Fglv5 is required for branched-chain amino acid biosynthesis and full virulence in *Fusarium graminearum*

Xin Liu, Jian Wang, Jianhong Xu and Jianrong Shi

Key Laboratory of Food Quality and Safety of Jiangsu Province-State Key Laboratory Breeding Base/Key Laboratory of Control Technology and Standard for Agro-product Safety and Quality (Nanjing), Ministry of Agriculture/Institute of Food Quality and Safety, Jiangsu Academy of Agricultural Sciences, 210014, Nanjing, PR China

In this study, we characterized Fglv5, a homologue of the *Saccharomyces cerevisiae* keto-acid reductoisomerase (KARI) from the important wheat head scab fungus *Fusarium graminearum*. KARI is a key enzyme in the branched-chain amino acid (BCAA, including leucine, isoleucine and valine) biosynthetic pathway that exists in a variety of organisms from bacteria to fungi and higher plants, but not in mammals. The FglLV5 deletion mutant ΔFglv5-4 failed to grow when the culture medium was nutritionally limited for BCAAs. When grown on potato-dextrose agar plates, ΔFglv5-4 exhibited a significant decrease in aerial hyphae formation and red pigmentation. Conidia formation was also blocked in ΔFglv5-4. Exogenous addition of 1 mM isoleucine and valine was able to rescue the defects of mycelial growth and conidial morphogenesis. Cellular stress assays showed that ΔFglv5-4 was more sensitive to osmotic and oxidative stresses than the wild-type strain. In addition, virulence of ΔFglv5-4 was dramatically reduced on wheat heads, and a low level of deoxynivalenol production was detected in ΔFglv5-4 in wheat kernels. The results of this study indicate that Fglv5 is involved in valine and isoleucine biosynthesis and is required for full virulence in *F. graminearum*.

INTRODUCTION

The filamentous ascomycete *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch], the primary causal agent of *Fusarium* head blight (FHB) (Dean et al., 2012), is a highly destructive pathogen of all cereal species (http://www.scabusa.org). Worldwide, *Fusarium* epidemics have occurred in all the major cereal-growing regions (Champeil et al., 2004). Devastating economic losses occur when the floral tissues become infected by the fungus. In addition to yield loss, FHB also reduces grain quality. Grains contaminated by mycotoxins [e.g. deoxynivalenol (DON), nivalenol and zearalenone] are unsafe for human consumption, animal feed or malting purposes and thus pose a serious threat to food safety.

Control of FHB remains problematic. As most wheat cultivars are susceptible to *Fusarium* species (Goswami & Kistler, 2004), the primary method for management of FHB is through fungicide application (Blandino et al., 2006; Lechocz-Krsjak et al., 2008). In the last two decades, benzimidazole fungicides, particularly carbendazim, have been extensively and consistently applied for the control of FHB in China. Due to its heavy application, carbendazim-resistant isolates have been frequently detected in wheat fields since 1992, and a decline in carbendazim control efficacy has been observed in some areas of China since 1998 (Chen et al., 2007; Liu et al., 2010; Wang & Zhou, 2002). Therefore, alternative fungicides are urgently needed for the management of FHB.

Certain biosynthetic pathways provide attractive targets for herbicides, fungicides and antimicrobial compounds given that they are absent in mammals. One such pathway is the branched-chain amino acid (BCAA) biosynthetic pathway, which synthesizes leucine (Leu), isoleucine (Ile) and valine (Val) in a variety of organisms from bacteria to fungi and high plants, but not animals. The biosynthesis of BCAAs consists of a common pathway that leads from pyruvate and α-ketobutyrate to Val and Ile and a branch that leads from the immediate precursor of Val, α-ketoisovalerate, to Leu. BCAA biosynthesis shares the raw material pyruvate, the final product of glycolysis, with several other amino acids. A unique feature of BCAA biosynthesis is that Val and Ile are synthesized in two parallel pathways each with a

Abbreviations: AHAS, acetohydroxyacid synthase; BCAAs, branched-chain amino acids; DHAD, dihydroxyacid dehydratase; DON, deoxynivalenol; FHB, *Fusarium* head blight; ILV, isoleucine, leucine and valine; KARI, keto-acid reductoisomerase; PIMG, percentage of inhibition of mycelial radial growth.

One supplementary table and two supplementary figures are available with the online version of this paper.
set of four identical enzymes catalysing the reactions with different substrates, including acetohydroxyacid synthase (AHAS, EC 2.2.1.6), ketol-acid reductoisomerase (KARI, EC 1.1.1.86), dihydroxyacid dehydratase (DHAD, EC 4.2.1.9) and branched-chain aminotransferase (EC 2.6.1.42). BCAAs are important precursors for protein synthesis. Besides this basic role, BCAAs, especially Leu, also function in the regulation of intracellular signal transduction pathways that control mRNa translation (Harris et al., 2004; Yoshizawa, 2004).

Many herbicides targeting the first common enzyme AHAS in the BCAA biosynthetic pathway have been developed successfully and used extensively for broad-spectrum weed control on major crops (McCourt & Duggleby, 2006). AHAS inhibitors are highly selective, potent and non-toxic to animals (Kohlhaw, 2003; Tan et al., 2006). To date, according to statistics by the Herbicide Resistance Action Committee, more than 50 AHAS inhibitors are available for weed control and making great contributions to world agricultural production. Several AHAS inhibitors have also shown bacteriostatic effects on some pathogenic microorganisms, including Burkholderia pseudomallei, Pseudomonas aeruginosa, Acinetobacter baumannii and Candida albicans, indicating a promising antimicrobial drug strategy targeting enzymes involved in the BCAA biosynthesis pathway (Kreiberg et al., 2013; Lee et al., 2013).

KARI, encoded by Ilv5, is one step downstream from AHAS, and catalyses the conversion of 2-acetolactate and 2-aceto-2-hydroxybutyrate to 2,3-dihydroxyisovalerate and 2,3-dihydroxy-3-methylvalerate, respectively, in the BCAA biosynthetic pathway. The success of AHAS inhibitors has also stimulated research into inhibitors of other enzymes in the pathway, including KARI. Currently, there are no commercial herbicides targeting KARI, although several compounds, including HOE 704 (Schulz et al., 1988), IpOHA (Aulabaugh & Schloss, 1990) and CPD (Lee et al., 2005; Liu et al., 2007), have been reported as potent competitive inhibitors of KARI in vitro. Morya et al. (2012) have modelled the Aspergillus KARI enzyme and the modelled structure was validated and used for docking study; six potent inhibitors were pharmacologically active agonists and antagonists of KARI. These results revealed the potential of KARI as a drug target. In this study, we characterized the gene encoding KARI, FgIlv5, by using a targeted disruption strategy in the wheat head scab fungus F. graminearum.

METHODS

Strains, growth conditions and phenotype assays. F. graminearum strain PH-1 was used as the parental wild-type. To assess mycelial growth and colony characteristics, strain PH-1 and mutant strains were cultured on potato-dextrose agar (PDA), yeast extract peptone glucose agar (YEPD), fructose gelatin agar (FGA) or minimal medium (MM) plates supplemented with different amino acids and incubated at 25 °C. Colony diameter was measured every 24 h. Fungal biomass was measured by collecting mycelia from 2-day-old liquid culture, and then washed with sterilized water, dried at 65 °C in an oven and determined by mycelia dry weight. Experiments were performed in triplicate. Escherichia coli DH5α was cultured at 37 °C and used for normal bacterial transformations.

For the conidiation assay, six 5 mm mycelial plugs of each strain taken from the edge of a 3-day-old colony were inoculated in a 50 ml triangular flask containing 20 ml MBL (40 g mung beans boiled in one litre of water for 20 min, and then filtered through cheesecloth) supplemented with different amino acids for conidiation. The flasks were incubated at 25 °C for 4 days in a shaker (180 r.p.m.) (Bai et al., 2002). The amount of conidia in the broth was determined for each sample using a haemocytometer. Additionally, conidia germination was assessed after resuspension in 2 % sucrose solutions amended with or without amino acids at 25 °C for 4 or 6 h; conidial germination was examined under a Nikon ECLIPSE E100 microscope. Each experiment was carried out with three replicates.

Expression analysis and intron verification of the FgIlv5 gene in F. graminearum. We identified the F. graminearum homologue FgIlv5 and its encoding gene FgIlv5 (Broad Institute Fusarium Comparative Database accession number FGSG_10118) via BLASTP searches at the Fusarium Comparative Database site (available at http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html) using the amino acid sequence of Saccharomyces cerevisiae Kari Ilv5 (Saccharomyces database accession number YLR355C) as a query.

To validate the existence of introns in the FgIlv5 gene, RNA was extracted from mycelia of the wild-type PH-1 using TaKaRa RNAiso Reagent (TaKaRa Biotech), and used for reverse transcription with the primer oligo(dT)18 using a PrimeScript RT reagent kit (TaKaRa Biotech). PCR amplification with primer pair Ilv5-all-F + Ilv5-all-R (Table S1, available in the online Supplementary Material) was performed for FgIlv5, using cDNA and genomic DNA as templates. PCR amplifications were conducted using the following parameters: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min. PCR products were separated on 1 % agarose gels in TAE buffer and photographed after staining with ethidium bromide. The resultant PCR product was purified, cloned and sequenced.

Construction of FgIlv5 gene deletion mutants. FgIlv5 deletion vector pBS-ilv5-Del was constructed by inserting two flanking sequences of the FgIlv5 gene into the left and right sides of HPH (hygromycin resistance gene) in the pBS-HPH1 vector (Liu et al., 2011). Briefly, by using primer pair A1 + A2 (Table S1), a 754 bp upstream flanking sequence fragment of FgIlv5 was amplified from PH-1 genomic DNA, and was inserted into KpnI–XhoI sites of the pBS-HPH1 vector to generate plasmid pBS-ilv5-up. Subsequently, a 1067 bp downstream flanking sequence fragment of FgIlv5 amplified from PH-1 genomic DNA using primers A3 + A4 (Table S1) was inserted into HindIII–BamHI sites of the pBS-ilv5-up vector to generate plasmid pBS-ilv5-Del. Finally, the 3324 bp fragment containing the ilv5-upstream–HPH–ilv5-downstream cassette was obtained by PCR amplification with primer pair A1 + A4 using diluted plasmid pBS-ilv5-Del as template. PCR products were separated on 1 % agarose gels in TAE buffer and photographed after staining with ethidium bromide. The resultant PCR product was purified and stored at −20 °C for protoplast transformation.

PEG-mediated protoplast fungal transformation was performed as described previously (Liu & Friesen, 2012). For selective growth of transformants, PDA medium supplemented with hygromycin (100 mg l−1) was used. After single spore isolation, transformants were kept at 4 °C for further experiments.
Genetic complementation of the FgILV5 deletion mutant. To confirm that the phenotype of the FgILV5 deletion mutant is due to disruption of the gene, genetic complementation was performed. A stretch of 2975 bp of the full-length FgILV5 including 1070 bp upstream and 548 bp terminator region was amplified using primer pair ilv5-com-F + ilv5-com-R (Table S1) from genomic DNA of wild-type strain PH-1. This fragment was cloned into the BamHI–PstI sites of pCAMBIA1300-NEO to generate the complement plasmid pCA-ilv5-Com. Transformation of sites of pCAMBIA1300-NEO to generate the complement plasmid was analysed using the ANOVA procedure within the program SAS (version 2011). The experiment was repeated three times, and data were 10 days after inoculation. The experiment was repeated three times. Levels of plant infection assays. Conidia harvested from 4-day-old cultures of the wild-type and mutant strains were resuspended in 0.01 % (v/v) Tween 20 with or without amino acids and adjusted to 10^5 conidia ml^{-1}. Pathogenicity tests were performed with the single floret infection injection method as described by Wu et al. (2005). Briefly, 10 μl of conidial spores (10^5 ml^{-1}) was injected into a single floret in the central section spikelet of single flowering wheat heads of susceptible cultivar Jimai 19 at early anthesis, and ten spikes were used for each strain. Infected spikelets in each inoculated wheat head were recorded 10 days after inoculation. The experiment was repeated three times. To examine the ability to colonize tomato, a 10 μl aliquot of the conidial suspension was injected into wounded tomato after surface sterilization. Five replicates were used for each strain. Inoculated tomatoes were incubated at 25 °C and 100 % humidity with 12 h of daylight, and were photographed 3 days after inoculation. The experiment was repeated three times.

Determination of sensitivity of the FgILV5 mutant to cell stresses and herbicides. Mycelial growth tests were performed on YEPD plates supplemented with the following chemicals: for osmotic stress NaCl, d-sorbitol, KCl and glycerol; for oxidative stress H_2O_2; cell-wall inhibitors congo red and caffeine; cell membrane damager SDS; and seven AHAS inhibitors, namely rimsulfuron, chlorimuron-ethyl, halosulfuron-methyl, tribenuron-methyl, metsulfuron-methyl, imazapyr and imazethapyr at the concentrations indicated in the figures. The percentage of inhibition of mycelial radial growth (PIMG) was calculated using the formula PIMG=\left[\frac{C-N}{C}\right]\times 100, where C is colony diameter of the non-treated control, and N is that of the chemical treatment. Each experiment was carried out with three replicates and the experiments were repeated twice.

Determination of DON production. Diseased wheat kernels were harvested from inoculated spikelets 20 days after inoculation and used for assays of DON production using a previously described protocol (Bluhm et al., 2007; Sasanya et al., 2008). The amount of DON in each sample was determined using an HPLC-MS/MS system (Agilent1100-6410; Agilent Technologies). MS parameters were according to Soleimany et al. (2012).

Additionally, the amount of F. graminearum DNA in each sample was determined using a quantitative real-time PCR method (Jiang et al., 2011). The experiment was repeated three times, and data were analysed using the ANOVA procedure within the program SAS (version 8.0; SAS Institute).

Analysis of gene expression level. Total RNA of the wild-type PH-1 and deletion mutants was extracted and cDNA was reverse transcribed using the protocol described above. Expression of the pigment genes and TRI genes was determined by quantitative real-time PCR (RT-PCR) with primer pairs Pks12-RT-F + Pks12-RT-R, Gip1-RT-F + Gip1-RT-R, Gip2-RT-F + Gip2-RT-R, AurF-RT-F + AurF-RT-R, AurR2-RT-F + AurR2-RT-R, AurO-RT-F + AurO-RT-R, TR14-RT-F + TR14-RT-R, TR15-RT-F + TR15-RT-R and TR16-RT-F + TR16-RT-R (Table S1). The RT-PCR amplifications were performed in a Rotor Gene 6000 analyser (Corbett Research) using SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20 μl volume containing 10 μl iQ SYBR Green Supermix (Bio-Rad Laboratories), 1 μl reverse transcription product and 0.5 μl of each primer (Table S1). There were two replicates for each sample. RT-PCR amplifications were performed with the following parameters: an initial preheat at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s and 75 °C for 3 s to quantify the fluorescence at a temperature above that for denaturation of primer-dimers. Once amplifications were completed, melting curves were obtained to identify PCR products. For each sample, PCR amplifications with primer pair Fgactin-F + Fgactin-R (Table S1) for quantifying expression of the actin gene were performed as a reference. The experiment was repeated three times. Levels of expression of the genes in the wild-type strain and deletion mutants were calculated using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001).

RESULTS

Identification of FgILV5 in F. graminearum

Homology searches using Ilv5 from S. cerevisiae and Candida albicans as query showed that the locus FGSG_10118.3 (Broad accession number) in the F. graminearum genome shared a high level of homology at the amino acid level with S. cerevisiae Ilv5 (72.24 %) and Candida albicans Ilv5 (71.60 %). The putative encoding gene was annotated as FgILV5. FgILV5 is 1357 bp in length and encodes 405 amino acids. Ilv5 is relatively conserved among Fusarium species (Fig. S1). FgILV5 has two predicted introns; one is 78 bp in length located between nucleotides 212 and 289 and the other is 61 bp in length located from 601 to 661 bp of the nucleotide sequence. Sequencing of the 1218 bp cDNA of FgILV5 verified the existence of the two introns.

Disruption and complementation of FgILV5 in F. graminearum

For a detailed functional analysis of FgILV5, we generated deletion mutants using a double homologous recombination strategy. Wild-type strain PH-1 was transformed with the 3324 bp fragment containing the ilv5-upstream-HPH-ilv5-downstream cassette by protoplast-mediated fungal transformation, resulting in more than 30 primary hygromycin-resistant transformants. We identified six FgILV5 deletion mutants by PCR analysis with primers...
A5 + A6 (Table S1). This primer pair amplified a 1801 bp fragment from the six FgILV5 deletion mutants including ΔFgIlv5-4, and a 1181 bp fragment from the wild-type strain PH-1, respectively. As shown in Fig. S2(b), reverse transcription PCR with the primer pair lv5-spantron-F + lv5-spantron-R amplified an expected 287 bp fragment from the wild-type strain PH-1 and complemented strain ΔFgIlv5-7C, but not from ΔFgIlv5-4 (Fig. S2b). PCR results were further verified by Southern blotting analysis. The genomic DNA of PH-1, ΔFgIlv5-4 and ΔFgIlv5-7C digested by NcoI were blotted and hybridized with a DIG-labelled FgILV5 downstream fragment (Fig. S2a). The wild-type strain had an expected hybridizing band of 2410 bp, while this band was replaced by a 1941 bp fragment in ΔFgIlv5-4. Additionally, the Southern blot hybridization pattern of ΔFgIlv5-7C confirmed that a single copy of the full-length FgILV5 was reintroduced into the genome of ΔFgIlv5-4 (Fig. S2c).

**FgILV5 is an essential component of the BCAA biosynthetic pathway in F. graminearum**

Deletion of FgILV5 resulted in BCAA auxotrophy in *F. graminearum*. As shown in Fig. 1, ΔFgIlv5-4 was unable to grow on MM or FGA containing no amino acids. By contrast, ΔFgIlv5-4 was able to grow on PDA medium despite showing reduced aerial hyphae. When cultured on YEPD plates, auxotrophy was fully recovered. The mycelial growth defects of ΔFgIlv5-4 could be restored by genetic complementation with the full-length FgILV5.

Given that FgILV5 encodes a key enzyme in the BCAA biosynthetic pathway, we further determined whether the growth defect of ΔFgIlv5-4 was due to individual or multiple amino acid starvation. Different amino acids were added to FGA and the results showed that exogenous supplementation of Val, Leu or Ile independently at 1 mM could not rescue the growth defect of ΔFgIlv5-4 (Fig. 2a). We next analysed how the mutant reacted to addition of two optional amino acids; the results showed that ΔFgIlv5-4 could only grow on FGA with Val and Ile each added at 1 mM – the combined addition of any two other amino acids failed to restore the growth defect of the mutant (Fig. 2a). Also, addition of 0.01, 0.05 and 0.25 mM Val and Ile in the medium did not rescue the growth defects of ΔFgIlv5-4, and we concluded that the concentration of Val and Ile required to reduce the growth defect is greater than 1 mM for each amino acid (Fig. 2b). Concurrent supplementation of three BCAAs at 1 mM could also rescue the phenotype defect in ΔFgIlv5-4 (Fig. 2a). Exogenous addition of non-BCAA glycine (Gly), methionine (Met), aspartic acid (Asp) or threonine (Thr) could not rescue the growth defect of ΔFgIlv5-4.

---

**Fig. 1.** *FgILV5* is required for mycelial growth in *F. graminearum*. (a) Colony morphology of the FgILV5 gene deletion mutant (ΔFgIlv5-4) compared with wild-type strain PH-1 and the complemented strain (ΔFgIlv5-7C) on PDA, YEPD, FGA or MM after 3 days of incubation at 25 °C. (b) Reduction of aerial hyphae production and red pigmentation of ΔFgIlv5-4 compared with PH-1 and ΔFgIlv5-7C on PDA medium after 3 days of incubation at 25 °C. (c) Reduced mycelial growth rate of ΔFgIlv5-4 compared with PH-1 and ΔFgIlv5-7C on PDA medium. (d) Reduced fungal biomass of ΔFgIlv5-4 in liquid PDB, YEPD, FGB and MM after 2 days of incubation at 25 °C.
Deletion of \textit{FgILV5} leads to reduced aerial hyphal growth and red pigmentation in \textit{F. graminearum}

Deletion of \textit{FgILV5} dramatically affected colony morphology of \textit{F. graminearum} on PDA plates. As shown in Fig. 1(b), Δ\textit{FgIlv5}-4 exhibited reduced aerial hyphal growth on PDA, although microscopy showed that the hyphal structure of Δ\textit{FgIlv5}-4 had no visible difference from the wild-type PH-1 (data not shown). In addition, deletion of \textit{FgILV5} resulted in a lower growth rate on PDA plates (Fig. 1c) and decreased mycelial dry mass in PDA broth (PDB) when compared with the wild-type PH-1 (Fig. 1d).

When cultured on PDA plates, Δ\textit{FgIlv5}-4 showed a complete loss of red pigment formation (Fig. 1b). To further confirm this observation, expression of six genes involved in aurofusarin biosynthesis that are necessary for red pigmentation in \textit{F. graminearum} were tested (Kim et al., 2005, 2006; Malz et al., 2005). Quantitative RT-PCR analyses showed that the level of expression of all six genes in...
ΔFgIlv5-4 was decreased by about 90% when compared with levels in the wild-type PH-1 (Table 1).

Again, the phenotypic defects of ΔFgIlv5-4 could be restored by exogenous supplementation of Val and Ile (1 mM each) or by genetic complementation with the full-length FgILV5 (Fig. 2a, b).

**Deletion of FgILV5 affected conidial morphogenesis in F. graminearum**

*F. graminearum* reproduces and propagates disease via conidia, which are produced by asexual development. The wild-type strain PH-1 and the mutants were cultured in MBL to test conidiation. Strikingly, in contrast to the wild-type strain PH-1 and the mutants, ΔFgIlv5-4 failed to produce conidia in MBL even at day 15 (data not shown). Exogenous supplementation of Val and Ile (1 mM each), but not of individual Ile, Val or Leu (1 mM), rescued the conidia formation defect of ΔFgIlv5-4.

To further examine the germination rate of the recovered conidia of ΔFgIlv5-4, conidia were resuspended in 2% sucrose solution; after 4 or 6 h of incubation at 25 °C, more than 90% of the wild-type strain and of the complemented strain conidia germinated normally. By contrast, less than 5% of ΔFgIlv5-4 conidia germinated. Again, as shown in Fig. 3, when cultured in 2% sucrose solution supplemented with Val and Ile (1 mM each), but not Ile, Val or Leu (1 mM) individually, nor the non-BCAAs (data not shown), recovered conidia of ΔFgIlv5-4 were able to germinate within 4 h of incubation at 25 °C.

**Deletion of FgILV5 causes increased sensitivity to osmotic and oxidative stresses and increased tolerance to AHAS inhibitors in F. graminearum**

Sensitivity tests were conducted in YEPD medium to allow auxotrophs to be completely restored in growth. As shown in Fig. 4(a, b), when compared with PH-1 and ΔFgIlv5-7C, ΔFgIlv5-4 did not show recognizable changes to cell-wall-damaging agents congo red (0.4 mg ml⁻¹) and caffeine (5 mM), or cell membrane inhibitor SDS (0.005%). However, ΔFgIlv5-4 showed increased sensitivity to four osmotic stress agents, namely NaCl (1.2 M), KCl (1.2 M), D-sorbitol (1.2 M) and glycerol (1.2 M), and oxidative stress mediated by H₂O₂ (24 mM).

Enzymes involved in the BCAA biosynthetic pathway are the target of several herbicides (AHAS inhibitors), and therefore we also tested the sensitivity of the wild-type strain and the mutants to seven AHAS inhibitors: rimsulfuron, chlorimuron-ethyl, halosulfuron-methyl, tribenuron-methyl, metsulfuron-methyl, imazapyr and imazethapyr. As shown in Fig. 5, ΔFgIlv5-4 showed significant resistance to six of the AHAS inhibitors tested (not metsulfuron-methyl) as compared with the wild-type strain PH-1.

**FgILV5 deletion mutants lost full virulence in F. graminearum**

Given that the conidia of ΔFgIlv5-4 could not germinate in 2% sucrose solution or sterilized water, we added 1 mM Val and Ile to the conidial suspension for plant infection assays. Pathogenicity results indicated that conidia of ΔFgIlv5-4 suspended in 2% sucrose solution could not colonize and generate any visible symptoms, whereas conidia of ΔFgIlv5-4 suspended in 2% sucrose solution supplemented with 1 mM Val and Ile could colonize in the inoculated spikelet but lose aggressiveness. As shown in Fig. 6(a), 10 days after inoculation, the wild-type PH-1 or the complemented strain caused the typical scab symptoms in the inoculated and nearby spikelets of flowering wheat heads. Under the same conditions, however, scab symptoms caused by ΔFgIlv5-4 were only observed in the inoculated spikelet but not in any nearby spikelets. As shown in Fig. 6(b), 3 days after inoculation on non-host

---

**Table 1. Expression changes of the genes involved in aurofusarin biosynthesis in *F. graminearum* FgILV5 deletion mutant ΔFgIlv5-4 detected by quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Gene (Broad accession number)</th>
<th>Putative function</th>
<th>ΔFgIlv5-4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKS12 FGSG_02324</td>
<td>Hypothetical protein similar to type I polyketide synthase</td>
<td>0.072 ± 0.05</td>
</tr>
<tr>
<td>Gip1 FGSG_02328</td>
<td>Hypothetical protein similar to brown 2 (dimerizes to 9-hydroxyrubrofusarin)</td>
<td>0.058 ± 0.06</td>
</tr>
<tr>
<td>Gip2 FGSG_02320</td>
<td>Hypothetical protein similar to C6 zinc finger protein (positive acting transcription factor for the aurofusarin gene cluster)</td>
<td>0.127 ± 0.09</td>
</tr>
<tr>
<td>AurF FGSG_02326</td>
<td>Conserved hypothetical protein (O-methyltransferase: converts nor-rubrofusarin to rubrofusarin)</td>
<td>0.053 ± 0.04</td>
</tr>
<tr>
<td>AurO FGSG_02321</td>
<td>Conserved hypothetical protein (monooxygenase: converts rubrofusarin to 9-hydroxyrubrofusarin)</td>
<td>0.047 ± 0.02</td>
</tr>
<tr>
<td>AurO FGSG_02321</td>
<td>Oxidoreductase that catalyses the conversion of dimeric 9-hydroxyrubrofusarin to aurofusarin</td>
<td>0.099 ± 0.03</td>
</tr>
<tr>
<td>AurR2 FGSG_02323</td>
<td>Hypothetical protein similar to AurR2</td>
<td>0.157 ± 0.09</td>
</tr>
</tbody>
</table>

*The relative expression of each aurofusarin biosynthetic gene in the deletion mutant ΔFgIlv5-4 was relative to the amount of cDNA of each corresponding gene in the wild-type progenitor PH-1. Data are mean ± SD.
tomato, the water-soaked rot lesion caused by ΔFgIlv5-4 was significantly smaller than that caused by the wild-type strain or the complemented strain.

Deletion of *FgILV5* leads to reduced DON accumulation in *F. graminearum*

As shown in Fig. 7, when cultured on sterilized wheat kernels for 20 days, the amounts of DON produced by the wild-type strain or the complemented strain were threefold higher than that produced by ΔFgIlv5-4. Genetic complementation with the full-length *FgILV5* in ΔFgIlv5-4 restored the ability of the fungus to produce the same amounts of DON.

To further confirm the results, we assayed the expression of *TRI4*, *TRI5* and *TRI6* by quantitative RT-PCR using RNA samples isolated from mycelia grown in minimal synthetic liquid medium (Merhej et al., 2010) for 4 days at 25 °C. The expression level of *TRI4*, *TRI5* and *TRI6* in the mutant ΔFgIlv5-4 was decreased by 92, 68 and 62 %, respectively, as compared with levels in the wild-type PH-1 (Fig. 7b).

**DISCUSSION**

Amino acids, the building blocks of proteins, are important components in the metabolism of a variety of pathogens, plants and animals. Animals do not synthesize all of the amino acids they require, but obtain some of them from the environment (e.g. BCAAs), making enzymes involved in the biosynthesis of these amino acids desirable targets for the discovery of new herbicide and antimicrobial agents. In fact, a significant proportion of newly commercialized herbicides are AHAS inhibitors, targeting the BCAA biosynthetic pathway of weeds (Tan et al., 2006).

Can similar pathways be targeted in the fight against microbial infections? Recent reports regarding the bacteriostatic effects of several AHAS inhibitors on some pathogenic bacteria (Kreisberg et al., 2013; Lee et al., 2013) have highlighted the potential of antimicrobial drug strategies targeting BCAA biosynthetic enzymes and the urgent need for identification of essential *ILV* genes in important human and plant pathogenic micro-organisms. Previous work in *S. cerevisiae* and *Cryptococcus neoformans* showed that AHAS-encoding gene *ILV2* deletion mutants exhibited amino acid starvation and are unable to survive *in vivo* and/or are avirulent (Kingsbury et al., 2004, 2006). Subsequently, other studies showed that *Candida albicans* *ILV2* deletion mutants die rapidly, are less virulent and are BCAA-auxotrophic (Kingsbury & McCusker, 2010). In the human pathogenic fungi *Aspergillus fumigatus*, of four paralogous *AfILV3* genes encoding DHAD, only *AfILV3A* is required for BCAA biosynthesis, and deletion of *AfILV3A* leads to reduced virulence (Oliver et al., 2012). In *S. cerevisiae*, an *ILV5* deletion mutant was non-viable and Ilv5 also was shown to be a biofunctional enzyme involved in mitochondrial genome maintenance (Macierzanka et al., 2008).

In this study, we found that *FgILV5* deletion mutants were BCAA-auxotrophic, indicating that *FgILV5* is an essential component of BCAA biosynthesis in *F. graminearum*. Phenotype assays showed that ΔFgIlv5-4 exhibited a series of growth defects as compared with the wild-type strain PH-1. Hyphal growth was attenuated on PDA medium as indicated by the reduced aerial mycelium, reduced growth rate and fungal mass when cultured in PDB (Fig. 1). Conidial formation was blocked in MBL and recovered conidia also could not germinate in 2 % sucrose solution (Fig. 3). No red pigment accumulated in ΔFgIlv5-4, even when cultured on PDA plates for 10 days (data not shown), which is in
agreement with the dramatically reduced expression level of six pigmentation-related genes in ΔFglv5-4. Simultaneously addition of Val and Ile at 1 mM could rescue all the above growth defects (Figs 2 and 3), indicating that a defect in BCAA biosynthesis could affect normal physiology of the pathogen.

In addition to the defects in hyphal and conidial development, pathogenicity and DON accumulation were also impaired in ΔFglv5-4. In the infection assays on flowering wheat heads and non-host tomatoes, the virulence of ΔFglv5-4 was completely lost (Fig. 6). Recovered conidia of ΔFglv5-4 with addition 1 mM Val and Ile to the suspension solution could successfully colonize the inoculated spikelet of wheat heads, but lost aggressiveness in plant tissues. However, the aggressiveness of the mutant was not recovered to the wild-type level, probably because the exogenous addition of BCAAs could meet the demands of conidial germination but the acquisition of BCAAs from plant tissues may be insufficient during mycelial spread of the mutant, thereby inhibiting the infection. This suggests that BCAA limitation is an environmental condition that F. graminearum encounters during infection, making the ability to synthesize BCAAs a crucial process for full virulence of the pathogen.

Reduced virulence could not be separated from the severe in vitro phenotype displayed by ΔFglv5-4. First, as compared with wild-type strain PH-1, the mycelial growth of ΔFglv5-4 was significantly slower and the aerial hyphae were reduced when cultured on medium using wheat head extracts and glucose as the main source of nutrition.
Second, it is well known that reactive oxygen species play essential roles in host–pathogen interactions. Under pathogen attack, plants use the oxidative burst as an early defence reaction. ΔFglLV5-4 showed significantly increased sensitivity to the oxidative agent H$_2$O$_2$, which may be related to the reduced virulence in plant tissues.

In addition to the increased sensitivity to H$_2$O$_2$ displayed by ΔFglLV5-4, the cellular stress tests also revealed increased sensitivity of ΔFglLV5-4 to osmotic stresses mediated by NaCl, KCl, D-sorbitol and glycerol. To our knowledge, this is the first report showing the involvement of an Ilv protein in the mediation of sensitivity to osmotic and oxidative stresses.

**Fig. 5.** The *FgILV5* deletion mutant is resistant to AHAS inhibitors. Comparisons of mycelial inhibition percentages of each strain grown on YEPD medium amended with various AHAS inhibitors at the concentrations indicated; line bars in each column denote standard errors of three repeated experiments.

**Fig. 6.** *FgILV5* is required for full virulence of *F. graminearum*. (a) Flowering wheat heads were point inoculated with a conidial suspension at 10$^5$ conidia per millilitre of PH-1, ΔFglLV5-4 and ΔFglLV5-7C and infected wheat heads were photographed 10 days after inoculation. (b) Tomatoes were inoculated with a conidial suspension at 10$^5$ conidia per millilitre of each strain and infected fruits were photographed 3 days after inoculation.
stresses in fungi. The growth defects of ΔFgIlv5-4 can be fully recovered in YEPD plates used for stress tests, indicating that the increased sensitivity of ΔFgIlv5-4 in YEPD plates amended with various osmotic and oxidative stresses may not be caused by depletion of BCAAs. The FgIlv5 gene is involved in the mediation of sensitivity to osmotic and oxidative stresses and this role is independent of BCAA biosynthesis.

ACKNOWLEDGEMENTS

The research was supported by the National Science Foundation of China (31301623), Jiangsu Provincial Natural Science Foundation (BK20130704) and Jiangsu Agricultural Science and Technology Innovation Fund (CX(13)5050) to X. L., and the Special Fund for Agro-scientific Research in the Public Interest (201303016) to J. S.

REFERENCES


Edited by: R. Oliver