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A Putative Transcription Factor *pcs1* Positively Regulates Both Conidiation and Sexual Reproduction in the Cereal Pathogen *Fusarium graminearum*

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The plant pathogen *Fusarium graminearum* causes Fusarium head blight in cereal crops and produces mycotoxins that are harmful to animals and humans. For the initiation and spread of disease, asexual and sexual reproduction is required. Therefore, studies on fungal reproduction contribute to the development of new methods to control and maintain the fungal population. Screening a previously generated transcription factor mutant collection, we identified one putative C₂H₂ zinc-finger transcription factor, *pcs1*, which is required for both sexual and asexual reproduction. Deleting *pcs1* in *F. graminearum* resulted in a dramatic reduction in conidial production and a complete loss of sexual reproduction. The pathways and gene ontology of *pcs1*-dependent genes from microarray experiments showed that several G-protein related pathways, oxidase activity, ribosome biogenesis, and RNA binding and processing were highly enriched, suggesting that *pcs1* is involved in several different biological processes. Further, overexpression of *pcs1* increased conidial production and resulted in earlier maturation of ascospores compared to the wild-type strain. Additionally, the vegetative growth of the overexpression mutants was decreased in nutrient-rich conditions but was not different from the wild-type strain in nutrient-poor conditions. Overall, we discovered that the *pcs1* transcription factor positively regulates both conidiation and sexual reproduction and confers nutrient condition-dependent vegetative growth.

Keywords : Conidiation, *Fusarium graminearum*, *Gibberella zeae*, sexual reproduction, transcription factor

The plant pathogenic fungus *Fusarium graminearum* (teleomorph *Gibberella zeae*) causes Fusarium head blight in small grains and infected grains are frequently contaminated with mycotoxins, such as trichothecenes and zearalenone (Leslie and Summerell, 2006). This fungus produces ascospores and conidia through sexual and asexual reproduction, respectively. Ascospores formed within perithecia are believed to be the primary inoculum of this disease, and conidia serve to disperse the disease (Fernando et al., 1997; Sutton, 1982).

F. graminearum is homothallic, but it can also cross with other strains in a heterothallic manner (Bowden and Leslie, 1999; Lee et al., 2003). In North America, high levels of *F. graminearum* genotypic diversity suggest that outcrossing often occurs in nature (Zeller et al., 2003, 2004). However, this may be a regional observation, as Korean populations show low genetic diversity (Lee et al., 2009, 2012). Many research groups have studied *F. graminearum* sexual reproduction because manipulation of the sexual stage is one of most efficient targets to decrease the primary inoculums and genetic diversity in field populations (Han et al., 2007; Hou et al., 2002; Lee et al., 2008, 2010; Son et al., 2011b).

Through asexual reproduction, *F. graminearum* produces macroconidia without microconidia, in contrast to many other *Fusarium* species that produce conidia with diverse shapes or sizes (Leslie and Summerell, 2006). A putative mitogen-activated protein kinase, Gpmk1, has been associated with conidial production (Zenczmionka et al., 2003), and gene expression profiles during conidial germination have been studied (Seong et al., 2008). However, despite the importance of conidia in disease dispersal, functional studies on conidiogenesis of *F. graminearum* are limited.

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F. graminearum undergoes vegetative growth and asexual reproduction in normal conditions, but sexual reproduction is only initiated under specific light and nutrient conditions. Because sexual reproduction requires many kinds of cellular differentiation, such as the development of antheridium, oogonium, crozier, ascus, and perithecia, asexual reproduction is blocked for *F. graminearum* in order to switch on sexual reproduction (Guenther and Trail, 2005; Hallen et al., 2007; Lee et al., 2010; Min et al., 2010; Qi et al., 2006; Yu et al., 2008). Recently, the genes for all annotated transcriptional factors were individually deleted and characterized for phenotypic changes (Son et al., 2011b), providing an in-depth study of each transcription factor responsible for specific developmental stages.

In this study, we screened the transcription factor deletion mutant library to identify genes related to both asexual and sexual reproduction. We identified a transcription factor named *pcs1* (positive regulator for conidiogenesis and sexual reproduction) and characterized the function of this gene by deletion, overexpression, and complementation. We found that *pcs1*-dependent genes are involved in basic biological processes, such as RNA processing and ribosome biogenesis, as well as in initial signaling cascades, such as signal receptors and G-protein coupled receptor

signaling. Mutants with a *pcs1* deletion exhibited several important phenotypic changes, including differential vegetative growth, conidia germination, and sexual and asexual production. Specifically, we identified that *pcs1* positively regulates both asexual and sexual reproduction in *F. graminearum*.

Materials and Methods

Fungal strains and media. The wild-type *F. graminearum* strain Z3639 (Bowden and Leslie, 1999) and mutants derived from it were stored as frozen conidial suspensions in 20% glycerol at -70°C . Fungal strains were maintained on potato dextrose agar (PDA), and the radial growth of strains was measured on PDA, minimal medium (MM), and complete medium (CM) (Leslie and Summerell, 2006). Sexual reproduction, including self, female, and male fertility, was induced on carrot agar as previously described (Lee et al., 2008) and carnation leaf agar (Leslie and Summerell, 2006). Asexual reproduction was induced both in carboxymethyl cellulose (CMC) (Capellini and Peterson, 1965) and on yeast malt agar (YMA) (Harris, 2005). Conidia germination was tested in MM and CM as previously described (Lee et al., 2009). All experiments were performed three

Table 1. Primers used in this study

Name	Sequence (5'-3')	Reference
Pcs-5F	AGTTGCTTGCTTCTCTGTCTATGTCTA	Son et al., 2011b
Pcs-5R	gcacaggtacactgttttagagCCACAATTGTAGGCTGGAGACC	Son et al., 2011b
Pcs-3F	ccttcaatatcatcttctgtcgCTTGCAAACAACACCAACACCACT	Son et al., 2011b
Pcs-3R	GCAATTGCGAAGATGGTAACT	Son et al., 2011b
Gen-F	CGACAGAAGATGATATTGAAGG	Min et al., 2010
Gen-R	CTCTAAACAAGTGACCTGTGC	Min et al., 2010
Pcs-5N	TTTCCCCTTCTTTCTTTTGGACCTAA	Son et al., 2011
Pcs-3N	ATCCACCAGCGTCACATAACACAATAC	Son et al., 2011
Pcs-rtF	CGCCAGGACTTCTACGAGCATCT	This study
Pcs-rtR	CGGTGGTGGTAGGGAGGTTGTTCT	This study
Pcs-5R gfp	gaacagctcctcgccctgtctacCAGAGTGGTGGTGGTGGTGGTGGC	This study
Pcs-3F gfp	cctccactagctccagccaagccCTTGCAAACAACACCAACACCACT	This study
pIGPAPA-sGFP F	GTGAGCAAGGGCGAGGAGCTG	Son et al., 2011a
HYG-F1	GGCTTGGCTGGAGCTAGTGGAGG	Son et al., 2011a
Pcs-5R OE	gatagtggaaaccgacgccccGAAGGGAGTGAAGCTTGTGTGAATC	This study
Pcs-3F OE	cacaaaaggaaccaatcttcaagCATCATGACAGCCTTGTCAGATACG	This study
Pcs-3F gfp OE	cggcatggacgagctgtacaagATGACAGCCTTGTCAGATACGTCC	This study
Pcs-3R OE	TGGTGAACGAATTGGCTGATACT	This study
Pcs-3N OE	TGGAAGTTGCTGGCCTGGAGAAAT	This study
Neo-for new	GGGGCGTCGGTTTCCACTATC	Lin et al., 2011
EFpro-Rev	TTTGAAGATTGGGTTTCTTTTGTG	Lin et al., 2011
eGFP-P1	CTTGACAGCTCGTCCATGCCG	Lin et al., 2011

times with three replicate plates.

Nucleic acid manipulation and polymerase chain reaction (PCR). Fungal genomic DNA was extracted from CM culture as previously described (Leslie and Summerell, 2006). Total RNA was extracted from mycelia or conidia using an RNA-spin total RNA kit (Intron Biotech., Seongnam, Korea). First-strand cDNA was synthesized with SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed with SYBR Green Supermix (Bio-Rad, Hercules, CA) and a 7500 real-time PCR system (Applied Biosystems). Elongation factor 1- β (Broad Institute; FGSG_01008.3) was used as an endogenous control for normalization. The PCRs were repeated twice with two replicates per run. Standard procedures were used for restriction endonuclease digestion, DNA ligation, gel electrophoresis, and gel blotting (Sambrook and Russell, 2001). The PCR primers (Table 1) used in this study were synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corp., Chungwon, Korea), diluted to 100 μ M in sterilized water, and stored at -20°C . General PCR reactions were performed as described previously (Lee et al., 2008).

Identification of *pcs1* gene. A previous study identified 41 transcription factor mutants that had a dramatic reduction or no conidial production in CMC (Son et al., 2011b). Each strain was re-activated on PDA and inoculated both in CMC medium and YMA for conidial production as described above. In addition, each strain was tested for sexual reproduction on carnation leaf agar and carrot agar.

Complementation and overexpression of *pcs1*. To complement the *pcs1* deletion mutant, the *pcs1* open reading frame (ORF), including its promoter region and excluding its stop codon, was amplified from the wild-type strain of the genomic DNA of Z3639 with primers Pcs-5F and Pcs-5R gfp. The GFP and hygromycin resistance gene cassette was amplified from pIGPAPA (Horwitz et al., 1999) with primers pIGPAPA-sGFP F and HYG-F1. This gene cassette was fused to the C-terminus of *pcs1* by double-joint PCR (Yu et al., 2004). The fused construct was amplified with primers Pcs-5N and Pcs-3N, and the amplicon was transformed into the *pcs1* deletion mutant. The phenotypes of the transformant were compared with those of the wild-type and *pcs1* deletion strains. Overexpression of *pcs1* was performed as previously described (Lin et al., 2011). Briefly, primer pairs Pcs-5F/Pcs-5R OE and Pcs-3F OE/Pcs-3R OE were used to amplify the 5' flanking region of

pcs1 and the *pcs1* ORF, respectively. A *gen-P_{EF1a}*, carrying the elongation factor 1 α promoter (*P_{EF1a}*) from *Fusarium verticillioides*, was amplified from Pskgen (Lee et al., 2011) with primers Neo-for new/EFpro-Rev. The three amplicons were fused as described above. Using this fusion fragment as a template, a final PCR product was amplified with the nested primers Pcs-5N and Pcs-3N OE. Insertion of the construct was confirmed by Southern hybridization. The transcript level in the mutants was checked by real-time PCR with the Pcs-rtF/Pcs-rtR primer pair.

Fertility test. For the self-fertilization test, mycelia of strains grown on carrot agar for 5 to 7 d were mock-fertilized, as previously described (Leslie and Summerell, 2006). For outcrossing tests, the heterothallic strain Δ *mat1-1::hH1::GFP* (Hong et al., 2010), which carries a *mat1-1* deletion and histone H1::GFP, was used as the female. The mutant strains generated in this study were used as males. Thus, all ascospore progeny resulted from these heterozygous crosses. Female mycelia on plates were fertilized with a suspension of conidia from the male strains grown on CMC medium, and the fertilized plates were incubated at 25°C . Ascospores and asci in perithecia were observed 7 to 14 d after fertilization under the Carl Zeiss microscope DE/Axio imager A1 (Carl Zeiss) equipped with a GFP filter (470/40 nm excitation, 495 nm dichroic, 525/50 nm emission). Images were analyzed with Axio vision software (Carl Zeiss).

cDNA synthesis and microarray analysis. Each strain was incubated in 50-ml CM for 72 h at 25°C on a rotary shaker (150 rpm). Subsequently, mycelia were harvested and washed twice with 50-ml sterile water. Mycelia were spread on YMA to induce conidiation (Lee et al., 2009). After 48 h, total RNA was extracted from cultures including mycelia and conidia. The cDNA synthesis and microarray hybridization were performed as previously described (Lee et al., 2010).

KEGG pathway and gene ontology (GO) enrichment analysis. All GO terms and KEGG pathway sources were downloaded using the Wget program and the direct downloading service of the web server from the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) and UniProt (<http://www.uniprot.org/>). In total, 1,280 *pcs1*-dependent genes were annotated with data from both KEGG pathway and GO terms. Common results of annotated genes from both databases were enriched by Python coding script. All results of enriched KEGG pathways or GO terms were compared to the original genome

according to the formula:

$$P \text{ value} = 1 - \sum_{x=0}^{m-1} \frac{\binom{M}{x} \binom{N-M}{n-x}}{\binom{N}{n}}$$

where N is the number of genes with annotation by KEGG pathways or GO terms annotation; n is the number of *pcs1*-dependent genes in N; M is the number of genes annotated to the specific KEGG pathway or GO term; and m is the number of *pcs1*-dependent genes in M. We chose only KEGG pathways and GO terms with a *P* value of < 0.05. To identify enriched GO gene categories, we classified all data by biological process, molecular function, and cellular component.

Results

Identification of *pcs1*. *F. graminearum* conidial development is suppressed during sexual reproduction; therefore, many genes related to sexual development work to suppress conidial production, and vice versa. However, we identified one deletion mutant that showed both dramatic reduction in conidial production and a complete loss in sexual development. The locus of this *F. graminearum* gene in the comparative *Fusarium* genome database is FGSG_01877.3, and it is annotated as a C₂H₂ zinc-finger transcription factor. The gene is 2881 bp with one intron, and the deduced amino acid size is 630. This hypothetical protein is highly conserved among the Ascomycota, but the gene's function has not been studied. Based on the charac-

teristics displayed by the deletion mutant, we designate this gene as *pcs1*.

***pcs1* is required for sexual development.** To confirm whether the phenotypic changes shown in *pcs1* deletion mutants were triggered by the deletion event of *pcs1*, the *pcs1* deletion mutant was complemented. The complemented strain was constructed by reintroducing the *pcs1* ORF, including its native promoter and terminator, into the deletion mutant (C-*pcs1*). In addition, to confirm the effect of *pcs1* overexpression, the construct with the *pcs1* ORF controlled under a constitutive promoter was introduced

Table 2. Mycelial growth and conidiation of *Fusarium graminearum* strains

	Radial growth of mycelia (mm) [*]		No. of conidia (×10 ⁵ /ml) [§]
	Minimal medium	Complete medium	
Z3639	76 ^{a,¶}	84 ^a	27 ^b
D- <i>pcs1</i> -1	83 ^b	85 ^a	0.6 ^a
D- <i>pcs1</i> -2	85 ^b	86 ^a	0.4 ^a
C- <i>pcs1</i> -1	77 ^a	82 ^a	32 ^b
C- <i>pcs1</i> -2	78 ^a	83 ^a	29 ^b
O- <i>pcs1</i> -1	75 ^a	63 ^b	45 ^c
O- <i>pcs1</i> -2	74 ^a	65 ^b	50 ^c

^{*}Each strain was grown on minimal medium and complete medium for 5 days at 25°C and radial growth was measured.

[§]Fungal strains were grown in 50 ml carboxymethyl cellulose medium for 5 days at 25°C in an orbital shaker (150 rpm), and conidial production was measured using a hemocytometer.

[¶]Values within a column not sharing a letter are significantly different according to Tukey's test (*P* < 0.05).

Fig. 1. Vegetative growth of *G. zeae* strains on CM and MM, and perithecia production on carrot agar. Z3639, wild-type strain; D-*pcs1*, *pcs1* deletion mutant; O-*pcs1*, mutant with constitutive expression of *pcs1*. Photos were taken after a 5-d incubation on CM and MM, and 7 d after sexual induction on carrot agar.

Fig. 2. Asci and ascospores produced by outcrossing between the heterothallic strain $\Delta mat1-1::hH1::GFP$ and $D\text{-}pcs1$. Photos were taken in an 8-day incubation after sexual induction. Only four out of eight mature ascospores in each ascus fluoresced as observed by a fluorescent microscopy with a GFP filter. Two images, differential interference contrast (left) and GFP fluorescence (center), were merged (right).

into the *pcs1* deletion mutant ($O\text{-}pcs1$).

As described in a previous study (Son et al., 2011b), the *pcs1* deletion mutant ($D\text{-}pcs1$) produced less pigment on both CM and MM (Fig. 1) and lost the ability to self-fertilize. In the $D\text{-}pcs1$ strain, the radial growth of mycelia on MM, but not on CM, was slightly increased compared to the wild-type strain (Table 2). In the $C\text{-}pcs1$ strain, all phenotypes observed in the $D\text{-}pcs1$ mutants were completely restored to wild-type phenotypes (Fig. 1 and Table

1), which confirmed that the phenotypic changes in $D\text{-}pcs1$ were triggered by the *pcs1* gene deletion. In $O\text{-}pcs1$, the strain with constitutive *pcs1* expression, the radial growth of mycelia on MM was not significantly different from the wild-type strain, but it was dramatically decreased on CM (Fig. 1 and Table 2).

For sexual development in the $O\text{-}pcs1$ strain, cirrhi were observed earlier than with the wild-type strain (Fig. 1). Although $D\text{-}pcs1$ lost self- and female fertility, it retained

Fig. 3. Conidia production of *F. graminearum* strains on YMA. Black arrows indicate conidia produced from intercalary phialides on hyphae, and black arrowheads indicate conidia produced from intercalary phialides in false heads. White arrowheads indicate conidia produced from terminal phialides.

male fertility, which resulted in normal sexual recombination when it was outcrossed (Fig. 2). The MIPS *F. graminearum* database (<http://mips.helmholtz-muenchen.de/>) reports that *pcs1* transcript gradually increases until 3 d after sexual induction. This expression profile combined with our results suggests that *pcs1* is responsible for earlier events of sexual reproduction.

Proper conidia production requires *pcs1*, which regulates conidia germination. In the submerged condition of CMC, D-*pcs1* mutants showed a dramatic reduction in conidial production compared to the wild type and C-*pcs1* (Table 2). The O-*pcs1* strain produced approximately two-fold more conidia compared to the wild type (Table 2). To verify conidia production in *F. graminearum*, we induced conidia production on YMA. In the wild-type strains, the majority of conidia were produced from terminal phialides on conidiophores and rarely produced from intercalary phialides on hyphae or intercalary phialides in false heads (Fig. 3). This observed conidia production in *F. graminearum* was similar to that described in *F. oxysporum* (Ohara and Tsuge, 2004), even though *F. graminearum* do not produce microconidia. In the D-*pcs1* strain, terminal phialides produced less conidia than those of the wild-type strain, and conidia produced from intercalary phialides on hyphae often formed hyphae before conidiogenesis (Fig. 3). Additionally, often conidiation in conidiophores was arrested in the D-*pcs1* strain, and conidiophores produced hyphae without conidiogenesis (Fig. 3). In the O-*pcs1* strain, the majority of conidia were produced from intercalary phialides in false heads, and the number of intercalary

Fig. 4. Conidia germination after 0, 4, and 8 h incubation in minimal medium.

phialides on hyphae was dramatically increased compared to wild type (Fig. 3).

Conidia germination in the D-*pcs1* mutants was faster than in the wild-type strain, while that of O-*pcs1* was delayed compared to the wild-type strain (Fig. 4). The germination rate of conidia was not different between the wild type and D-*pcs1*, as in both strains approximately 80% of conidia were germinated after a 4-h incubation, and 100% of conidia were germinated after an 8-h incubation. In contrast, only 30% and 70% of the O-*pcs1* conidia were germinated after 4- and 8-h incubations, respectively. The germination rate in each strain was not significantly differ-

Fig. 5. Pathway enrichment of *pcs1*-dependent genes. Red and blue bars represent level of enrichment in positive and negative *pcs1*-dependent genes, respectively. We set a cut-off with a *P* value of < 0.05.

Fig. 6. Gene ontology (GO) enrichment of *pcsI*-dependent genes. (A) Cellular component, (B) biological process, (C) molecular function. Red and blue bars represent level of enrichment in positive and negative *pcsI*-dependent genes, respectively. We set a cut-off with a *P* value of < 0.05.

ent between CM and MM (data not shown).

Analysis of *pcsI*-dependent genes. Generally, the destruction of important transcription factors results in differential expression of genes involved in biological processes regulated by those transcription factors. Thus, to characterize *pcsI* function, we examined *pcsI* transcription factor-dependent genes, using both the KEGG pathway and GO. First, we selected differentially expressed genes that had greater than two-fold changes between D-*pcsI* and wild

type. We detected 1,280 *pcsI*-dependent genes out of a total of 11,624 genes. Enrichment analysis of KEGG pathways indicated that ribosome biogenesis, RNA polymerase, MAPK signaling pathway, and nucleotide metabolism are highly enriched in positively regulated *pcsI*-dependent genes. Additionally, biotin, nitrogen, and several amino acid metabolisms are enriched in negatively regulated *pcsI*-dependent genes (Fig. 5). Furthermore, GO enrichment analysis indicated that G-protein signaling pathways in both cellular component and biological process, and

RNA binding and processing in both biological process and molecular function are enriched in positively regulated *pcs1*-dependent genes (Fig. 6).

Discussion

In this study, we identified that *pcs1*, a transcription factor, is responsible for conidia production, especially conidia generated from intercalary phialides on hyphae, and sexual reproduction. The observed overexpression of *pcs1* confirmed its role in the regulation of asexual and sexual reproduction.

In CMC, conidia of the wild-type strain mainly were produced from intercalary phialides on hyphae, but terminal phialides and intercalary phialides in false heads were rarely observed (data not shown). Therefore, the dramatic reduction of D-*pcs1* in conidia production in CMC may be triggered by inadequate generation of intercalary phialides and *pcs1* is especially required for intercalary phialides formation on hyphae. In addition, conidia germination in the D-*pcs1* mutants was faster than in the wild-type strain, while that of O-*pcs1* was delayed compared to the wild-type strain. These results suggest that *pcs1* also regulates conidia germination, and is partly responsible for the phase shift from conidia dormancy to germination.

Comparison of gene expression profiles between wild type and D-*pcs1* revealed that *pcs1* regulates genes involved in basic biological processes, such as RNA processing, ribosome biogenesis, and initial signaling cascades. Interestingly, we identified that *pcs1* positively regulates both asexual and sexual reproduction in *F. graminearum*. Particularly, G-protein signaling pathways in both cellular component and biological process are enriched. heterotrimeric G protein $G\alpha$ subunit, *GzGPA1*, responsible for sexual reproduction in *F. graminearum* (Yu et al., 2008), was highly down-regulated in D-*pcs1* where mutants carrying deletion did not produce protoperithecia, indicating the loss of ability for sexual reproduction observed in D-*pcs1* is closely related to down-regulation of *GzGPA1* gene in the mutant. Global regulators, velvet family proteins, are involved in diverse development stages including sexual and asexual reproduction. Under sexual development condition, *velB* deletion mutants do not produce protoperithecia but produce large numbers of conidia (Lee et al., 2012). However, *velB* expression was not changed in the D-*pcs1* and *pcs1* gene expression was not affected by *velB*, indicating *pcs1* is independent of *velB*.

In conclusion, the *pcs1* transcription factor regulates genes involved in basic biological processes and initial signaling cascades such as G-protein signaling pathways

as well as phenotypically regulates both conidiation and sexual reproduction in a positive manner and confers nutrient condition-dependent vegetative growth.

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