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# The *Fusarium Graminearum* virulence factor FGL targets an FKBP12 immunophilin of wheat

Xiao-Wei Niu [b,c], Zi-Yang Zheng [a], Yi-Gao Feng [b], Wang-Zhen Guo [b], Xin-Yu Wang [a,*]

[a] College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
[b] State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China
[c] Institute of Vegetables, Academy of Zhejiang Agricultural Sciences, Hangzhou 310021, China

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ABSTRACT

Wheat scab, caused by the fungal pathogen *Fusarium graminearum* is a devastating disease worldwide. Despite an extensive and coordinated effort to investigate this pathosystem, little progress has been made to understand the molecular basis of host–pathogen interactions, for example how the pathogen causes disease in plant. Recently, a secreted lipase (FGL1) has been identified from the fungus and shown to be an important virulence factor; however, the intrinsic function of FGL1 in plant is unknown. Here, we report the identification of the molecular components that may possibly be involved in the FGL virulence pathway using yeast two hybrid system. FGL gene was amplified from a local virulent strain (F15) and shown to be 99.5% identical to the original published FGL at the amino acid level. We showed that transient expression of this FGL gene by Agroinfiltration in tobacco leaves causes cell death further implicating the role of FGL in virulence. To identify FGL initial physical target in plant, we screened two wheat cDNA libraries using the FGL protein as the bait. From both libraries, a small FKBP-type immunophilin protein, designated wFKBP12, was found to physically interact with FGL. The direct interaction of FGL with wFKBP12 was confirmed in living onion epidermal cells by biomolecular fluorescence complementation (BiFC) assay. To investigate further, we then used wFKBP12 protein as bait and identified an elicitor-responsive protein that contains a potential $Ca^{2+}$ binding domain. Semi-quantitative PCR showed that this elicitor-responsive gene is down-regulated during the *F. graminearum* infection suggesting that this protein may be an important component in FGL virulence pathway. This work serves as an initial step to reveal how fungal lipases act as a general virulence factor.

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## 1. Introduction

Interactions between plants and fungal pathogens require a complex interplay at the plant–fungus interface. Molecules produced by both the pathogen and the host are critical factors determining the outcome of the interaction. Recent studies have revealed that secreted fungal effector proteins play a crucial role in establishing a successful infection. These effector proteins can act in apoplast or inside plant cells, where most of them interact with host factors to suppress or elicit host defense responses, resulting in plant susceptibility or resistance. Therefore, isolation and characterization of fungal effectors and their host targets benefit the elucidation of molecular basis of host–fungi interactions and the outcomes of these interactions. In recent years, a number of examples for effector–host interactions that result in plant resistance or susceptibility are reported in plant–biotroph pathosystems. For example, Avr2, an effector secreted by the bitrophic fungal pathogen *Cladosporium fulvum*, interacts with an apoplastic tomato Cys protease Rcr3, which is required for Cf-2-mediated immunity. The molecular basis for this immune reaction is that Avr2-Rcr3 complex enables resistance protein Cf-2 to activate a hypersensitive reaction (HR) since Avr2 may modify the structure of Rcr3 protein which is sensed by Cf-2 and then trigger a Cf-2-mediated defense signaling (Rooney et al., 2005). This kind of immune response has been referred to as effector triggered immunity (ETI). *Magnaporthe grisea*, the causal agent of rice blast, secretes a preprotein of 233 aa designated Avr-Pita during its infection of rice. This protein is further processed into an active 176 aa mature protein (Avr-Pita176) that is dispensable for virulence on rice. Avr-Pita176 interacts directly with the cognate Pi-ta resistance protein, a predicted 928 aa receptor like protein with a central nucleotide-binding site (NBS) and a C-terminal leucine-rich repeat (LRR) domain. Direct interaction between the two proteins has been demonstrated by yeast two-hybrid assays and by in vitro binding assays that showed binding

*Abbreviations:* BiFC, Biomolecular fluorescence complementation; DON, Deoxynivalenol; ERG, Elicitor-responsive gene; ETI, Effector-triggered immunity; ETS, Effector-triggered susceptibility; FHB, Fusarium head blight; FKBP, FK506-binding protein; FKBP12, 12-kDa FK506-binding protein; FGL, Lipase of *Fusarium graminearum*; HR, Hypersensitive reaction; LRR, Leucine-rich repeat; NBS, Nucleotide-binding site; PVX, Potato virus X.

* Corresponding author.
*E-mail address:* xywang@njau.edu.cn (X.-Y. Wang).

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of the Avr-Pita176 to the LRR domain of Pi-ta. Furthermore, coexpression of the two proteins in rice cells elicited plant defense responses, suggesting that physical interaction inside host cells is required for activation of Pi-ta-mediated defense (Jia et al., 2000). In *Phytophthora infestans*-tomato pathosystems, to promote pathogenesis, the pathogenic oomycete uses effectors EPI1 and EPI10 to bind and inhibit pathogenesis-related protease P69B (Tian et al., 2004), and effector EPI2B to interact and inhibit a papain-like extracellular cystein protease, termed phytophthora inhibited protease1 (PIP1), which is a PR protein closely related to Rcr3 of tomato (Tian et al., 2007).

In contrast to reports on host–biotrophic effector interactions, few host–necrotrophic effector interactions are reported. However, several recent studies of necrotrophic effectors from two wheat pathogens, *Pyrenophora tritici-repentis* (Died.) Drechs. and *Stagonospora nodorum* (Berk.) Castell. & Germano, have shed light upon how these effector proteins serve to disable the host defense. PtrToxA is a small (13.2 kDa) secreted protein produced by *P. tritici-repentis*. It causes necrosis in sensitive wheat genotypes containing *Tsn1*, a gene with nucleotide-binding site (NBS), leucine-rich repeat (LRR) and serine/threonine protein kinase domains, common features of plant disease R genes involved in defense against biotrophic pathogens (Ciuffetti et al., 1997; Faris et al., 2010). *S. nodorum*, the causal agent of wheat *S. nodorum* blotch (SNB), produces multiple necrotrophic effectors (also called host-selective toxins) that promote disease by interacting with corresponding host sensitivity gene products. To date, six interactions have been reported including SnTox1-Snn1 (Liu et al., 2004), SnToxA-Tsn1 (Liu et al., 2006), SnTox2-Snn2 (Friesen et al., 2007), SnTox3-Snn3-B1 (Friesen et al., 2008), SnTox4-Snn4 (Abeysekara et al., 2009), and SnTox3-Snn3-D1 (Zhang et al., 2011). *SnToxA* was the first reported necrotrophic effector gene identified in *S. nodorum* by BLAST searches of the *S. nodorum* genome sequences with the *PtrToxA* gene as a query (Friesen et al., 2006). The SnToxA gene is essentially identical to the PtrToxA gene (99.7% similarity). Several lines of evidence have been published confirming that SnToxA interacts (directly or indirectly) with Tsn1 (Friesen et al., 2006). SnToxA and PtrToxA possess the same mode of action. Both effectors cause necrosis on wheat carrying *Tsn1* in a light-dependent manner (Friesen et al., 2006; Manning and Ciuffetti, 2005). Based on these new findings, it is suggested that R genes could paradoxically play a role in disease susceptibility by serving as targets for necrotrophic effectors such as PtrToxA, resulting in plant susceptibility that can be described as effector-triggered susceptibility (ETS).

The filamentous fungus *Fusarium graminearum* sensu stricto is one of the most destructive plant pathogens of small grain cereals. It causes Fusarium head blight (FHB, also known as wheat scab) on wheat and barley and ear rot disease of maize (Goswami and Kistler, 2004). FHB has great economic impact for cereal farmers and industry due to yield and quality losses and production of several harmful mycotoxins such as the trichothecene deoxynivalenol (DON) that contaminate the grain, rendering it unsafe for human and livestock consumption. Major FHB epidemics that have occurred in the last two decades have evoked extensive studies on *F. graminearum* as a major global pathogen of cereals. The genome of *F. graminearum* has been sequenced, annotated, and compared with other organisms (Ma et al., 2010). In the last few years, significant progress has been made towards a better understanding of the processes involved in *F. graminearum* pathogenesis. Several virulence factors had been identified and the mechanisms of their actions had been elucidated. For example, DON is an intensively studied virulence factor. DON inhibits protein synthesis in eukaryotes and prevents polypeptide chain initiation or elongation by binding to the 60S ribosomal subunit (Kimura et al., 1998). During infection, DON is proposed to be involved in disease spread but not required in initial infection of wheat heads since tri5 mutant strains deficient in DON production exhibit infection at the site of inoculation but lack symptom spread and infection structures related to initial infection such as 'bulbous infection hyphae' and lobate appressoria and infection cushions were not affected in tri5 mutant. DON also exhibit a tissue-specific manner in inoculated wheat heads since the DON biosynthesis in the pathogen is specifically elicited in rachis tissue. Another identified key virulence factor is topoisomerase. The top1 deficient *F. graminearum* strain was unable to colonize the wheat ear despite producing wild-type DON levels and infections were restricted to just below the surface of the floral brackets (Baldwin et al., 2010). To date, there are 49 pathogenic genes of *F. graminearum* that were verified by biological experiments and stored in PHI-base database (http://www.phi-base.org/query.php). Based on molecular interaction network and gene expression data of *F. graminearum*, a pathogenic network was established that consists of 127 genes which are potential virulence factors, providing target genes for further analysis (Liu et al., 2010). Over the years, one contentious issue has been whether *F. graminearum* exhibits a biotrophic lifestyle during the initial stages of infection of floral tissues (Brown et al., 2010; Trail, 2009). A recent detailed microscopic study of the *F. graminearum* infection process in wheat heads found no indication of necrotrophy at the initial stages of infection, as the advancing *F. graminearum* hyphae remained in the intercellular spaces of wheat rachis cells before subsequent intracellular growth, which presumably leads to subsequent cell death and necrosis (Brown et al., 2010). Therefore, *F. graminearum* may be classified as a hemibiotrophic pathogen. In view of these findings, it is proposed that extracellular communication between live pathogen and host cells must occur, implying a role for secreted fungal proteins in this event. Pathogen effectors usually contain amino-terminal secretion signals and, after secretion by pathogens, either remain in the extracellular spaces of the host plant or enter host cells and have the ability to modify (i.e. suppress) host defenses by interacting with specific host factors. Recently, the predicted secretome of the plant pathogenic fungus *F. graminearum* contains 574 secreted proteins (Brown et al., 2012). But at present, how these effectors act and what host factors they interact with are largely unknown.

Voigt et al. (2005) reported that a secreted lipase (FGL1) of *F. graminearum* is a virulence factor (Voigt et al., 2005). Disruption of FGL1 led to reduced virulence to both wheat and maize. In contrast to wild-type infection, only directly inoculated and adjacent spikelets were bleached during Δfgl1 strain infection. The rachis of the wild type infected spikes was completely bleached while almost no symptoms of bleaching were detectable at the rachis of spikes infected with theΔfgl1 strains, suggesting that FGL1 could be involved in disease spread. In this study, to investigate the molecular basis of FGL in virulence function, we cloned a homolog of FGL1, designated FGL15, from a local *F. graminearum* strain F15. Transient expression of FGL15 by Agroinfiltration in tobacco leaves cause cell death, further implicating a role of FGL15 in virulence. Then, FGL15 was used as bait to screen its host virulence target with yeast two-hybrid. The results show that FGL15 interacts with an FKBP-type immunophilin of wheat designated wFKBP12. We further demonstrate that wFKBP12 interacts with an elicitor-responsive protein containing a C2 domain, a potential $Ca^{2+}$ binding domain and that the elicitor-responsive gene is down-regulated upon *F. graminearum* infection. Our study provides an initial step to dissect the components of FGL virulence pathway.

## 2. Materials and methods

### 2.1. Plant material

Two wheat cultivars, Sumai3 and Wangshuibai with partial resistance to *F. graminearum* were used to construct cDNA expression

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