농학석사학위논문

Functional analysis of two host genes that were up-regulated upon Fusarium graminearum virus1 infection

Fusarium graminearum virus1 에 감염된 붉은 곰팡이에서 발현량이 증가하는 유전자들에 관한 기능분석

2015 년 2 월

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박진만

A THESIS FOR DEGREE OF MASTER SCIENCE

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BY

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Department of Agricultural Biotechnology

The Graduate School of Seoul National University

February 2015

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UNDER THE DIRECTION OF ADVISER
DR. KOOK-HYUNG KIM

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY
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Abstract

Functional analysis of two host genes that were up-regulated upon Fusarium graminearum virus 1 infection

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Major in Plant Microbiology

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Fusarium graminearum virus 1(FgV1) is a mycovirus that isolated from *Fusarium boothii*. It has 6.6kb double stranded RNA as a genome, including putative four open reading frames. Along with FgV1, three other mycoviruses have been reported including FgV2, FgV3, and FgV4. Among four reported viruses, FgV1 and FgV2 infection shows hypovirulence such as a reduction in growth rate, conidiation, and virulence to their host fungi. But infection with the other two viruses, FgV3 and FgV4, makes no dramatic morphological change.

Based upon RNA-sequence analyses of the host transcriptome upon combination of those four mycovirus infection, ten genes that might have relationship with mycovirus infection and/or defense responses of the host fungi are selected. These genes were assorted into four groups depending on their changes of expression level upon virus infection. To investigate possible functional roles of these genes involved in relationship between host fungi and virus, target gene deletion mutants were generated. FgV1 infected deletion mutants were constructed using hyphal fusion mediated mycovirus transmission. While deletion mutants did not show significant alteration in colony morphology and radial growth on solid comparing with wild-type strain, viral RNA accumulations in *FGSG_05076* and *FGSG_10551* deletion mutants were decreased comparing with those of virus infected wild-type strain. This study can provide experimental support for RNA-sequence analysis and investigation of individual gene functions which related to FgV1 accumulation in host fungi, *F. graminearum*.

Keywords: *Fusarium graminearum*, Fusarium graminearum virus 1 (FgV1), host factor, gene deletion, mycelia growth, viral RNA accumulation.

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CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

Since the first report of fungal virus isolated from mushroom in 1962 (Hollings, 1962), many fungal viruses (mycoviruses) have been reported in filamentous fungi, but also been reported in various fungal species. Most mycoviruses infections are cryptic to their host or express symptom under specific condition (Lemke, 1979; Rogers, 1986). Some mycovirus infections, however, show diverse symptoms. Double-stranded RNA (dsRNA) containing *Cryphonectria parasitica,* chestnut blight fungus, showed decreased level of virulence compared to the fungus which does not have the dsRNA. These phenomenon were referred as a hypovirulence-associated traits and the mycovirus denominated Cryphonectria hypovirus 1 (CHV1) (Hilman et al., 1990). In addition to hypovirulence to host fungus, CHV1 infection can induced suppressed speculation, alteration of colony morphology, reduced pigmentation, oxalate accumulation, decreased cellulase and laccase activity (Anagnostakis et al, 1979; Hilman et al., 1990). In addition, it is also reported that dsRNA mycovirus is transmitted from one strain to another strain through hyphal anastomosis. These traits suggested that mycovirus can be used as a biological control of chestnut blight (Anagnostakis et al., 1979).

Chestnut blight fungus and CHV1 also provided an opportunity for understanding host-virus interaction. Recently, several host factors were recognized as key genes for mycovirus induced alterations of host biology. *NAM-1* gene is responsible for

symptom induction in CHV1 infected *C. parasitica* (Faruk et al., 2008). Host transcription factor *pro1,* that is required for female fertility of the host fungus, reported as a crucial gene for developing asexual spores and the maintaining CHV1 infection (Sun et al., 2009). Cpbir, functioning in fungal conidiation and virulence, is down-regulated when fungus is infected with CHV1, and it affects transmission of CHV1 (Gao et al., 2013). Hsp24, acid-inducible small heat-shock protein, also down-regulated CHV1 infection, and it related fungal stress response and virulence (Baek et al., 2014). These studies suggested that functional analysis of host genes can provide the proper access to understand how mycovirus interacts with the host fungus. Because previous studies showed many genes changed their expression level upon virus infection, these genes might have crucial role(s) in virus-host interactions.

Fusarium graminearum is well known fungus as plant pathogen. The fungus causes head blight in small grains such as wheat and barley and stalk and ear rot on maize (Cook et al., 1981;Kommedahi et al., 1981; Sutton et al., 1982). The disease by *F. graminearum* also can cause reduced yield and contamination of harvested grains with mycotoxins such as trichothecenes and zearalenone (Marasas et al., 1984). In *Fusarium species,* dsRNA mycoviruses have been reported in *F. poae* and, *F. solani* f. sp. *robiniae* (Compel et al., 1999; Nogawa et al., 1993; Nogawa et al., 1996). In *F. graminearum* several mycoviruses also have been reported (Aminian et al., 2011; Chu et al., 2002; Chu et al., 2004; Darissa et al., 2011; Kwon et al., 2009; Wang et al., 2013; Yu et al., 2009; Yu et al., 2011). Among them, some viruses that infect *F.*

graminearum have shown to hypovirulence (Aminianet al., 2011; Chu et al., 2002; Darissa et al., 2012; Wang et al., 2013). Fusarium graminearum virus 1 strain DK21 (currently named FgV1), which has dsRNA as genome, infects *F.* graminearum, reduces the virulence of *F.* graminearum and production of mycotoxin, delays mycelia growth, and increases pigmentation (Chu et al., 2004).

Proteomic and transcriptomic analysis based approaches have been employed to identify host factors required for FgV1 infection cycle (Cho et al., 2012; Kwon et al., 2009; Lee et al., 2014). Continued research on revealing function(s) of host factor involved in FgV1-*F. graminearum* interaction was also conducted. Recently, study about *HEX1* gene of *F. graminearum* showed validity of proteomic analysis based research. Hex1, a major protein of woronin body, plays crucial roles in maintaining the cellular integrity and pathogenicity of *F. graminearum* and it also affect viral RNA accumulation (Son et al., 2013). *FgHal2* gene was also reported as a host factor that affects viral RNA accumulation and virus transmission (Yu et al., 2014).

In this study, ten *F. graminearum* genes were selected as perspective host factors according to previous study (Lee et al, 2014). Ten genes were divided into four groups depending on different expression level (up or down) by different mycovirus (FgV1, 2, 3, and 4) infections (Table 1). This study might provide basic knowledge for understanding mycovirus-host fungus interactions which can influence hypovirulence of mycovirus or pathogenicity of the host fungus.

Table 1. List of selected target genes. Blast result based on NCBI database. Length means number of gene sequence nucleotide. FgV1, FgV2, FgV3, FgV4 indicate Fusarium graminearum virus 1,2,3, and 4 infections. Values below each infection are converted transcriptomic reads value of each gene.

			Log2 ratio (* /PH-1) [UP & DOWN]									
Ref	Blast result	Length	FgV1	FgV1-2	FgV1-3	FgV1-4	FgV2	FgV2-3	FgV2-4	FgV3	FgV3-4	FgV4
FGSG_06952T0	Hypothetical protein	1539	7.15	5.36	5.91	2.36	3.70	2.21	5.30	-0.13	0.35	-0.30
FGSG_06989T0	Lipase like protein	1530	2.72	2.73	2.03	3.46	3.14	1.14	3.87	0.78	-1.32	-2.37
FGSG_05076T0	ABC transporter or ATP relate gene	3024	4.48	3.16	6.53	4.58	1.24	1.17	2.86	0.91	0.61	0.40
FGSG_01872T0	DUF3455 family	795	-3.59	-1.51	-5.16	-3.24	-2.92	-1.45	-1.60	-0.96	1.82	2.15
FGSG_09110T0	Monooxygenase, FAD-binding, Aromatic-ring hydroxylase, Thioredoxin-like fold,	2004	-3.95	-1.68	-2.11	-1.66	-2.26	-1.07	-2.32	-0.54	0.14	-0.61
FGSG_00130T0	Heterokaryon incompatibility	1818	6.81	5.19	7.66	5.28	3.89	3.65	5.76	1.27	4.32	2.46
FGSG_10551T0	Killer toxin, KP4	825	3.56	4.11	7.86	3.76	2.89	2.03	5.27	2.31	2.17	1.28
FGSG_11564T0	Only exist in F.graminearum	411	7.17	5.22	5.84	5.40	3.29	2.43	4.66	1.37	3.11	2.82
FGSG_04917T0	Hypothetical protein	414	-4.71	-3.79	-4.35	-2.43	-1.79	-2.44	-4.08	-2.41	-3.37	-2.72
FGSG_07861T0	Hypothetical protein	669	-6.35	-5.50	-8.11	-4.86	-2.26	-1.47	-9.36	-2.83	-2.43	-2.02

MATERIAL AND METHODS

1. Fungal strains and culture condition

All strains used in this study (Table 2) were stored in 25% (v/v) glycerol at -80^{o}C and were reactivated on potato dextrose agar (PDA; Difco). For nucleic acid manipulation, all strains of *F. graminearum*PH-1 were grown in 50 ml of a liquid complete medium [CM, (Lee et al., 2011)] at 25^{o}C with shaking (150 rpm) for 5 days. Mycelia were harvested by filtration through miracloth (Calbiochem) and ground to a fine powder with liquid nitrogen in a mortar and pestle.

2. Computational analysis

Nucleotide sequence of target genes in *F. graminearum* strain PH-1 were obtained mainly from *Fusarium* comparative database (http://www.broadinstitute.org). NCBI database was also used as a reference. Blast research program CFGP 2.0 (Choi et al., 2013) are used to find out predicted functions of genes and whether they conserved in filamentous fungi.

3. Construction of target gene deletion and complementation mutants

For extraction of genomic DNA, a previously described procedure was used (Lee et al., 2011). To construct a PCR fragment for deletion and complementation, a slightly modified double-joint (DJ) PCR strategy was applied for fusion of PCR products (Son et al., 2013).

Table 2. List of fungal strains used in this study.

Strain	Description	Reference
WT-VF	Wild-type *F. graminearum* (lineage7), *G. zeae* PH-1	Lee *et al.*, 2014
WT-VI	Wild-type PH-1 infected with FgV1	Lee *et al.*, 2014
⊿*fgsg_05076*-VF	*fgsg_05076* deletion mutant in WT-VF genetic background	This study
⊿*fgsg_05076*-VI	FgV1 introduced ⊿*fgsg_05076*-VF by hyphal anastomosis	This study
⊿*fgsg_06952*-VF	*fgsg_06952* deletion mutant in WT-VF genetic background	This study
⊿*fgsg_06952*-VI	FgV1 introduced ⊿*fgsg_06952*-VF by hyphal anastomosis	This study
*fgsg_06952*C-VF	*fgsg_06952* complement mutant in WT-VF genetic background	This study
*fgsg_06952*C-VI	FgV1 introduced *fgsg_06952*C-VF by hyphal anastomosis	This study
⊿*fgsg_06989*-VF	*fgsg_06989* deletion mutant in WT-VF genetic background	This study
⊿*fgsg_06989*-VI	FgV1 introduced ⊿*fgsg_06989*-VF by hyphal anastomosis	This study
*fgsg_06989*C-VF	*fgsg_06989* complement mutant in WT-VF genetic background	This study
*fgsg_06989*C-VI	FgV1 introduced *fgsg_06989*C-VF by hyphal anastomosis	This study
⊿*fgsg_09110*-VF	*fgsg_09110* deletion mutant in WT-VF genetic background	This study
⊿*fgsg_09110*-VI	FgV1 introduced ⊿*fgsg_09110*-VF by hyphal anastomosis	This study
⊿*fgsg_01872*-VF	*fgsg_01872* deletion mutant in WT-VF genetic background	This study
⊿*fgsg_01872*-VI	FgV1 introduced ⊿*fgsg_01872*-VF by hyphal anastomosis	This study
*fgsg_01872*C-VF	*fgsg_01872* complement mutant in WT-VF genetic background	This study
*fgsg_01872*C-VI	FgV1 introduced *fgsg_01872*C-VF by hyphal anastomosis	This study

Table 2. Continued.

Strain	Description	Reference
∠*fgsg_00130*-VF	*fgsg_00130* deletion mutant in WT-VF genetic background	This study
∠*fgsg_00130*-VI	FgV1 introduced ∠*fgsg_00130*-VF by hyphal anastomosis	This study
∠*fgsg_10551*-VF	*fgsg_10551* deletion mutant in WT-VF genetic background	This study
∠*fgsg_10551*-VI	FgV1 introduced ∠*fgsg_10551*-VF by hyphal anastomosis	This study
fgsg_10551 C-VF	*fgsg_10551* complement mutant in WT-VF genetic background	This study
fgsg_10551 C-VI	FgV1 introduced *fgsg_10551* C-VF by hyphal anastomosis	This study
∠*fgsg_11564*-VF	*fgsg_11564* deletion mutant in WT-VF genetic background	This study
∠*fgsg_11564*-VI	FgV1 introduced ∠*fgsg_11564*-VF by hyphal anastomosis	This study
∠*fgsg_04917*-VF	*fgsg_04917* deletion mutant in WT-VF genetic background	This study
∠*fgsg_04917*-VI	FgV1 introduced ∠*fgsg_04917*-VF by hyphal anastomosis	This study
∠*fgsg_07861*-VF	*fgsg_07861* deletion mutant in WT-VF genetic background	This study
∠*fgsg_07861*-VI	FgV1 introduced ∠*fgsg_07861*-VF by hyphal anastomosis	This study
∠*fgsg_Cluster*-VF	*fgsg_00060*, *fgsg_00061*, *fgsg_00062* cluster deletion mutant in WT-VF genetic background	This study
∠*fgsg_Cluster*-VI	FgV1 introduced ∠*fgsg_Cluster*-VF by hyphal anastomosis	This study

For the complementation of deletion mutants, the hygromycin resistance gene cassette (*hph*) was amplified from the pIGPAPA plasmid. General PCR was performed following the manufacturer's instructions (TaKaRa). The PCR primers used in this study were produced at an oligonucleotide synthesis facility (Bioneer).

4. Fungal transformation

Preparation and inoculation of fungal protoplasts were described previously. *F. graminearum* strain PH-1 was used to perform transformation. In shaking incubator (150 rpm, 25°C), fungi were grown in 250 ml flask containing 50 ml of CMC media. 5 days post inoculation, 10 ml of CMC media which contain conidia sub cultured in 40 ml of YPG media for overnight at 25°C in shaking. Germinated mycelia were harvested with filter paper and washed with 1M NH_4Cl, and mixed with 0.8% driselase enzyme solution. After incubating in shaking incubator (50 rpm, 30°C) for 3 hours, mixture were filtered with gauze. Filtered mixtures were centrifuged 5000 rpm for 10 min at 4°C. Protoplasts were washed with 1 ml of 1M NH_4Cl and further re-suspended in STC buffer (1.2 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$). DNA fragment was added to protoplast suspension in STC buffer and incubation on ice for 10 min. After incubation 10 min, PEG solution [60% polyethylene glycol in 10 mM Tris (pH7.5), 10mM $CaCl_2$] was added and then 1 ml of STC buffer was added. Mixture was gently mixed and incubated on ice for 15 min. On 1 5ml of regeneration medium, 300 μl of mixture was poured and incubated at 25℃ for overnight. Incubated plated was covered with water agarose containing 150 ppm of geneticin. Transgenic fungal isolates were sub cultured two times on geneticin containing CM media.

5. Genomic DNA extraction

Fungal strains were incubated for 3 days in CM broth in shaking incubator (150 rpm, 25°C). 100 ml flask and 20 ml of CM media were used to incubate mycelia. Incubated mycelia were collected with filter paper and distilled water, and deposited at –80°C until extraction procedure. From deposited mycelia genomic DNA were extracted with following procedure. Mycelia were grinded with liquid nitrogen and CTAB buffer solution (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA and 1.4 M NaCl) was added. Mixture of grinded mycelia and CTAB solution was incubated at 65°C for 30 min. After incubation, chloroform and iso-amyl alcohol (24:1) were added to mixture. Mixture was centrifuged 13000 rpm for 10minand than upper layer transferred to new tube. Transferred solution was cleaned up two times with PCI solution (Phenol : chloroform : iso-amyl alcohol= 25 : 24 : 1). After all clean up step, DNA were precipitated with iso-propanol and re-suspended with TE buffer (10 mM Tris Base, 1 mM EDTA).

6. Southern blot hybridization

For the Southern blot hybridization of transgenic mutants and wild type, 10 μg of those sample's genomic DNA was used. Genomic DNAs were digested with proper restriction enzyme through overnight incubation. Digested DNA was loaded in 0.8% agarose gel at low voltage (50~60 V) and agarose gel went through following step. The agarose gel was incubated in 0.2 N HCl solution 10min, and flow discarded. In denaturation buffer (1.5 M NaCl, 0.5 N NaOH), the agarose gel was soaking for 35min. After all procedure, digested DNAs were transferred from agarose gel to nylon membrane with capillary transfer. DNA

probes for southern blot hybridization were generated with fragment in 20 ml of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM DTT, 30 μCi [α-^{32}P] dCTP, 3 mM dNTP mix, 10 p moles of random primers and 2 U klenow fragment (TaKaRa). Membrane and probe were incubated at 65°C with hybridization buffer. Hybridized membrane was exposed to phosphor imaging screens (BAS-IP MS 2040, Fuji Photo Film, Japan).

7.Total RNA extraction

Total RNAs of all strain that used in this study were extracted following same protocol. Mycelia incubated on 50 ml of CM liquid media in 250 ml flask. After 5 days in shaking incubator (150 rpm, 25°C), mycelia were harvested with filter paper (Hyundai-micro) and H_2O. Harvested mycelia were deposited at -80°C until extraction. Frozen samples are grinded with liquid N_2, and add Iso-RNA Lysis reagent (5 PRIME, Gaithersburg, USA) and chloroform. After vortex the mixture enough, centrifuge the mixture 10 min in 12000rpm at 4°C. In centrifuged mixture, upper layer transferred new tube and add iso-propanol. Mixtures were incubated in ice 30minitues and centrifuged 10 min 10,000 rpm at 4°C. Centrifuged tube cleaned up with 70% ethanol and the pellet re-suspended with DEPC treated 3°H_2O. To remove genomic DNA, DNase I (Takara Bio Inc, Japan) was treated for 1hours at 37℃. Total RNA samples were cleaned up with PCI and synthesized to cDNA with reverse-transcriptase.

8. Semi quantitative RT-PCR

Viral RNA accumulation was speculated with semi quantitative RT-PCR. 5 μg of total RNA

were extracted from mycelia at 5 dpi and used for cDNA synthesis. cDNA was diluted (0.05 μg/μl) for PCR amplification. In 15, 20, 25 cycles of PCR amplification were distinguishable viral RNA accumulation when comparing wild type and mutant.

9. Colony morphology and radial growth test

Mycelia growth test was performed in CM agar media (1.5% agarose gel). 35 ml of media was used in assay. 0.5 mm square agar block was transferred on the center of Petri dish. Mycelia growth was measured in four directions and colony edge of each direction was checked and measured as data. Growth test was performed at 25°C for 5 days. Colony morphology was pictured after 5days incubation. Especially, in two gene MM and PDA agar media were used in colony morphology test.

Table 3. Primer sets for this study. Gene indicates target gene number. DEL means primer for deletion mutant and COM means primer for complementation mutant. 5' and 3' indicate each flanking region previously described in transformation strategy. FW and RV are directions of primers.

Gene	primers	sequence
fgsg_05076	DEL 5' FW	AGACCGCTCAAGAGAAAAAAAG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGTGCGTCTTCTGGTCACAGTTTC
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGTAGATGAGTTTCTTGGGTATAC
	DEL 3' RV	GGTTGCCAAAGTCTTCAACGTG
	NESTED FW	TGCTGACAGGAACATCCAAGAT
	NESTED RV	GTGTGGGGGGAACAACCGAGGA
fgsg_06952	DEL 5' FW	ACCCAAGTTGAGCTAGACCGAG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGAGAGTCTGATAGATTCTTGCGA
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGTTTCTGCCAGCGAGCTACCGCG
	DEL 3' RV	CCTTCTCGGGAGATCACATACG
	NESTED FW	GATCGAATCTACAAGTTGATGCG
	NESTED RV	ATTCTACCCAAGCTGAAACACGG
	COM 5' RV	ACAGCTCCTCGCCCTTGCTCACAAGGAAAAACATGATGACAGCA
	COM 3' FW	CTCCACTAGCTCCAGCCAAGCCTTTCTGCCAGCGAGCTACCGCG
fgsg_06989	DEL 5' FW	CAGTCACATGTGTGGTCTCACG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGGGTGTTCTGTTGAGTCGATATT
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGGAACTCTTGGATTGGAGAAGCG
	DEL 3' RV	GGTTACGCAACTGGAGATGTCAG
	NESTED FW	TTGCCTCATAGTCAAGGTCACG
	NESTED RV	AGATCCTCGGCAACTCTATCCC
	COM 5' RV	AGAGCTCCTCGCCCTTGCTCACTTCCGGCACCCGAGGCTGACAG
	COM 3' FW	CTCCACTAGCTCCAGCCAAGCCGAACTCTTGGATTGGAGAAGCG

Table 3. Continued.

Gene	primers	sequence
fgsg_00130	DEL 5' FW	GAGGTAATATCGGTGGAGAGAG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGCTTGAGGAATGGTTTGTTGATG
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGATGTCTTTTTCGGTGAGGGTAT
	DEL 3' RV	GCCAAAATAGAAGTCAAGCATG
	NESTED FW	TCTCAGTGAGGAGAGTGGTAGA
	NESTED RV	TCTGGAAGAGGGGCCGCGGTAC
fgsg_10551	DEL 5' FW	AGATGCATACCCCGGACTAGAA
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGGTTGTTGGAATGAGAGTTTGTA
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGACAAGGCTTTGGATGCCGAAAA
	DEL 3' RV	CAGTGGCTAATCATCCATAATG
	NESTED FW	TCGGAGCTAGACTTGTATCACG
	NESTED RV	ACTCGATTCAAAAGACTCTTTT
fgsg_11564	DEL 5' FW	GCAGCAAGACTAACTCATCTCG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGATTGAAGAGATGAGTAAATGAG
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGGGAGTCAAGACTGGAATCTCCC
	DEL 3' RV	GTGTTTGGGGAGACCCTTGATG
	NESTED FW	GCGGATTCTTTCATGACTAGGG
	NESTED RV	ATTTCAATATGCCTGTCAGAGC

Table 3. Continued.

Gene	primers	sequence
fgsg_09110	DEL 5' FW	TAAAGTTCAGGACGTTCCATGC
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGAGTTGCGGCTAAGTACGGTGAT
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGAAAGAAGACACTTATTAGATAG
	DEL 3' RV	TGGTCTCCAGGCATGGTCCAGG
	NESTED FW	CCACTTGAGCTGATAAATGTAA
	NESTED RV	TATTGGAACTACTCTGAGTTTG
fgsg_01872	DEL 5' FW	ATAGCCCGTCCCAATTAAGAGG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGCTTAAAGTGGTGTATATGTGTG
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGACTTATTAGGAAGACTTTAGGA
	DEL 3' RV	AGATGGACCTTGTGCCGTGTTC
	NESTED FW	GACCCTGGTTGTATTGAAGATGG
	NESTED RV	AAGTGTTGGTGGGTGATTGCC
	COM 5' RV	ACAGCTCCTCGCCCTTGCTCACAGCCTCAGCATCATCAGTTTTG
	COM 3' FW	CTCCACTAGCTCCAGCCAAGCCACTTATTAGGAAGACTTTAGGA

Table 3. Continued.

Gene	primers	sequence
fgsg_04917	DEL 5' FW	CAACCCTGACGGCGTGCTAAGC
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGTGTGGTGATGTTTGTATGTGTG
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGGACGATATAATTTGAAGTGTTT
	DEL 3' RV	CTGCAACTGACTGCAGACTGTA
	NESTED FW	ATAGCGGGAAGAAAGTGACAGGA
	NESTED RV	AACGCCAAGGAGAAGATTTTT
fgsg_07861	DEL 5' FW	CCAGAGCCAGAGACCGGGGAAG
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGTTTGAAGGTGTTGTAAATGAAT
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGGGGTTTTGCTGGGATGAATCGG
	DEL 3' RV	TCGGCACAGATGATGTGCTCGC
	NESTED FW	AACCTTCTCGGACTTCTTTTTT
	NESTED RV	CATCGATATGCTCCTCAACGTG
fgsg_cluster	DEL 5' FW	AGATTATTTCGTGCGCACTCAT
	DEL 5' RV	GCACAGGTACACTTGTTTAGAGGATTACTAAGCAGTCGGAGGCA
	DEL 3' FW	CCTTCAATATCATCTTCTGTCGATCTTTTCATGTATTTAGGGAA
	DEL 3' RV	AAGTACCTACTTCAAGACCACG
	NESTED FW	CATGGAATAGCCTACGTCATTG
	NESTED RV	AACAATCTGGATGACATAAGTG
gen	FW	CTCTAAACAAGTGTACCTGTGC
	RV	CGACAGAAGATGATATTGAAGG

Table 3. Continued.

Primer name	sequence
FgV1 RdRp region (1411) FW	TGTGGGAGAAGAAGTATGGCCT
FgV1 RdRp region (2889) RV	ATCAGGAACCATTGAAAGAGTCC
FgV1 ORF4 region (5286) FW	ATGAGCGCAACCAGCAACAG
FgV1 ORF4 region (6556) RV	TTAGGCGTTTGTTGCCGGAA

RESULTS

1. Selection of target gene

Ten genes of the *F. graminearum* were selected as suspect host factors that related to mycovirus-host fungus interaction according to previous RNA-sequence analysis (Lee et al., 2014). Ten genes were divided into four groups according to their expression characteristics (Table 1). Group 1 for *FGSG_06952*, *FGSG_06989* and *FGSG_05076* showed highly expressed only in FgV1 and FgV2 infected *F. graminearum* PH-1 strain (hypovirulent strains). Group 2 for *FGSG_01872* and *FGSG_09110* was down-regulated in hypovirulent strains. Group 3 for *FGSG_00130*, *FGSG_10551* and *FGSG_11564* showed highly expressed in all FgVs infected strains. Group 4 for *FGSG_04917* and *FGSG_07861* was down-regulated in all FgVs infected strains.

2. Prediction of gene function using blast tools

Functions of ten genes were predicted using the NCBI blast and CFGP 2.0 software (Choi et al., 2013). *FGSG_05076* has ATP binding site domain, and it has similar sequence of ATP binding cassette group G2. *FGSG_10551* has two killer toxin 4 (KP4) domains. In *F. graminearum*, there are four KP4 similar genes and *FGSG_10551* constitute as a cluster (*FGSG_00060, FGSG_00061 and FGSG_00062*). Excepting*FGSG_10551*, other genes have only one domain.*FGSG_00130* is related with heterokaryon incompatibility which affects undergoing fusion of vegetative fungal cells. *FGSG_09110*, which FAD-binding aromatic-ring

hydroxylase related gene, is widely distributed in filamentous fungi. *FGSG_11564*presents only in *F. graminearum. FGSG_09110*, whcih FAD-binding aromatic-ring hydroxylase related gene, is widely distributed in filamentous fungi. Prediction of other genes functions did not available because they are hypothetical gene or have low ortholog similarity.

3. Generation of gene deletion mutants

Target gene-deletion mutants were generated to investigate the role(s) of each gene. All genes were successfully replaced with *gen* by homologous recombination (Fig. 1A, Fig. 2, and Fig. 3). All of gene deleted strains were confirmed by southern blot hybridization (Fig. 2 and Fig. 3).

4. Generation of complementation mutants

In pre-performed mycelia radial growth test, three genes deletion mutants, ∆*FGSG_06952*-VF, ∆*FGSG_06989*-VF and ∆*FGSG _06952*-VF, showed different growth pattern (data not shown). They grew faster than WT-VF. In FgV1 infected strains, ∆*FGSG_06952*-VI and ∆*FGSG_01872*-VI also grew faster comparing with WT-VI strain. These genes were successfully complemented by homologous recombination (Fig. 1B). All of the gene complementation strains were confirmed by southern blot hybridization (Fig. 4).

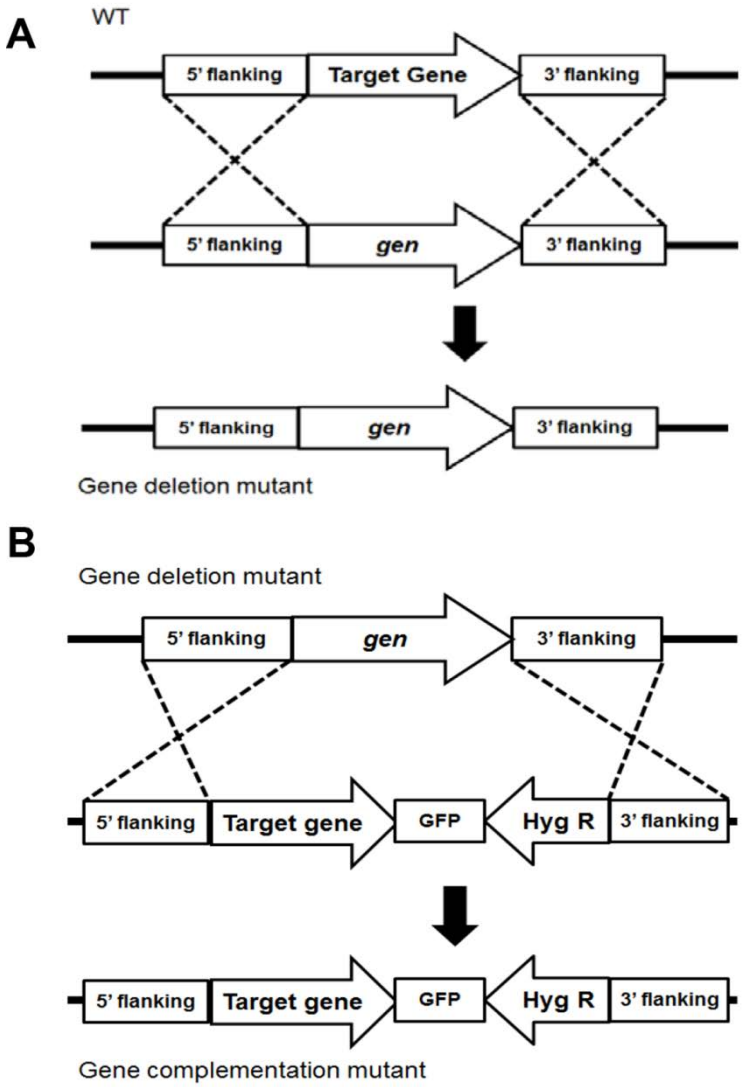
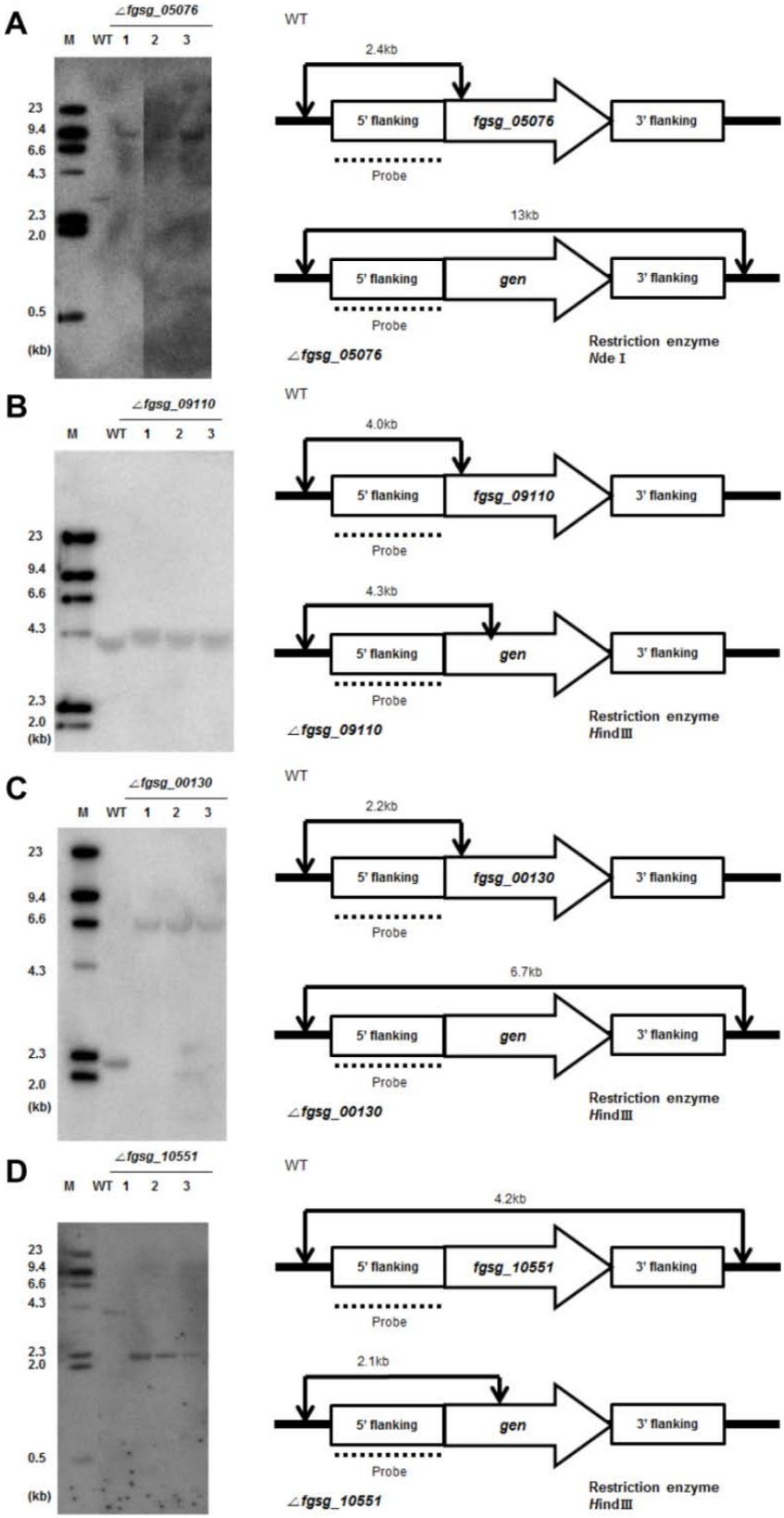


Fig.1 Representative strategy for generating gene deletion and complementation mutants. (A) Generation of gene deletion mutant using WT back bone. (B) Generation of complementation mutants using gene deletion mutant back bone. Both strategies have been designed based upon homologous recombination which occurs at 5' and 3' flanking regions.


A
⊿fgsg_05076
M WT 1 2 3
23
9.4
6.6
4.3
2.3
2.0
0.5
(kb)
WT
2.4kb
5' flanking
fgsg_05076
3' flanking
Probe
13kb
5' flanking
gen
3' flanking
Probe
⊿fgsg_05076
Restriction enzyme
Nde I
B
⊿fgsg_09110
M WT 1 2 3
23
9.4
6.6
4.3
2.3
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(kb)
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4.0kb
5' flanking
fgsg_09110
3' flanking
Probe
4.3kb
5' flanking
gen
3' flanking
Probe
⊿fgsg_09110
Restriction enzyme
HindIII
C
⊿fgsg_00130
M WT 1 2 3
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9.4
6.6
4.3
2.3
2.0
(kb)
WT
2.2kb
5' flanking
fgsg_00130
3' flanking
Probe
6.7kb
5' flanking
gen
3' flanking
Probe
⊿fgsg_00130
Restriction enzyme
HindIII
D
⊿fgsg_10551
M WT 1 2 3
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9.4
6.6
4.3
2.3
2.0
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(kb)
WT
4.2kb
5' flanking
fgsg_10551
3' flanking
Probe
2.1kb
5' flanking
gen
3' flanking
Probe
⊿fgsg_10551
Restriction enzyme
HindIII

Fig. 2 Construction strategy for target gene deletion mutant and Southern blot hybridization. Deletion mutants were confirmed by DNA gel blot analysis. When 5' flanking regions were used as a probe for southern blot hybridization, the genomic DNAs digested with restriction enzymes from each deletion strains generated distinguishable hybridized DNA fragments of various sizes. All strains were constructed using a PCR-based strategy as described in the Materials and Methods. (A) Deletion for *FGSG_05076*, (B) *FGSG_09110*, (C) *FGSG_00130*, and (D) *FGSG_10551*.

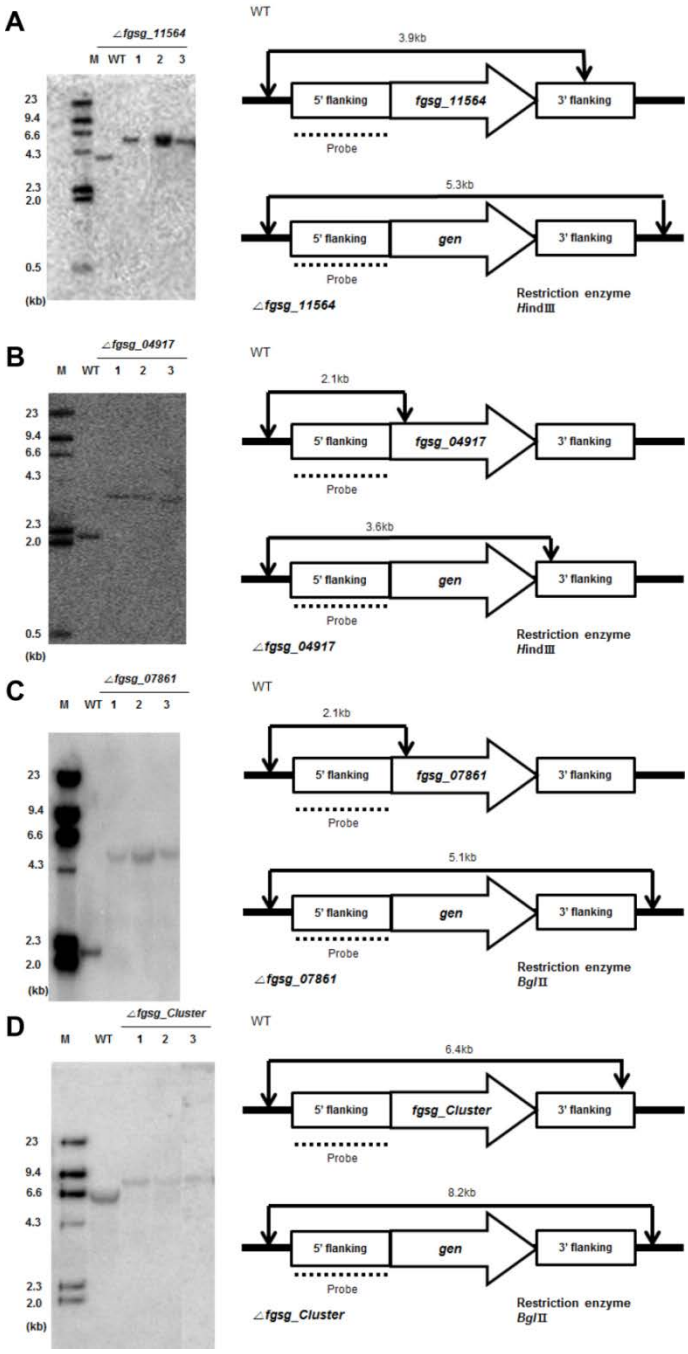

Fig.3 Construction strategy of target gene deletion mutant and Southern blot hybridization. Deletion mutants were confirmed by DNA gel blot analysis. When 5' flanking regions were used as a probe for southern blot hybridization, the genomic DNAs digested with restriction enzymes from each deletion strains generated distinguishable hybridized DNA fragments of various sizes. All strains were constructed using a PCR-based strategy as described in the Materials and Methods. (A) Deletion for *FGSG_11564*, (B) *FGSG_04917*, (C) *FGSG_07861*, and (D) *FGSG_cluster*.

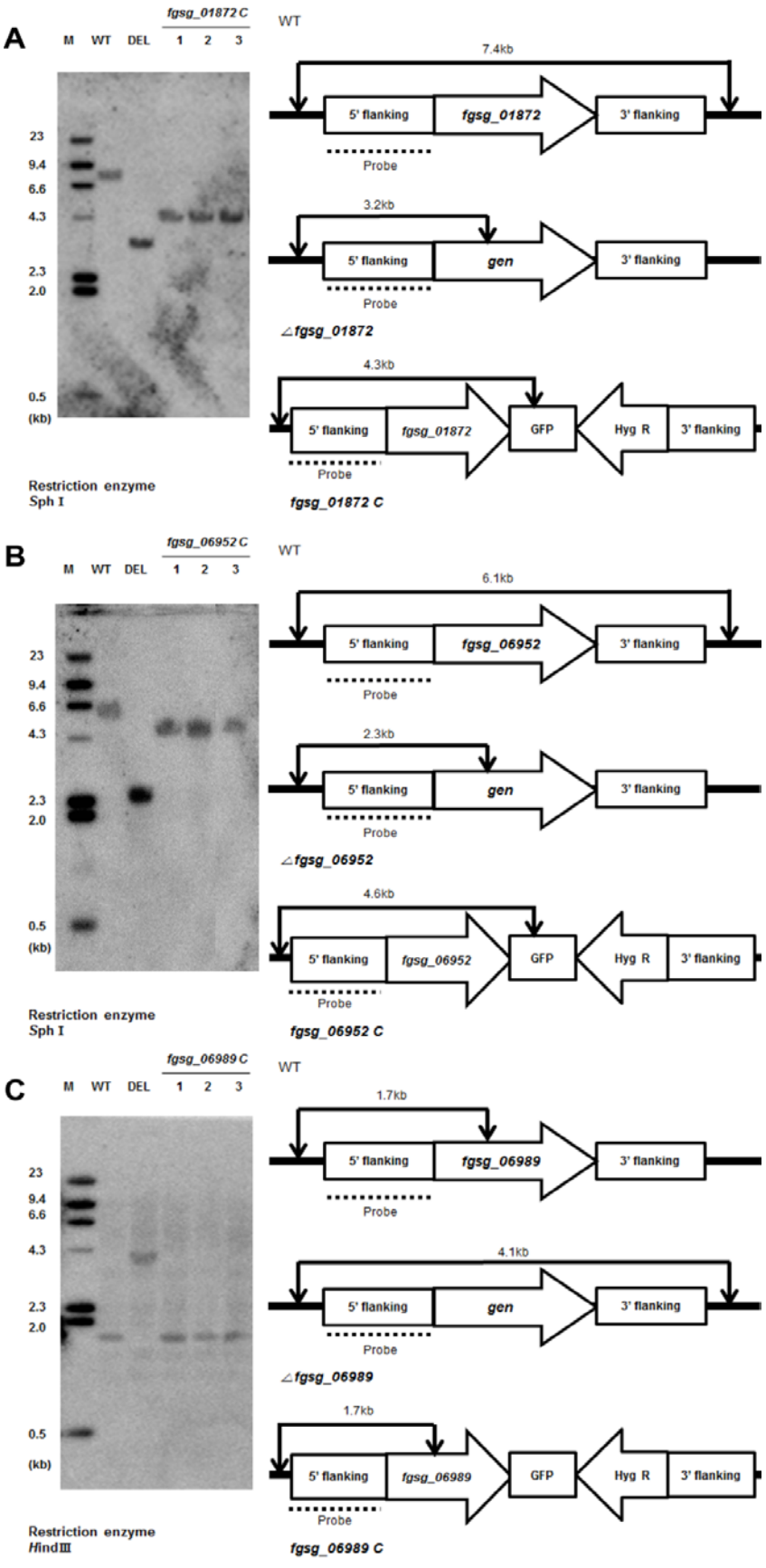
A
fgsg_01872 C
M WT DEL 1 2 3
23
9.4
6.6
4.3
2.3
2.0
0.5
(kb)
Restriction enzyme
Sph I
WT
7.4kb
5' flanking
fgsg_01872
3' flanking
Probe
3.2kb
gen
⊿fgsg_01872
4.3kb
GFP
Hyg R
fgsg_01872 C
B
fgsg_06952 C
6.1kb
fgsg_06952
⊿fgsg_06952
4.6kb
fgsg_06952 C
C
fgsg_06989 C
1.7kb
fgsg_06989
4.1kb
⊿fgsg_06989
fgsg_06989 C
HindⅢ

Fig.4 Construction strategy of complementation mutant and Southern blot hybridization. Complementation mutants were confirmed by DNA gel blot analysis. When 5' flanking regions were used as a probe for southern blot hybridization, the genomic DNAs digested with restriction enzymes from each deletion strains generated distinguishable hybridized DNA fragments of various sizes. All strains were constructed using a PCR-based strategy as described in the Materials and Methods. (A) Complementation for *FGSG_01872* C, (B) *FGSG_06952* C, and (C) *FGSG_06989*C

5. Colony morphology test

To investigate effect of genes deletion and FgV1 infection, colony morphologies were analyzed using PDA, CM and MM. In all virus-free deletion strains, deletion of target genes did not cause any dramatic morphological change compare to WT-VF (Fig. 5A, top row). However, in virus infection, several mutants cause slight difference in their morphology (Fig. 5A, bottom row). First, in ∆*FGSG_09110*-VI, ∆*FGSG_11564*-VI and ∆*FGSG_04917*-VI strain, pigmentation of their colony were decreased. Comparing with WT-VI, the portions of red colored area in colony were small. Second, *FGSG_11564* and *FGSG_04917* deletion mutants show very slow growth and their mycelia were not much as WT-VI strain.

And Second morphology test that target on *FGSG_05076* and *FGSG_10551* deletion mutant had more distinguishable changes occurred (Fig. 5B). In FgV1 infected strains, both genes deletion mutants grew faster than WT-VI in MM and CM (Fig. 5B, right side) Especially, WT-VI grow just little while both genes deletion mutants grew faster and widely on MM media.

6. Vegetative growth of deletion mutants

The radial growths on PDA, CM, and MM were measured to support colony morphologies using quantitative data. Among all the virus-free strains, radial growth of ∆*FGSG_01872*-VF, ∆*FGSG_06952*-VF and ∆*FGSG_06989*-VF strains were faster than WT-VF. Radial growths of virus-free *FGSG_09110*, *FGSG*_10551, and *FGSG_11564* deletion strains were slower

than WT-VF (Fig. 6A). Interestingly, ∆*FGSG_01872*-VI and ∆*FGSG_06952*-VI strains also grew faster comparing with WT-VI strain (Fig.6B).

The radial growths of *FGSG_01872*, *FGSG_06952*, and *FGSG_06989* deletion and complementation mutants were measured day by day to investigate detailed growth pattern (Fig. 7). Deletion and complementation mutant of those three genes increased their growth in without FgV1 infection (Fig. 7A). However, there were not significant differences among three different mutants comparing with FgV1 infected strains (Fig. 7B). *FGSG_01872* and *FGSG_06952* gene deletion mutants showed increased mycelia growth when compared to WT and complement mutants. Otherwise, *FGSG_06989* deletion mutant showed decreased mycelia growth when comparing with WT and complement mutants.

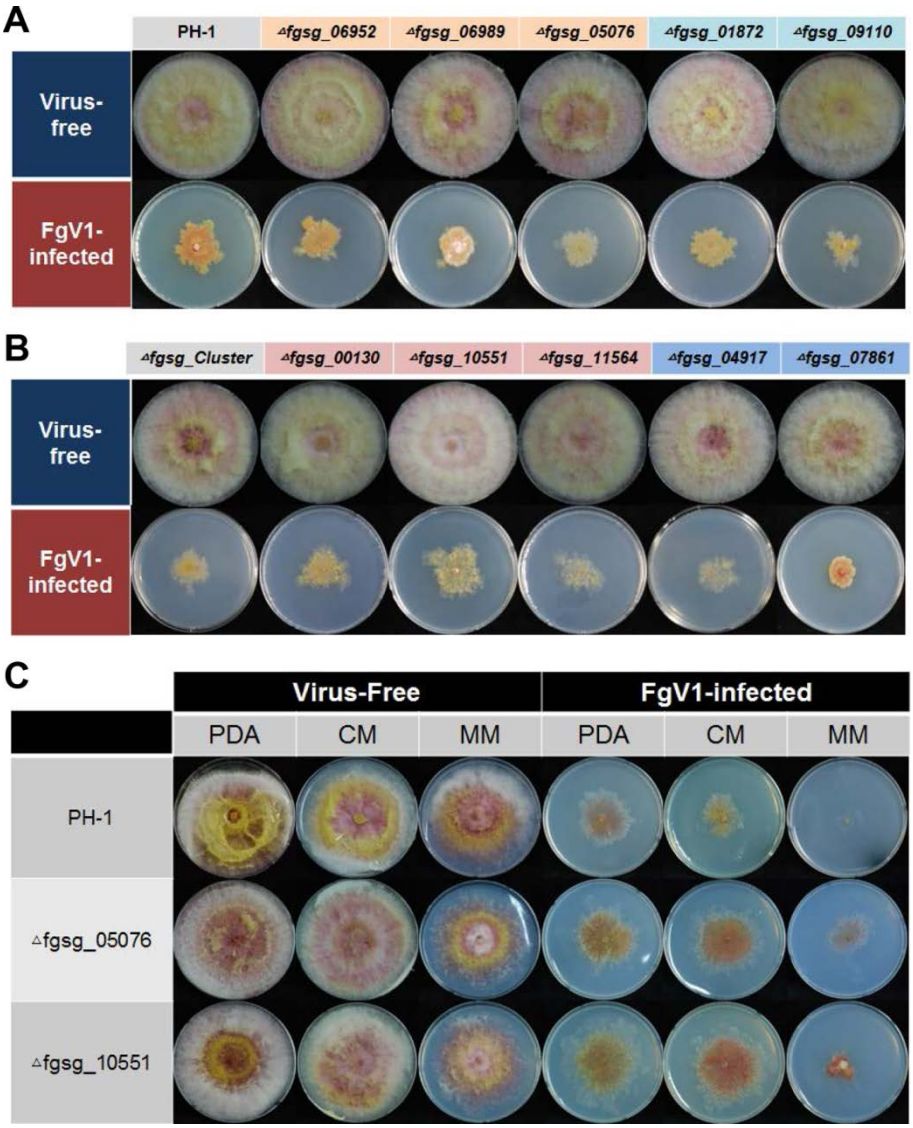

Fig.5 Colony morphologies of all deletion mutants. Colonies were photographed after 5 days on PDA, CM, and MM.

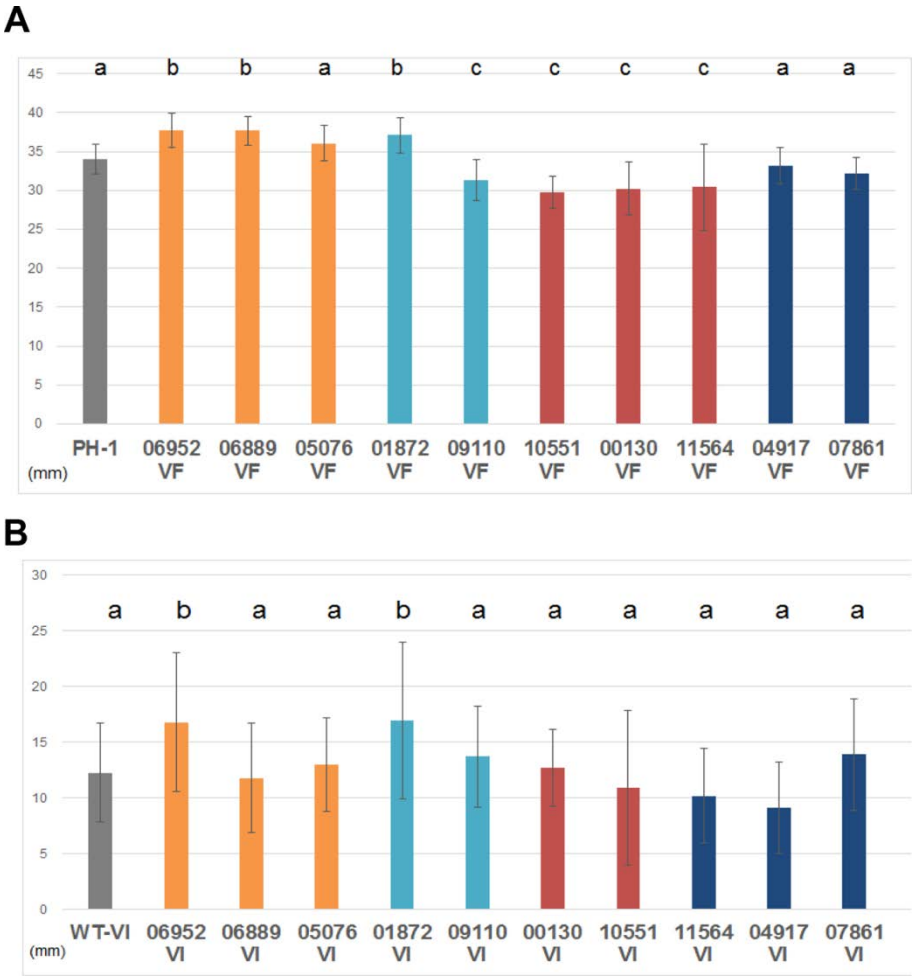

Fig.6 Radial growth after 5 days on CM. Error bars indicate the standard deviation, and values with different letters are significantly different at $p<0.05$ based on the Tukey test. (A) Virus-free strains (B) Virus-infected strains.

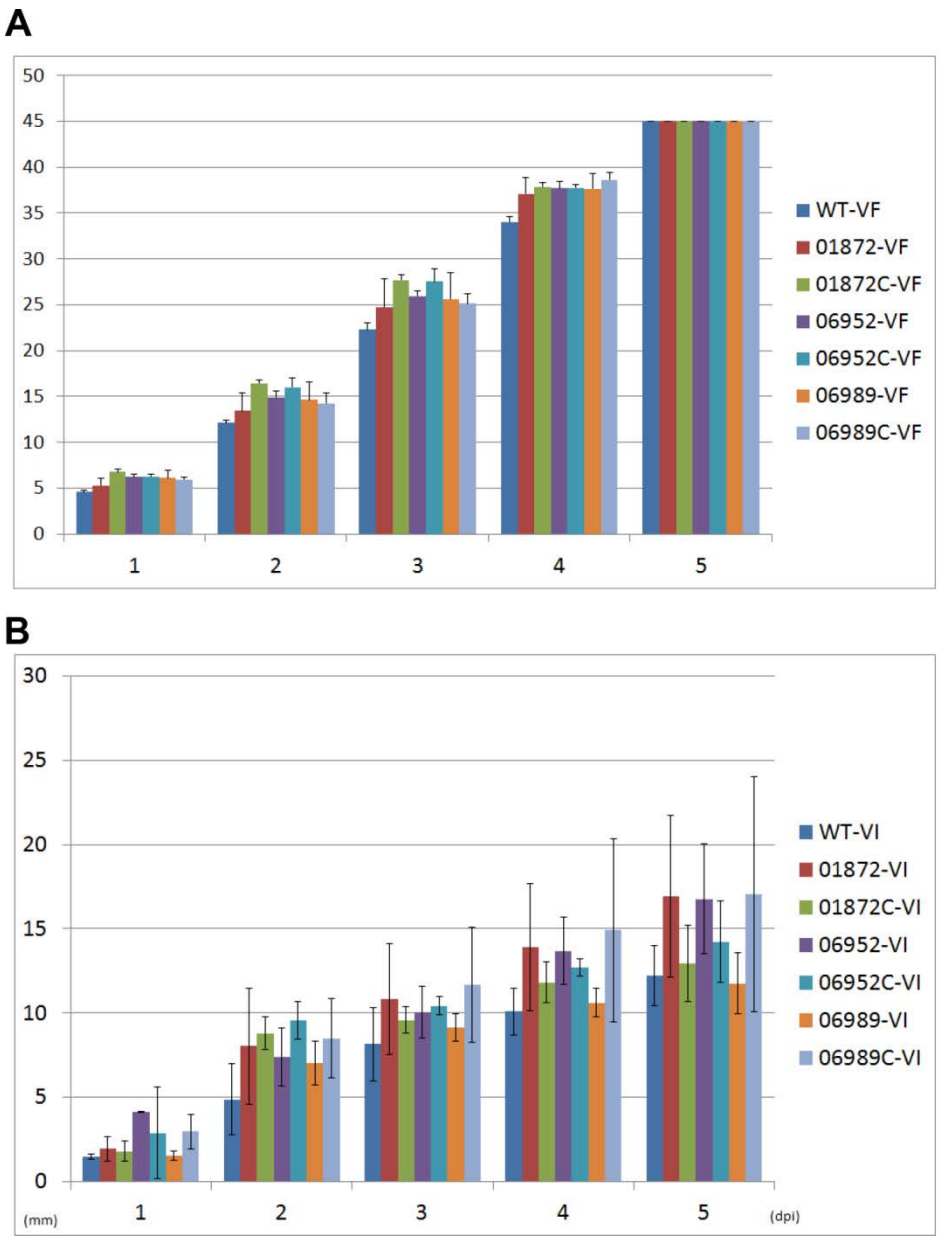

Fig.7 Radial growth of 5 days on CM. Error bars indicate the standard deviation, (A) Virus-free strains (B) Virus-infected strains, Numbers indicate days of incubation.

6. Quantification of FgV1 viral RNA accumulation using Semi-quantitative RT-PCR

To investigate effect of genes deletion on viral RNA accumulation, semi-quantitative RT-PCR was performed. Viral RNA accumulations in gene deletion mutants are not significantly changed when compare with WT-VI strain excluding two different deletion strains. In ∆*FGSG_05076*-VI and ∆*FGSG_10551*-VI strain, viral RNA accumulations were decreased comparing with that of WT-VI strain (Fig. 8).

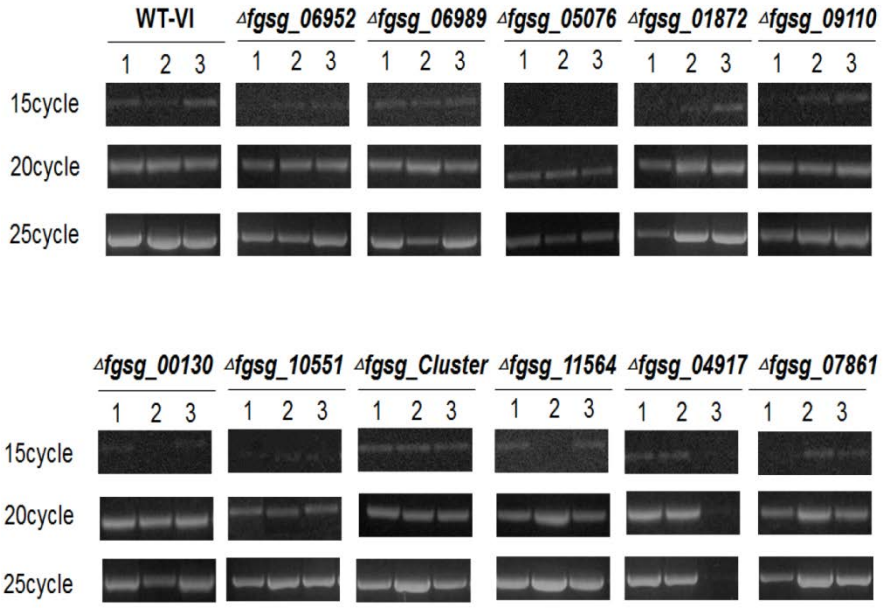

Fig.8 Semi-quantitative RT-PCR result. 15, 20, 25 indicate numbers of PCR amplification cycles. Three isolates of WT and each gene deletion mutants were assayed.

Discussion

In this study, ten genes were selected according to previous transcriptome analysis of four different mycoviruses infected *F. graminearum* to verify the analysis from biological point of view. The genes were selected by different expression levels in different mycoviruses infected strains. Then, deletion and complementation mutants were generated to examine putative role(s) of these genes in relationship between mycoviruses from *F. graminearum* and host fungus. From this study, five candidate genes seem to affect host fungi in virus infection. In colony morphology test, all of deletion mutants showed similar morphology without FgV1 infection (Fig. 5). These results indicated that deletion of each ten genes do not affect colony morphology. However, in FgV1 infected deletion strains, some strains showed different pigmentation comparing with WT-VI strain. In ∆*FGSG_09110*-VI, ∆*FGSG_11564*-VI, and ∆*FGSG_04917*-VI strains, FgV1 induced typical dark pigmentation was slightly decreased (Fig. 5A). From this data, we could assume that these genes are involved in modification of pigmentation pathway caused by FgV1. Although deletion of each ten genes did not much affect colony morphology, deletion of some genes leads to different growth pattern. In virus-free ∆*FGSG_01872*, ∆*FGSG_06952* and ∆*FGSG_06989* gene deletion mutants, radial growth at 4 d.p.i. were increased comparing with WT-VF (Fig.6A). These analyses demonstrated that these three genes might be involved in vegetative growth of *F. graminearum.* In virus infected ∆*FGSG_10551*, ∆*FGSG_00130* and ∆*FGSG_11564* gene deletion mutants, radial growths of each virus-infected strain showed normal pattern (Fig.

6B). These result suggested that these genes seem to relate with virus-specific controlling of vegetative growth in *F. graminearum.*

During the process of experiment, virus-infected ∆*FGSG_01872* and ∆*FGSG_06952* deletion mutants showed irregular growth pattern (Fig.8). These mutants showed hybridized mycelia growth form that mixed with typical virus-free and virus-infected mycelium. Based on semi-quantitative RT-PCR results, *FGSG_05076* and *FGSG_10551* are further selected as suspected genes which affect viral RNA accumulation, these two genes deletion mutants showed significantly decreased viral RNA accumulation. In colony morphology test and measurement of radial growth experiment, these two gene deletion mutants grew faster than WT in CM and MM upon FgV1 infection. These results suggested that *FGSG_05076* and *FGSG_10551* might affect FgV1 viral RNA accumulation directly or indirectly.

FGSG_10551 gene has high similarity of gene sequence with KP4 toxin. KP4 toxins are well known toxin which is produced by *Ustilago maydis* as a mycovirus encoded toxin (Koltin, 1988). Interestingly, it encoded by mycovirus, and functions like a killer toxin of *Saccharomyces cerevisiae* (Bostianet et al., 1984; Dignard et al., 1991; Park et al., 1994; Skipper et al., 1984). KP4 toxin in and a/b sandwich protein with a relatively compact structure (Gu et al., 1995) and inhibits fungi growth by blocking calcium uptake (Gage et al., 2001). It not only functions as fungal toxin but also effects on plant root (Allen et al., 2008). In Fusarium species, clustered genes are conserved and have similar sequence identity. Figure (Fig. 10) shows example of previously described gene cluster (Brown, 2011). In *F. graminearum, FGSG_00060, FGSG_00061* and *FGSG_00062* are constituent of KP4 toxin gene cluster.

Future research should include the construction of each target gene over-expression mutant and test effect(s) of over-expression to further support above described data. Although there are still a lot of works needed, results obtained from this study provide interesting enough information for understanding mycovirus-host fungus interaction especially in *F. graminearum.*

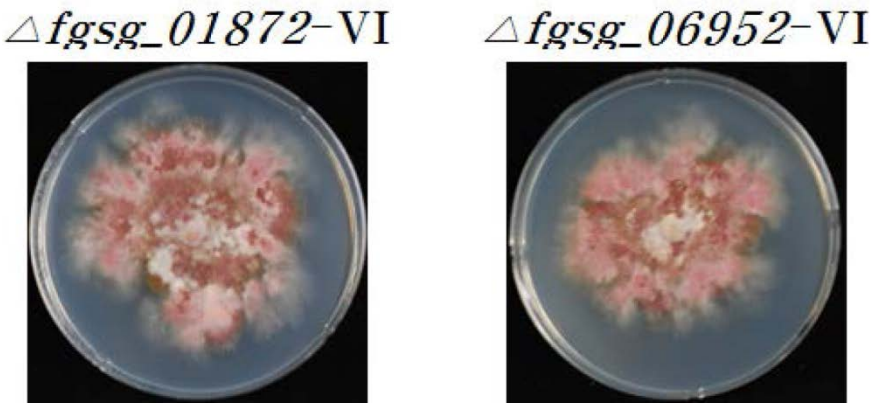

Fig. 9 Abnormal colony morphology of *FGSG_01872* and *FGSG_06952* deletion mutants upon FgV1 infection

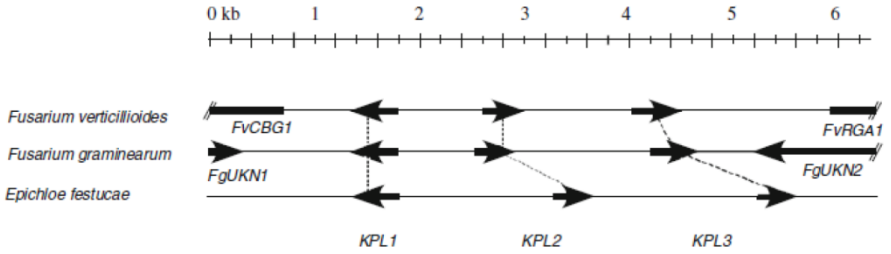

Fig. 10 Conserved KP4 toxin orthologs in *F. graminearum, F. verticillioides, E. festucae.*

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Fusarium graminearum virus 1 에 감염된 붉은 곰팡이에서 발현량이 증가하는 유전자들에 관한 기능 분석

박진만

초록

Fusarium graminearum virus 1 (FgV1)은 붉은 곰팡이를 기주로 삼는 mycovirus 이며 *Fusarium boothii* 에서 분리, 동정 되었다. 이 바이러스는 약 6.6kb 의 double stranded RNA 를 genome 으로 갖고 있으며 네 개의 ORF 를 포함하고 있을 것이라 추정된다. 붉은 곰팡이를 감염하는 바이러스는 FgV1 을 제외하고 FgV2,FgV3, 그리고 FgV4 도 존재한다. 이들 바이러스 중 FgV1 과 FgV2 는 기주 곰팡이의 생장을 억제하거나 무성생식포자를 감소시키고 병원성을 약화시키는 hypovirulence 한 특성을 보인다. 하지만, 다른 두 종의 바이러스는 기주의 형태와 생활사에 큰 변화를 주지 않는 것으로 알려져 있다. 우리 실험실의 선행연구에 의해 이러한 mycovirus 감염 시 기주 곰팡이에서 그 발현량이 증가하거나 감소하는 유전자들에 관한 연구가 이루어졌다. 단일감염과 두 종의 바이러스를

기주 곰팡이에 복합 감염시키고 그 때 발생하는 transcriptome 을 분석하여 일련의 유전자들에서 그 발현량이 크게 변화함을 확인하였다.

이 유전자들 중 10 개의 유전자를 바이러스와 상호작용 할 것이라 추측하고 각각의 유전자가 바이러스 감염 시 미치는 영향력을 조사하였다. 각각 유전자들의 유전자 결손 돌연변이체를 생성하여 바이러스 감염 시 colony 의 형태변화, 균사의 생장 그리고 바이러스 RNA 의 축적량 변화를 관찰하였다. 유전자 삭제 돌연변이체는 DJ-PCR 과 homologous recombination 을 통해 제작되었고 southernblot 을 통해 모든 유전자들의 유전자삭제 돌연변이체가 성공적으로 생성되었음을 확인하였다. 균사생장을 조사하면서 *FGSG_01872, FGSG_06952, FGSG_06989* 세 유전자 중 앞의 두 유전자가 바이러스 감염 시 균사생장이 증가하는 것에 연관성이 있고 나머지 한 유전자는 균사생장을 감소시킨다는 사실을 확인했다. 또한 바이러스 RNA 축적량 변화를 관찰하여 FGSG_05076 과 FGSG_10551 이 바이러스 RNA 의 축적량을 감소시킨다는 사실을 확인했고 이는 PDA,CM, 그리고 MM 에서 WT 에 비해 증가한 균사생장을 통해 그 사실을 뒷받침해줄 유사성을 발견하였다. 이를 통해 바이러스 감염 시 균사생장의 변화를 야기하는 세 유전자를 확인하였고, 특히 바이러스 감염 시 발현량이 증가하는 두 유전자가 바이러스의 RNA 축적에 관여함을 확인하였다. 이들을 통해 특정 유전자가 바이러스와 상호작용할 것이라 추측되는 증거를 찾았다는 것에 이 실험의 의의를 둔다.

주요어 :*Fusarium graminearum*, Fusarium graminearum virus 1 (FgV1), host factor, gene deletion, mycelia growth, viral RNA accumulation.

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