also indicated that FgV1 infection did not affect conidial production (Lee *et al*., 2014).

We have shown previously that FgV1 infection affects conidium morphology, i.e. FgV1-infected *F. graminearum* produce swollen and short conidia (Lee *et al*., 2014). In the current study, all virus-infected strains also produced swollen and short conidia (Fig. 4B). With respect to virus-free strains, Δ*FgHal2*-VF produced shorter conidia than WT-VF, *FgHal2C*-VF or *FGHAL2* OE-VF (Fig. 4B). Overall, these results suggest that the *FgHal2* gene is related to conidial production and development.

Effect of *FgHal2* deletion and over-expression on virulence

To determine the effect of *FGHAL2* on *F. graminearum* virulence, we inoculated wheat heads with conidial suspensions of virus-free and virus-infected WT or mutant *F. graminearum* strains, including the deletion mutant Δ*FgHal2*. FHB symptoms appeared on wheat heads inoculated with the virus-free WT (WT-VF), virus-free over-expression strain (*FGHAL2* OE-VF) and virus-free complementation strain (*FgHal2C*-VF) (Fig. 5A). However, inoculation with Δ*FgHal2*-VF did not result in bleaching of the head or even of the inoculated spikelet. Because the growth rate of Δ*FgHal2*-VF was severely decreased relative to that of the WT, we monitored inoculated wheat heads for a prolonged period. Even at 21 days post-inoculation, however, no symptoms were caused by Δ*FgHal2*-VF.

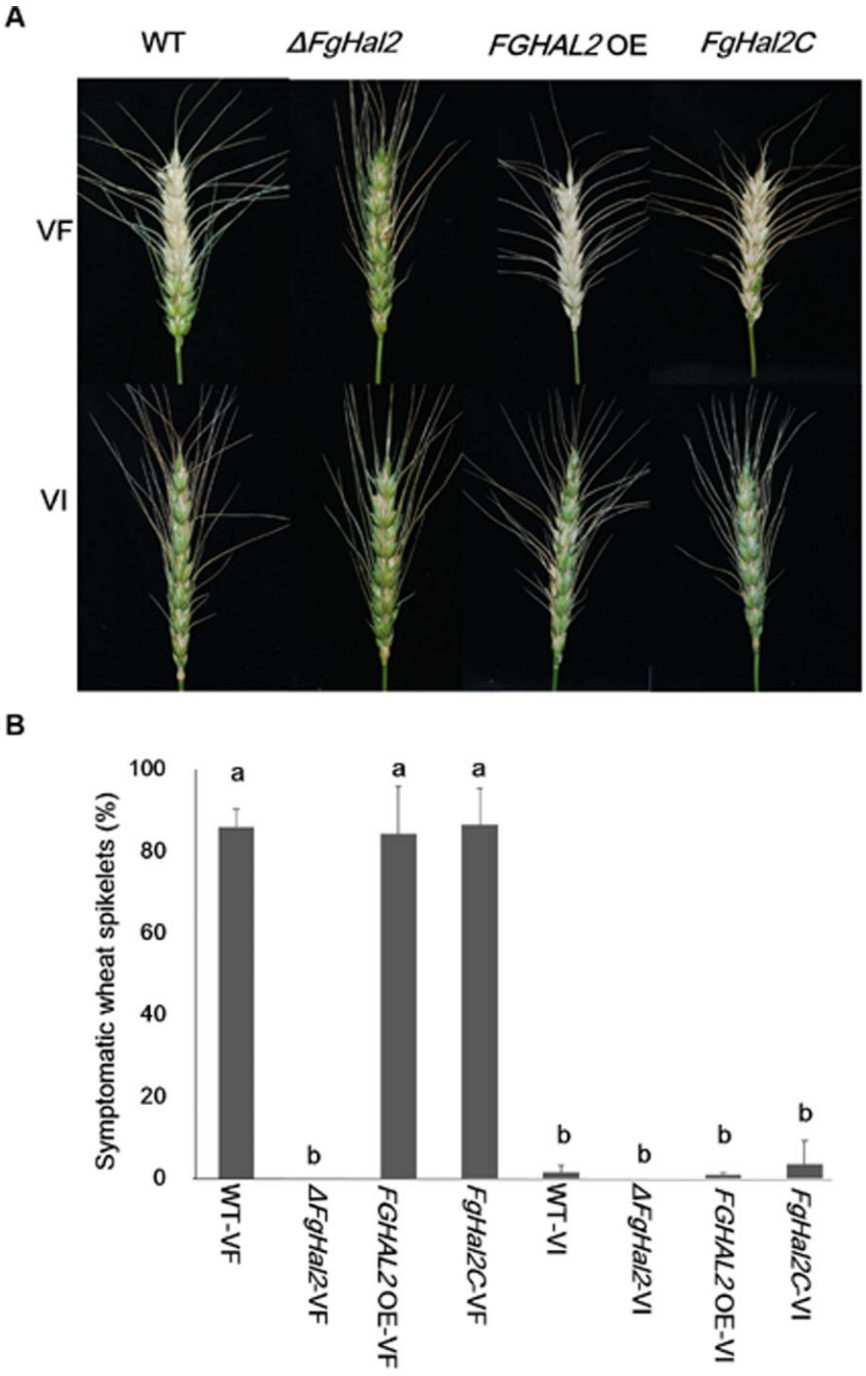

Fig. 5 Virulence of virus-free and *Fusarium graminearum virus 1* (FgV1)-infected *F. graminearum* strains [wild-type (WT) and *FgHal2* gene deletion, complementation and over-expression mutants] on wheat. (A) Representative wheat heads 14 days after they were inoculated with conidia. (B) The percentage of symptomatic wheat spikelets 14 days after inoculation. Means and standard deviations were calculated from independent repeated experiments. Error bars indicate standard deviation. Bars with different letters are significantly different ($P < 0.05$).

None of the virus-infected strains caused visual symptoms on the wheat heads or inoculated spikelets (Fig. 5B).

Effect of *FgHal2* deletion and over-expression on vertical transmission

To assess the stability of the FgV1 virus in *F. graminearum*, we quantified the vertical transmission of FgV1 through the asexual spores of the WT and mutant strains (Table 2). Transmission to the first, second and third generations of conidia was 100% for the WT and complementation strain *FgHal2C*. Transmission for the over-expression strain *FGHAL2* OE was somewhat reduced in the first generation, but was 100% for the second and third generations. Transmission for the deletion strain *ΔFgHal2* decreased substantially with each generation. These data indicate that deletion of the *FgHal2* gene reduces the vertical transmission via conidia or reduces the maintenance of FgV1 in infected host cells.

Table 2 Vertical transmission of *Fusarium graminearum virus 1* (FgV1) between generations of *Fusarium graminearum* strains.

Strain	Vertical transmission rate (%)		
	1st generation	2nd generation	3rd generation
WT-VI	100	100	100
Δ*FgHal2*-VI	63	45	15
FGHAL2 OE-VI	84	100	100
FgHal2C-VI	100	100	100

Vertical transmission was measured as the percentage of FgV1-positive isolates among the total number of single-conidium isolates. Each value is based on ≥30 single-conidium isolates.

Effect of *FgHal2* deletion and over-expression on the accumulation of FgV1 RNA

We performed qRT-PCR to determine whether the deletion or over-expression of *FgHal2* affects viral RNA accumulation (Fig. 6). At 48 hpi, viral RNA levels were significantly greater in the WT than in the deletion mutant, over-expression mutant or complemented mutant. At 72 hpi, viral RNA levels had significantly increased in all strains except the deletion mutant. At 120 hpi, viral RNA levels remained low in the deletion mutant, had decreased substantially in the over-expression mutant and had dropped only moderately in the WT and complemented mutant. These results suggest that *FgHal2* may affect FgV1 RNA accumulation either directly or indirectly.

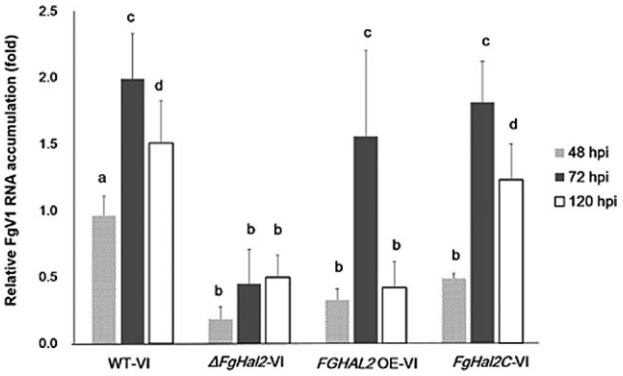

Fig. 6 Accumulation of *Fusarium graminearum virus 1* (FgV1) viral RNA in FgV1-infected wild-type (WT), Δ*FgHal2*, *FgHal2C* and *FGHAL2* OE strains of *F. graminearum*. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to quantify FgV1 viral RNA at 48, 72 and 120 h post-inoculation (hpi). Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on Tukey's test.

DISCUSSION

On the basis of computational sequence analysis, we assumed that *FgHal2* encodes a 3'-nucleotidase which is highly conserved in fungi. This nucleotidase is a member of the family of phosphatases that share the properties of sensitivity to Li^+ and Na^+ ions, metal dependence and substrate selectivity (Hudson and York, 2012). Only one gene encodes for the 3'-nucleotidase in *S. cerevisiae*, but two or more genes code for this enzyme in other fungi and plants, including *Arabidopsis*. The expression of isoforms of pAp phosphatase enzymes may be differentially regulated during organ differentiation and stress responses, and such enzymes usually exhibit substrate stringency and sensitivity to toxic cations (Gil-Mascarell *et al*., 1999; Hudson and York, 2012). *Fusarium graminearum* has three putative proteins with pAp phosphatase-conserved domains. Interestingly, only *FgHal2* is significantly down-regulated by FgV1 infection. This result suggests that *FgHal2* may be closely related to certain regulatory processes that are induced by FgV1 infection.

As mentioned earlier, *Hal2* (*Met22*) has been identified and characterized in yeasts (Gläser *et al*., 1993; Vaupotič *et al*., 2007) and plants (Gil-Mascarell *et al*., 1999). The function of this gene in plant-pathogenic fungi, however, has not been determined. Because the inhibition of Hal2 causes PAP accumulation in the cell, it also reduces sulfotransferase activity, RNA-processing enzymes and the sulfur assimilation pathway (Belles and Serrano, 1995; Gašparič *et al*., 2013). Accordingly, these effects impede growth. FRY1 in *Arabidopsis* is a homologue of MET22 in yeast, and the *fry1* mutant generates fewer lateral roots and shows stunted growth, short hypocotyls, curly leaves and a retarded phase transition and leaf initiation (Chen and Xiong, 2010). In this study, deletion of the *FgHal2* gene significantly reduced the growth of hyphae in and above the agar medium. Deletion of *FgHal2* also reduced conidial production and resulted in abnormal conidial morphology. These results suggest that *FgHal2* is involved in the regulation of *F. graminearum* growth and development. In addition, *FgHal2* deletion reduced the hydrophobicity of the fungal colony, suggesting that *FgHal2* contributes to cell wall integrity.

The colonies of Δ*FgHal2*-VF and Δ*FgHal2*-VI growing on CM agar showed a dramatic decrease in red pigmentation (Fig. 3A). This indicates that deletion of *FgHal2* may affect secondary metabolism in *F. graminearum*. *Fusarium graminearum* produces diverse secondary metabolites, including the polyketide pigments aurofusarin (AUR) and rubrofusarin (Kim *et al*., 2006). To date, few genes have been characterized that participate in the biosynthesis of AUR (Geng *et al*., 2014). *Gip2* is a putative positive regulator of the AUR biosynthetic gene cluster, and polyketide synthase 12 (*Pks12*) lies upstream of *Gip1* and is responsible for changing a precursor to AUR (Geng *et al*., 2014). Furthermore, an osmosensor histidine kinase (*FgOs1*) and an osmoregulatory mitogen-activated protein kinase (MAPK) pathway (consisting of *FgOs4*, *FgOs5* and *FgOs2*) are associated with the regulation of AUR in *F. graminearum* (Ochiai *et al*., 2007). Among the genes related to secondary metabolite synthesis in *F. graminearum*, *FgOs2*, *Gip2*, *Pks12* and *AURj* were not detected by qRT-PCR in Δ*FgHal2*-VF (Fig. S3, see Supporting Information). In addition, qRT-PCR indicated that the transcript levels of *FgOs2* and *Pks12* were significantly different in *FGHAL2* OE-VI and WT-VI than in WT-VF

(Fig. S3). These results support the inference that FgV1 infection and *FgHal2* may regulate AUR biosynthesis so as to affect osmoregulation by the MAPK signalling pathway. Together, the morphological phenotype of Δ*FgHal2*-VF and the alteration in the transcript levels of secondary metabolite synthesis genes in *FGHAL2* OE and WT-VI suggest that *FgHal2* is involved in secondary metabolite production. For this reason, the suppression of *FgHal2* in WT-VI may be involved in the biological and physiological changes caused by FgV1 infection. Presumably, mycovirus infection and fungal secondary metabolism are related, sharing various regulatory pathways. Previous research has shown that CHV1-EP713 hypovirus alters host G-protein signal transduction (Dawe *et al*., 2004) and the *Hog1*-homologue *cpmk1* gene of the MAPK pathway of *C. parasitica* (Park *et al*., 2004). These signalling pathways are associated with the regulation of secondary metabolite synthesis. In this regard, the expression levels of genes in the osmotic stress signal transduction pathway (*FgOs5* and *FgOs2*) and those related to polyketide synthase (*Pks12*, *Gip4* and *Gip5*) were significantly different in the FgV1-infected WT than in the virus-free WT (Lee *et al*., 2014). It would be useful to determine how *FgHal2* affects secondary metabolite synthesis pathways that may be related to FgV1 infection.

We used qRT-PCR to assess FgV1 RNA accumulation in our *F. graminearum* strains at 48, 72 and 120 hpi. Because *FgHal2* was repressed by FgV1 infection, we expected that the viral RNA accumulation level would be similar or higher in virus-infected Δ*FgHal2* than in the virus-infected WT. However, we found that significantly less FgV1 RNA accumulated in Δ*FgHal2*-VI than in WT-VI. This suggests that the *FgHal2* gene may also directly or indirectly affect virus RNA accumulation. The FgV1 RNA level in *FGHAL2* OE-VI was low relative to that in WT-VI at 48 hpi, increased at 72 hpi and then returned to a low level at 120 hpi (Fig. 6). The *FgHal2* expression level, however, was approximately nine-fold greater in *FGHAL2* OE-VI than in WT-VI. A possible explanation for this decrease in the FgV1 RNA level in *FGHAL2* OE-VI is that over-expression of *FgHal2* may help the fungus to overcome osmotic and other stresses, as demonstrated for the orthologue of *FgHal2*, *Hal2* (Gašparič *et al*., 2013; Shinozaki and Yamaguchi-Shinozaki, 1999). This result suggests that *FgHal2* may affect the host defence response. If FgV1 infection is considered as a biological stress to *F. graminearum*, fungal stress-responsive pathways or elements may also be affected by virus infection. As noted earlier, the small heat shock protein CpHsp24 contributes to stress adaptation, but not to hypoviral replication, in *C. parasitica* (Baek *et al*., 2014). Perhaps the reduced expression level of *FgHal2* in response to virus infection does not protect the fungal host in some respects, but does generate unsuitable conditions for FgV1 persistence in host cells.

The FgV1 transmission experiment in the current study suggests that *FgHal2* may help to maintain FgV1 infection. Relative to the vertical transmission in the FgV1-infected WT, the vertical transmission in Δ*FgHal2*-VI decreased gradually and was only 15% in the third generation (Table 2). This failure to maintain FgV1 was also observed during serial mycelial subcultures on agar. During repeated subculture on potato dextrose agar (PDA), WT-VI, *FgHal2C*-VI and *FGHAL2* OE-VI did not produce virus-cured isolate under typical conditions. Unlike these strains, Δ*FgHal2*-VI rapidly produced a colony with virus-free and virus-infected sectors (Fig. S4, see Supporting Information). These observations, which indicate that FgHal2 is necessary for the maintenance of FgV1 infection, may explain why *F. graminearum* down-regulates *FgHal2* expression after FgV1 infection. The *C. parasitica pro1* gene, which encodes the $Zn(II)_2Cys_6$ transcription factor, is also down-regulated in response to CHV1 infection (Sun *et al*., 2009). The hypovirus-infected *pro1*-disruption mutant was frequently recovered from hypovirus and produced colony coexisting the virus-free sector and virus-infected sectors on PDA. Like *pro1*, *FgHal2* levels in WT were reduced, but not eliminated, by FgV1 infection. The decrease in vertical transmission and low stability after successive subcultures on agar for the *FgHal2* deletion mutant indicate that FgV1 requires *FgHal2* to maintain itself in the host. Therefore, the down-regulation of the expression of the *FgHal2* gene might be harmful for the fungal host, but this reduction is an inevitable consequence to provoke the persistence of the virus and to induce the defence response. However, the association between *FgHal2* and the maintenance of FgV1 infection requires further investigation.

Taken together, our results suggest that *F. graminearum* requires the putative 3'(2'),5'-bisphosphate nucleotidase gene *FgHal2* for normal mycelial growth, conidial production, conidial maturation and secondary metabolite synthesis. When challenged by FgV1, however, *F. graminearum* represses *FgHal2* expression, possibly to reduce viral RNA accumulation and to generate unstable conditions for the maintenance of FgV1.

EXPERIMENTAL PROCEDURES

Fungal strains and media

The PH-1 strain of *F. graminearum* was used as the WT in this study (Lee *et al*., 2014). Transgenic mutants were generated from the WT, and FgV1 was transferred to each strain by hyphal anastomosis with the PH-1/FgV1 strain, as described previously (Lee *et al*., 2014). All strains used in this study are listed in Table 1. For extractions of total RNA and genomic DNA, strains were grown in 50 mL of liquid CM at 25 °C for 5 days on a rotary shaker at 150 rpm. The WT and mutant strains were stored in 15% (v/v) glycerol at −80 °C and were reactivated on CM agar medium.

Sequence analysis

Nucleotide sequence and amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) database and Fusarium comparative database (Fusarium Comparative

Sequencing Project, Broad Institute of Harvard and MIT; http://www.broadinstitute.org). Amino acid sequences were aligned using the MegAlign program in DNASTAR and GeneDoc programs (http://www.nrbsc.org/gfx/genedoc/). A conserved domain search and sequence similarity analysis were conducted with the NCBI BLAST program.

Construction of *FgHAL2* gene deletion, over-expression and complementation mutants

DNA constructs for targeted gene deletion, complementation and over-expression were amplified by the double-joint (DJ) PCR method, as described in a previous study, with slight modifications (Son *et al*., 2013). The 5'- and 3'-flanking regions of the *FgHal2* gene and a gen resistance cassette were amplified from *F. graminearum* PH-1 and pII99, and fused by DJ PCR under the PCR conditions described previously (Lee S. *et al*., 2011b; Lin *et al*., 2011). The primer sets used in this study are listed in Table S1 (see Supporting Information). To complement the *FgHal2* gene deletion mutant, the DNA fragment carrying the native promoter and the *FgHal2* ORF was amplified with 9532 5F2/9532 5' RC primers and then fused with the green fluorescent protein gene (*gfp*) and the hygromycin (hyg) resistance cassette, which was amplified from the pIGPAPA vector (Lin *et al*., 2011) by DJ PCR. To generate over-expressing *FgHal2* mutants, the 5'- and 3'-ends of the *FgHal2* region were amplified by the primer pairs 9532 5F/9532 5F Gen-R and EF1 9532-3F/9532 OE-3R, respectively. The gen-$P_{ef1\alpha}$ construct, containing the gen resistance cassette and the EF1α promoter ($P_{ef1\alpha}$) from *F. verticillioides*, was amplified from pSKGEN (Lee S. *et al*., 2011b) with Gen_EF1 F/EF1 pro R primers. Amplicons were joined by DJ PCR as described above, and a final PCR product was generated using the nested primers. These three final DNA constructs were used for fungal transformation. Fungal protoplasts were transformed with polyethyleneglycol (PEG) as described previously (Son *et al*., 2013). Transformants were selected on PDA supplemented with 50 μg/mL of hyg or gen. After analysis of all transgenic strains using Southern blot (described below), FgV1 was introduced into the virus-free transformant strains through hyphal anastomosis. Viral infection was confirmed by RT-PCR using FgV1-specific primer pairs.

DNA extraction and Southern blot hybridization

After fungal strains had been incubated for 5 days on CM liquid medium, mycelia were collected by filtration through Whatman 3MM filter paper (GE Healthcare, Uppsala, Sweden), washed with distilled water, pressed between paper towels to remove excess water and stored at −80 °C. Genomic DNA was extracted as described previously (Lee K.M. *et al*., 2011a). For the Southern blot hybridization of WT and transgenic mutants, 10 μg of genomic DNA were digested with the appropriate enzyme. Agarose gel electrophoresis, capillary blotting and hybridization were performed following standard protocols, as described previously (Lee K.M. *et al*., 2011a). Labelled probes were generated by standard reaction with DNA fragments in 20 mL of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 30 μCi [α-^{32}P]dCTP, 3 mM deoxynucleoside triphosphate (dNTP) mix, 10 pmol of random primers and 2 U Klenow fragment (TaKaRa Bio Inc., Ohtsu, Japan). Hybridized blots were exposed to phosphoimaging screens (BAS-IP MS 2040, Fuji Photo Film Co., Tokyo, Japan) and analysed using a BAS-2500 image analysis system (Fuji Photo Film Co).

Mycelial growth and conidiation test

The morphologies of the virus-free and virus-infected WT and transformed mutant strains were observed after the strains had grown for 5 days at 25 °C on PDA, CM and minimal medium (MM; 0.05% KCl, 0.2% $NaNO_3$, 3% sucrose, 1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.02% trace elements, 2% agar). The radial growth was measured on PDA after 3, 4 and 5 days at 25 °C. The experiment was repeated three times independently. To assess colony surface hydrophobicity, a 15-μL droplet of dH_2O coloured with bromophenol blue was added to the top of 4-day-old fungal colonies grown on CM agar; the colonies were photographed 10 min later.

For the assessment of conidial production, the virus-free and FgV1-infected strains were incubated for 3 days on CM liquid medium. Mycelia were collected by filtration through Whatman 3MM filter paper and washed twice with distilled water. The harvested mycelia were spread on yeast malt agar (YMA) and incubated for 48–72 h at 25 °C under near-UV light (wavelength, 365 nm; HKiv Import and Export Co., Ltd, Xiamen, China). Conidia were collected in distilled water, filtered through cheesecloth and suspended in distilled water. A 10-μL volume of this suspension (1×10^5 conidia/mL) was placed in 5 mL of CMC medium (1.5% CMC, 0.1% yeast extract, 0.05% $MgSO_4{\cdot}H_2O$, 0.1% NH_4NO_3 and 0.1% KH_2PO_4) for 120 h at 25 °C on a rotary shaker (150 rpm). The conidia produced in the CMC culture were filtered through six layers of sterilized gauze, collected by centrifugation and counted using a haemocytometer. The experiment was repeated independently three times.

Microscopic observation of conidial and hyphal morphology

To assess conidial morphology and length, the conidia produced by each strain on YMA or CMC were harvested and examined with differential interference contrast (DIC) optics using a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany). The localization of GFP in the complementation strain was determined with a DE/Axio imager A1 microscope and a GFP filter (470/40 nm excitation, 495 nm dichroic, 525/50 nm emission).

Virulence assay on flowering wheat heads

A virulence test was performed on wheat cv. Jokyoung as described previously (Son *et al*., 2013). To prepare the conidial inoculum, mycelial plugs were incubated in CMC liquid culture for 5 days. To harvest conidia, the cultures were filtered through six layers of sterilized gauze and washed with distilled water; conidia in the filtrate were collected by centrifugation. A 10-μL volume of the conidial suspension (1×10^5 conidia/mL) of each strain was injected into 10 replicate wheat head florets at early–mid anthesis. Virulence was assessed 14 days after inoculation by quantifying the percentage of spikelets with head blight symptoms. Statistical analysis was conducted with PASW statistics software 20.0 (IBM SPSS Inc., Armonk, NY, USA).

Total RNA extraction and gene expression level analysis

Total RNAs of the virus-free or virus-infected WT and mutant strains were extracted from the mycelia incubated on CM liquid for 48, 72 and 120 h.

Total RNA was extracted and cDNA was synthesized as described previously (Lee *et al*., 2014). qRT-PCR was performed on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using SsoFast™ EvaGreen® Supermix (Bio-Rad) according to the manufacturer's instructions. After initial denaturation at 95 °C for 5 min, 40 cycles consisting of 5 s at 95 °C and 5 s at 58 °C were performed. Two endogenous reference genes, ubiquitin C-terminal hydrolase (UBH, locus FGSG_01231; Kim and Yun, 2011) and EF1α (locus FGSG_08811), were used as internal controls to normalize qRT-PCR results.

Vertical transmission

Vertical transmission of FgV1 by virus-infected WT and mutant strains was measured on the basis of conidial sporulation and subculturing on PDA for three generations. Conidia were harvested from FgV1-infected strains as described above, and 10–15 spores of this first generation were spread onto one PDA plate. At least 50 conidia of each strain were spread onto PDA plates per generation. The plates were cultured for 2–3 days to allow the spores to germinate. Germinated single spores were transferred to new PDA plates, each containing 30 independent germinates. These were incubated at 25 °C for an additional 5 days. The morphology and pigmentation of the colonies that developed were assessed, and each colony of this first generation was then assessed for the presence of FgV1 RNA using RT-PCR, virus-specific primers and treatment with DNase I and S1 nuclease (TaKaRa Bio Inc.). The colonies were then used to generate a second generation of conidia, and the process was repeated to generate and assess a third generation. The percentage of conidia that produced colonies containing FgV1 RNA was determined for each generation.

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SUPPORTING INFORMATION

Additional Supporting Information is provided in the online version of this article at the publisher's website:

Fig. S1 (A) Amino acid sequence alignment of the 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain in *Fusarium gramineraum*. The predicted FgHal2 protein, hypothetical protein FGSG_07103 and FGSG_01708 were used for this analysis. The positions of the amino acid sequences are indicated on the right. Protein names and GenBank accession numbers are as follows: *Fusarium graminearum* FGSG_09532 (XP389708.1); *Fusarium graminearum* FGSG_07103 (XP387279.1); *Fusarium graminearum* FGSG_01708 (XP381884.1). (B) Relative mRNA expression in the virus-free and virus-infected *F. graminearum* PH-1 wild-type (WT) strain. Relative transcript levels of *fg07103*, *fg01708* and *FgHal2* were normalized to elongation factor 1α and ubiquitin C-terminal hydrolase. cDNAs were generated from total RNA extracts obtained after 120 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on Tukey's test. VF, virus-free; VI, virus-infected.

Fig. S2 Effect of *FgHal2* gene deletion on the hydrophobicity of the surface mycelium. The wild-type (WT), *FgHal2* gene deletion mutant and *Fusarium graminearum virus 1* (FgV1)-infected WT were grown on complete medium (CM) for 3 days. Each colony was treated with 15 μL of a 2.5% bromophenol blue solution. Colonies were photographed 10 min later. VF, virus-free; VI, virus-infected.

Fig. S3 Confirmation of the relative expression of genes related to secondary metabolite synthesis. (A) Relative mRNA expression in the *FgHal2* over-expression strain. Relative transcript levels of *FgOs-2* and *Pks12* were normalized to *elongation factor 1*α (*EF1*α) and *ubiquitin C-terminal hydrolase* (*UBH*). cDNAs were generated from total RNA extracts after 72 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on Tukey's test. VF, virus-free; VI, virus-infected. (B) Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of *FgOs-2*, *Gip2*, *Pks12*, *AURj* and *FgHal2* genes using *Fusarium graminearum* strains. cDNAs were generated from total RNA extracts obtained after 72 h of incubation. After 25 cycles, amplified DNA was analysed by 1.5% agarose gel electrophoresis. The *EF1*α gene-specific primer set was loaded as a control.

Fig. S4 The effects of subculturing on the morphology of virus-infected *Fusarium graminearum* strains growing on potato dextrose agar (PDA). Single spores of all virus-infected strains were inoculated on PDA containing appropriate antibiotics. After 5 days, a mycelial plug from each plate was transferred to fresh PDA. Δ*FgHal2*-VI was transferred once more and photographed 5 days later.

Table S1 Oligonucleotide primers used in this study.