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 trichothecences 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., 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 times.

<table>
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<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
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<td>AGTTGCTTCTCTCTTGTATGTCTA</td>
<td>Son et al., 2011b</td>
</tr>
<tr>
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<td>Son et al., 2011b</td>
</tr>
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<td>Son et al., 2011b</td>
</tr>
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<td>Min et al., 2010</td>
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<tr>
<td>eGFP-P1</td>
<td>TTGTACAGGCTCTGCCTGACG</td>
<td>Lin et al., 2011</td>
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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 μM 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-3R. 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_EF1α, carrying the elongation factor 1α promoter (P_EF1α) 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 Δmat1-1::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:

$$P \text{ value} = 1 - \sum_{x=0}^{m-1} \left( \frac{M}{n} \right)^x \left( \frac{N - M}{n} \right)^{n-x}$$

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 $C_2H_2$ 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 characteristics 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 complimented. The complimented 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

<table>
<thead>
<tr>
<th></th>
<th>Radial growth of mycelia (mm)</th>
<th>No. of conidia ($\times 10^5$/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimal medium</td>
<td>Complete medium</td>
</tr>
<tr>
<td>Z3639</td>
<td>76$^{a,\dagger}$</td>
<td>84$^a$</td>
</tr>
<tr>
<td>D-$pcs1$-1</td>
<td>83$^b$</td>
<td>85$^a$</td>
</tr>
<tr>
<td>D-$pcs1$-2</td>
<td>85$^b$</td>
<td>86$^a$</td>
</tr>
<tr>
<td>C-$pcs1$-1</td>
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<td>83$^a$</td>
</tr>
<tr>
<td>O-$pcs1$-1</td>
<td>75$^a$</td>
<td>63$^b$</td>
</tr>
<tr>
<td>O-$pcs1$-2</td>
<td>74$^a$</td>
<td>65$^b$</td>
</tr>
</tbody>
</table>

*Each strain was grown on minimal medium and complete medium for 5 days at $25^\circ C$ and radial growth was measured.

$^\dagger$Fungal strains were grown in 50 ml carboxymethyl cellulose medium for 5 days at $25^\circ C$ 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.
into the pcs1 deletion mutant (O-pcs1).

As described in a previous study (Son et al., 2011b), the pcs1 deletion mutant (D-pcs1) produced less pigment on both CM and MM (Fig. 1) and lost the ability to self-fertilize. In the D-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-pcs1 strain, all phenotypes observed in the D-pcs1 mutants were completely restored to wild-type phenotypes (Fig. 1 and Table 1), which confirmed that the phenotypic changes in D-pcs1 were triggered by the pcs1 gene deletion. In O-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-pcs1 strain, cirrhi were observed earlier than with the wild-type strain (Fig. 1). Although D-pcs1 lost self- and female fertility, it retained

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**Fig. 2.** Asci and ascospores produced by outcrossing between the heterothallic strain ∆mat1-1::hH1::GFP and D-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).

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**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 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. 4. Conidia germination after 0, 4, and 8 h incubation in minimal medium.](image-url)

![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.](image-url)
between CM and MM (data not shown).

**Analysis of pcs1-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 pcs1 function, we examined pcs1 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-pcs1 and wild type. We detected 1,280 pcs1-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 pcs1-dependent genes. Additionally, biotin, nitrogen, and several amino acid metabolisms are enriched in negatively regulated pcs1-dependent genes (Fig. 5). Furthermore, GO enrichment analysis indicated that G-protein signaling pathways in both cellular component and biological process, and

![Gene ontology (GO) enrichment of pcs1-dependent genes.](image_url)

**Fig. 6.** Gene ontology (GO) enrichment of pcs1-dependent genes. (A) Cellular component, (B) biological process, (C) molecular function. 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 $<0.05$. 

**References:**

Jung et al.
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. hetrotrimeric G protein Gα 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.

Acknowledgments

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References


