Fusarium graminearum Possesses Virulence Factors Common to Fusarium Head Blight of Wheat and Seedling Rot of Soybean but Differing in Their Impact on Disease Severity

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Accepted for publication 22 April 2014.

ABSTRACT

Sella, L., Gazzetti, K., Castiglioni, C., Schäfer, W., and Favaron, F. 2014. *Fusarium graminearum* possesses virulence factors common to Fusarium head blight of wheat and seedling rot of soybean but differing in their impact on disease severity. *Phytopathology* 104:1201-1207.

Fusarium graminearum is a toxigenic fungal pathogen that causes Fusarium head blight (FHB) and crown rot on cereal crops worldwide. This fungus also causes damping-off and crown and root rots at the early stage of crop development in soybean cultivated in North and South America. Several *F. graminearum* genes were investigated for their contribution to FHB in cereals but no inherent study is reported for the dicotyledonous soybean host. In this study we determined the disease severity on soybean seedlings of five single gene disrupted mutants of *F. graminearum*, previously characterized in wheat spike infection. Three of these mutants are impaired on a specific function as the production of deoxynivalenol (DON, $\Delta tri5$), lipase ($\Delta Fgl1$), and xylanase ($\Delta xyl03624$),

while the remaining two are MAP kinase mutants ($\Delta FgOS-2$, $\Delta gpmk1$), which are altered in signaling pathways. The mutants that were reduced in virulence ($\Delta tri5$, $\Delta Fgl1$, and $\Delta FgOS-2$) or are avirulent ($\Delta gpmk1$) on wheat were correspondingly less virulent or avirulent in soybean seedlings, as shown by the extension of lesions and seedling lengths. The $\Delta xyl03624$ mutant was as virulent as the wild type mirroring the behavior observed in wheat. However, a different ranking of symptom severity occurred in the two hosts: the $\Delta FgOS-2$ mutant, that infects wheat spikelets similarly to $\Delta tri5$ and $\Delta Fgl1$ mutants, provided much reduced symptoms in soybean. Differently from the other mutants, we observed that the $\Delta FgOS-2$ mutant was several fold more sensitive to the glyceollin phytoalexin suggesting that its reduced virulence may be due to its hypersensitivity to this phytoalexin. In conclusion, lipase and DON seem important for full disease symptom development in soybean seedlings, OS-2 and Gpmk1 MAP kinases are essential for virulence, and OS-2 is involved in conferring resistance to the soybean phytoalexin.

Fusarium graminearum (teleomorph *Gibberella zeae* (Schwein.) Petch) is a necrotrophic fungal pathogen that causes Fusarium head blight (FHB) and crown rot (CR) on cereal crops growing in different regions of the world (14,21). In temperate environments, wet and warm weather conditions during flowering provide favorable conditions for FHB epidemics (8). The disease decreases cereal yield and quality mostly because during the infection process the fungus secretes mycotoxins, such as the trichothecenes deoxynivalenol (DON) and nivalenol (NIV) and zearalenone, which accumulate in the seeds (41). These mycotoxins are a major concern for consumers and food producers (5).

In wheat, the fungus infects spikelets at anthesis and then can systemically colonize the entire spike producing extensive blight symptoms. Many studies have focused on the early stages of wheat spike infection. Several genes have been characterized as essential for spike infection (21) but few are strictly related to the secretome of the fungus. The *tri5* gene, for instance, is involved in the production of the secreted trichothecene toxins since it encodes trichodiene synthase, the first enzyme in the DON and NIV biosynthetic pathway. The *tri5* disrupted mutants ($\Delta tri5$) of *F. graminearum* are unable to synthesize DON and have impaired virulence on wheat (16,24,33). These mutants fail to cross the transition zone at the rachis node and are unable to infect spikelets adjacent to that initially infected (17). Other mutants, dis-

rupted for the stress-activated protein kinase OS-2, also have impaired DON production during wheat infection and cannot cross the rachis node at the base of spikelets (29). A strain with a disrupted lipase gene ($\Delta Fgl1$), but not impaired in DON biosynthesis, also fails to overcome the rachis node (39); while a MAP kinase-disrupted mutant ($\Delta gpmk1$) is avirulent (18,34,38) probably because of profoundly altered production of lipase, endoglucanase, and xylanolytic activity (19). However, a recent mutant ($\Delta xyl03624$) disrupted for an endoxylanase gene highly expressed during wheat infection did retain virulence on wheat, showing that activity of this endoxylanase was not necessary or essential for plant infection (36).

Some of these mutants have been assayed in infection experiments on other hosts. While $\Delta FgOS-2$ mutants were also impaired in virulence in maize spike infection (29), the $\Delta tri5$ mutants maintained their virulence in barley and maize (24). Thus, the trichothecenes are not general virulence factors of *F. graminearum* (24) and DON mycotoxin production is not required for *F. graminearum* to colonize maize and barley floral tissues. However, it is worth noting that in barley only the spikelets are colonized and *F. graminearum* cannot grow inside the rachis, as it does in wheat, and the diffusion of infection occurs along the rachis surface and not inside as in wheat (17).

F. graminearum was also reported as a pathogen of soybean in North and South America (3,7,26,32,40). Although able to experimentally infect soybean pods (26), all other cited studies describe *F. graminearum* as a cause of damping-off or of crown and root rots of soybean. Cultural practices such as early sowing, maize-wheat-soybean succession, and high inoculum levels associated

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<http://dx.doi.org/10.1094/PHYTO-12-13-0355-R>
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with no tillage or reduced tillage practices may favor the seed and seedling disease of soybean (10,11). The need for high inoculum levels to induce soybean seedling rot has been demonstrated by a rolled-towel assay used to compare virulence of different *F. graminearum* strains and to establish fungicide effectiveness against this pathogen (10).

As reported above, many studies on the mechanisms of *F. graminearum* infection were performed on cereals and no study was carried out on soybean, the main cultivated legume worldwide. It would be interesting to ascertain if the fungal factors shown to be involved in pathogenesis of wheat could have a role in soybean seedlings infection.

To this aim it was decided to analyze the disease severity on soybean seedlings of five *F. graminearum* disrupted mutant strains obtained at the author's laboratory and previously shown to be differently virulent on wheat plants (Table 1). Three strains are impaired for a specific function such as production of DON ($\Delta tri5$), lipase ($\Delta Fgl1$), and endoxylanase ($\Delta xyl03624$) and two are MAP kinase mutants ($\Delta FgOS-2$ and $\Delta gpmk1$) altered in signaling pathways (Table 1). In addition, to assess if DON and phytoalexin produced in plant are related to symptom severity, we determined the amount of DON produced by the fungal strains in soybean seedlings and the accumulation of glyceollin I, the major phytoalexin in soybean seedlings (2,9). An assay was also performed to estimate possible differences in strain sensitivities to glyceollin.

MATERIALS AND METHODS

Fungal strains and culture conditions. The *F. graminearum* strains 8/1 and PH1, isolated from rye and corn, respectively (27,37), and demonstrated to be highly virulent on wheat (16,36), were used in this study. The mutants $\Delta Fgl1$, $\Delta tri5$, and $\Delta gpmk1$ of the 8/1 strain and $\Delta FgOS-2$ and $\Delta xyl03624$ of the PH1 strain were from the authors' laboratories (Table 1). The strains were cultured at 25°C on complete medium (22) supplemented with 1.5% (wt/vol) granulated agar (Difco, Becton, Dickinson and Company, Sparks, MD) or on potato dextrose agar (Difco Laboratories, Detroit, MI).

Conidia were obtained by culturing *F. graminearum* wild type (WT) and mutant strains in 50 ml of carboxymethyl cellulose liquid medium (4) at 25°C and 100 rpm. After 5 to 7 days, the liquid cultures were filtered with sterile gauze, and conidia were collected, counted four times with a Thoma chamber, and diluted with sterilized water to approximately 2×10^5 conidia ml⁻¹.

Soybean infection experiments. The rolled-towel assay was performed according to Ellis et al. (10) with some modifications. In each infection experiment 20 soybean seeds (*Glycine max* (L.) Merr., 'Demetra') were inoculated with fresh macroconidia of *F. graminearum* WT or mutant strains. Each soybean seed was inoculated with 200 µl containing, in a first set of experiments, 2×10^4 spores; then, in a second group of experiments, 1×10^4 , 5×10^3 , and 2×10^3 spores. Both sets of experiments were repeated at least three times with two replicates each performed with different conidial preparations. Dilution of spores was performed with sterilized water and the suspensions were maintained at room temperature (22 to 24°C) for 16 h before inoculation. Preliminary experiments with the WT strain showed that this time interval improved uniformity in the seedling infection. Seeds used

as a control were mock-inoculated with sterilized water. Seeds were incubated in the dark at 24°C and 6 days postinoculation (dpi) symptom severity was recorded as the ratio between lesion length and total seedling length $\times 100$ (disease severity index, DSI). An index rating of 100% was attributed to nongerminating diseased seeds. Arcsine-transformed data were statistically analyzed by applying the Tukey-Kramer's test. To establish the effect of fungal infection on seedling growth, the length of each inoculated seedling was expressed as percentage compared with the length of healthy noninoculated seedlings used as control.

DON mycotoxin quantification. The concentration of DON produced by *F. graminearum* WT and mutant strains in the infected soybean seedlings 6 dpi was estimated using the AgraQuant deoxynivalenol enzymatic immunoassay (Romer Labs, Tull, Austria).

Infected seedling tissue (about 0.5 g) was homogenized with mortar and pestle, and the tissue was diluted 20-fold (wt/vol) with water. The homogenized tissues were then centrifuged at $10,000 \times g$ for 5 min and supernatants were further diluted with water. Different volumes were tested and at least four replicates were analyzed. Data were statistically analyzed by applying the Tukey-Kramer's test.

Two further samples of soybean seedlings infected with WT and $\Delta tri5$ mutant, respectively, were also analyzed by high-resolution LC-MS with Orbitrap technology at ERS Agency Analyses Services (Pozzuolo del Friuli, Udine, Italy). Chromatographic separation was achieved on a ACE 3 C18 column (100 mm $\times$ 2.1 mm) connected to Orbitrap. DON was eluted with methanol/water (5 mM ammonium formate) 10:90 (vol/vol) for 1 min and then with 100% methanol for 15 min. For LC-HRMS analysis the ESI interface was used in negative ionization mode.

Extraction and purification of glyceollin. Glyceollin used in the bioassays was extracted from soybean hypocotyls treated for 24 h with 5 mM oxalic acid as previously reported (12). Glyceollin was extracted from hypocotyls with 25 ml g⁻¹ fresh weight (f.w.) of ethanol, which was successively eliminated by Rotavapor (Büchi, Italy). The residue was suspended in ethyl acetate and after centrifugation the supernatant was brought to dryness and finally suspended in water/ethanol (97:3, vol/vol). This material was loaded onto a 6 ml SPE-C18 column (Sigma Baker, Milano, Italy) equilibrated, washed, and eluted with pure methanol. The solvent was eliminated by Rotavapor and the residue diluted in ethanol represented the stock solution of glyceollin used for the treatment of *F. graminearum* strains (see below). The amount of glyceollin was determined by loading 25 µl of a 25-fold water-diluted sample of the stock solution on an ODS-Hypersil C-18 column (100 $\times$ 2.1 mm) connected to an Akta system (GE-Healthcare, Milano, Italy). The phytoalexin peak was eluted isocratically with water/acetonitrile (60:40) and detected at 280 nm. Glyceollin I was verified spectrophotometrically and the concentration was determined ($\epsilon = 10,300$ at A₂₈₆).

Determination of glyceollin in the inoculated soybean seedlings. The glyceollin I amount in soybean seedlings was determined after 3 days from inoculation with the WT and $\Delta Fgl1$, $\Delta tri5$, $\Delta FgOS-2$, and $\Delta gpmk1$ strains. The phytoalexin was extracted with 15 ml g⁻¹ f.w. of ethanol from 36 seedlings per strain divided in six samples each containing tissue fragments with visible lesions taken from six seedlings. A comparable amount of tissue was extracted also from noninfected control seedlings and

TABLE 1. List and description of gene functions disrupted in the mutants used in this paper

Gene name	Protein name	Function	Wild type of knock-out mutants	Reference
<i>Fgl1</i>	Extracellular lipase	Lipid metabolic process	8/1	39
<i>tri5</i>	Trichodiene synthase	First enzyme in the DON and NIV biosynthetic pathway	8/1	16
<i>FgOS-2</i>	Protein-kinase	Stress-activated protein kinase	PH1	29
<i>Gpmk1</i>	MAP kinase	Regulates the secretion of cell wall degrading enzymes	8/1	19
<i>xyl03624</i>	Endoxylanase	Degradation of xylan components of plant cell wall	PH1	36

from seedlings inoculated with the $\Delta gpmk1$ strain, which at this time did not display visible symptoms. A 250- μ l aliquot of each sample extract was dried, the residue suspended in 25 μ l of ethanol, diluted fourfold with water and a 25- μ l aliquot was loaded onto the reverse phase column (ODS-Hypersil C-18) as above reported. The glyceollin I peak was largely predominant and its level was estimated by plotting the area of the peak of each sample against a calibration curve obtained with known amounts (range 0.25 to 20 μ g) of glyceollin I purified as above reported. Data were statistically analyzed by analysis of variance (ANOVA) and Student-Newman-Keuls test.

Treatment of *F. graminearum* strains with glyceollin. The experiments were performed in wells of microtiter plates each filled with 200 μ l of PDB at pH 6.85 and glyceollin at 15, 30, 45, and 60 μ g ml⁻¹. The final concentration of ethanol in each well was at 2% (vol/vol). After addition of macroconidia of each strain to obtain a final concentration of 2×10^5 spores ml⁻¹, 68 μ g ml⁻¹ of resazurin dye (Sigma, Milano, Italy) was added to each well. Resazurin, which is the active component in Alamar Blue dye (30), is reduced to pink color in presence of actively growing cells and was previously used to evaluate *Botrytis cinerea* conidial germination (31). After 48 h of incubation in the dark at 24°C, the absorbance was read at 578 nm. The absorbance of the control treatment performed without glyceollin was subtracted from each value, and the growth inhibition was expressed as the percentage

of the ratio between net absorbance values of each sample and net absorbance of the medium with the resazurin dye but without the fungus. Every treatment with glyceollin was replicated from three to six times for each *F. graminearum* strain. Arcsine-transformed data were statistically analyzed by applying the Tukey-Kramer's test.

RESULTS

Soybean infection experiments. In a first set of experiments, soybean seeds were inoculated with 2×10^4 conidia per seed. After 6 days, the growth of seedlings inoculated with the WT strains was strongly compromised and extensive brown soft lesions affected seedlings for almost all their length (Fig. 1). Seedlings infected with the $\Delta tri5$ and $\Delta Fgl1$ mutants showed symptoms similar to those caused by the WT strain although the average length of lesions was 25% less (Figs. 1 and 2). Instead, the $\Delta FgOS-2$ mutant caused dark brown lesions with more defined borders or small scattered brown lesions, and the lesion length was about 70% less than that obtained with the WT. The $\Delta gpmk1$ mutant showed sporadic lesions and, when present, they were extremely restricted in size; therefore, this strain was considered avirulent (Figs. 1 and 2). The $\Delta xyl03624$ mutant was as virulent as the WT (Figs. 1 and 2) and therefore was not used in further experiments.

Fig. 1. Disease symptoms of representative soybean seedlings 6 days after inoculation. Two hundred microliters of spore suspension containing 2×10^4 conidia of the *Fusarium graminearum* wild type or mutant strains was dropped on each seed. The assay was performed with the rolled towel method.

Fig. 2. Disease severity index (DSI) determined on soybean seedlings 6 days after inoculation. Two hundred microliters of spore suspension containing 2×10^4 conidia of the *Fusarium graminearum* wild type or mutant strains was dropped on each seed. Experiments were repeated at least three times with two replicates each performed with 20 seeds and different conidial preparations. Therefore, each value is the mean of at least six repeats $\pm$ standard deviation performed with the rolled towel method. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

In order to determine if a reduced inoculum level could better highlight any differences among the mutant strains, experiments were performed with decreasing spore amounts: 1×10^4 , 0.5×10^4 , and 0.2×10^4 spores. At every spore dose the ranking of disease susceptibility was the same as obtained with 2×10^4 conidia per seed, i.e., $WT > \Delta tri5 = \Delta Fgl1 > \Delta FgOS-2 > \Delta gpmk1$ (Fig. 3). However, the greatest difference among the strains occurred for 1×10^4 conidia. With this dose, compared with WT, the $\Delta tri5$ and $\Delta Fgl1$ mutant strains showed a reduction of about 40% of symptom severity and $\Delta FgOS-2$ showed a reduction of about 85% (Fig. 3). The inoculation with the $\Delta gpmk1$ strain at 1×10^4 conidia showed no visible disease symptoms (data not shown).

Overall, as expected, extended lesions drastically reduced seedling growth and there was a close inverse relationship between the disease severity index and seedlings length, expressed as a percent of that of the noninoculated control (Fig. 4).

DON measured in infected tissues. DON concentration was determined on infected soybean seedlings by an enzymatic immunoassay at 6 days after inoculation with 2×10^4 conidia per seed. Compared with WT, which produced about 45 ppm of DON, tissues infected with the $\Delta Fgl1$ and $\Delta FgOS-2$ strains had about 60 and 85% decreases in DON content, respectively (Fig. 5). The $\Delta tri5$ mutant, which is unable to synthesize the trichothecene toxins, appeared positive for the DON assay although with very low readings at about 1 ppm (Fig. 5). However, this result was due to an incomplete specificity of the antibody of the immunoassay kit used since, as inferred by a high resolution LC-MS analysis, the DON level in a soybean seedling sample inoculated with the $\Delta tri5$ mutant was below the limit of quantification of the method (<0.025 ppm) while it was at 30 ppm in a WT inoculated sample.

Glyceollin determinations in infected seedlings. The glyceollin I content in soybean seedlings was determined at 3 days after inoculation of soybean seeds with *F. graminearum* strains. This time was chosen because, at a visual examination, the inoculated soybean began to show symptoms and appreciable differences in seedling elongation. Glyceollin strongly accumulated in hypo-

cotyls inoculated with the WT and with the $\Delta Fgl1$, $\Delta tri5$, and $\Delta FgOS-2$ mutated strains with concentration values of 440 to 724 $\mu\text{g g}^{-1}$ f.w. (Table 2). A very low amount of glyceollin was detected in the control and in seedlings inoculated with the avirulent $\Delta gpmk1$ strain. The high level of variability among the replicates, as indicated by the large SD values, was likely due to the different ratios of injured and healthy tissue in the soybean fragments harvested for glyceollin extraction and determination.

Effect of glyceollin on growth of *F. graminearum* strains. An experiment performed in vitro to observe possible differences in sensitivities to glyceollin showed that growth of WT, $\Delta tri5$, $\Delta Fgl1$, and $\Delta gpmk1$ strains was almost unaffected by glyceollin below 45 $\mu\text{g ml}^{-1}$ in culture, while a decrease in growth of 10 to 45% was observed only with the highest dose of 60 $\mu\text{g ml}^{-1}$ (Fig. 6). The $\Delta FgOS-2$ strain was most sensitive to glyceollin since its

Fig. 4. Relationship between the disease severity index (DSI) and the seedling length (expressed as percentage of the length of control seedlings) obtained from data of soybean infection experiment with *Fusarium graminearum* wild type and mutant strains shown in Figures 2 and 3. Pearson's correlation coefficient $r = -0.9864$.

Fig. 3. Disease severity index (DSI) determined on soybean seedlings 6 days after inoculation. Two hundred microliters of spore suspension containing 1×10^4 , 0.5×10^4 , or 0.2×10^4 conidia of the *Fusarium graminearum* wild type or mutant strains was dropped on each seed. Experiments were repeated at least three times with two replicates each performed with 20 seeds and different conidial preparations. Each value is the mean of at least six repeats $\pm$ standard deviation performed with the rolled towel method. At each conidial dose, data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

growth was reduced by 60% with 30 $\mu\text{g ml}^{-1}$ and was completely inhibited at 45 and 60 $\mu\text{g ml}^{-1}$ (Fig. 6).

DISCUSSION

A main issue with polyphagous fungal plant pathogens is assessing the relevance of pathogenicity or virulence factors on different hosts and plant tissues. For example, the mycotoxin DON is important for infection of *F. graminearum* in wheat spikes by favoring the fungus crossing through the rachis node; however, it seems dispensable for complete expression of symptoms in barley and maize inflorescences (24) and also in the model plant *Arabidopsis* (6). These findings may have practical implications since the increase of type II resistance (in contrast to

TABLE 2. Glyceollin I (μg of glyceollin g^{-1} fresh weight [f.w.]) measured in soybean tissues 3 days after inoculation with *Fusarium graminearum* wild type and mutant strains

<i>F. graminearum</i> strain	μg of glyceollin g^{-1} f.w. ^z
WT-8/1	690 ± 187 a
WT-PH1	670 ± 91 a
$\Delta Fgl1$	724 ± 135 a
$\Delta tri5$	440 ± 96 b
$\Delta FgOS-2$	588 ± 119 ab
$\Delta gpmk1$	34 ± 43 c
Mock-inoculated seedlings (control)	16 ± 12 c

^z Mean value ± standard deviation of six determinations, each performed on extracts from fragments collected from six soybean seedlings. Data were statistically analyzed by analysis of variance. Different letters indicate statistically significant differences at $P < 0.01$ (Student-Newman-Keuls test).

Fig. 5. Deoxynivalenol (DON) content (ppm) measured in the soybean seedlings 6 days after inoculation performed with 2×10^4 conidia/seed of the *Fusarium graminearum* wild type or mutant strains. Each DON value, measured by using the AgraQuant deoxynivalenol enzymatic immunoassay (Romer Labs), is the mean of at least four replicates ± standard deviation, each performed with about 0.5 g fresh weight of seedling tissue. Data were statistically analyzed by applying the Tukey-Kramer’s test. Different letters indicate significant differences at $P < 0.05$.

Fig. 6. Growth inhibition of the *Fusarium graminearum* wild type and mutant strains by a purified preparation of glyceollin at 15, 30, 45, and 60 $\mu\text{g ml}^{-1}$. Two hundred microliters of potato dextrose broth in wells of microtiter plates and containing the vital dye resazurin was inoculated with 2×10^5 spores ml^{-1} and incubated at 24°C for 48 h. Growth was determined by measuring spectrophotometrically the absorbance at 578 nm and subtracting the value of the correspondent control well without glyceollin. Growth inhibition was expressed as the percentage of the ratio between net absorbance values of each sample and net absorbance of the culture medium with the resazurin dye but without the fungus. Data are the mean of three to six experiments ± standard deviation. At each glyceollin dose, the asterisk indicates a significant statistically different value at $P < 0.05$ by applying the Tukey-Kramer’s test.

infection spreading in the rachis [35]) may be useful against FHB in wheat but not in other crops. DON also contributes to infection of rye and oat seedlings while its effect seems dispensable in development of seedling blight and crown rot disease in maize and wheat (28,33). However, Mudge et al. (28) inoculated wheat plantlets with *tri5* *F. graminearum* mutants and demonstrated that DON contributed to asymptomatic stem colonization.

In North and South America there is evidence of soybean as a host of *F. graminearum*. In this plant the fungus causes damping-off and crown and root rots at the early phase of seedling development, and diffusion of the fungus inoculum is favored by cultural practices (3,7,32,40).

The aim of this paper was to establish the behavior in soybean of some *F. graminearum* mutants disrupted in specific gene function and previously characterized for virulence in wheat spike infection. The outcome was unpredictable as soybean and wheat belong to different classes of plants, and also because in wheat most studies with *F. graminearum* mutant strains were performed during spike infection. However, when wheat spike and seedling infections were done concurrently, some differences in virulence were observed in relation to the organ infected (42).

The disrupted mutants used in the soybean assay were affected in DON ($\Delta tri5$), lipase ($\Delta Fgl1$), or xylanase ($\Delta xyl03624$) production, or in two MAP kinase (MAPK) signaling pathways regulating production of cell wall degrading enzymes ($\Delta gpmk1$) and osmotic balance ($\Delta FgOS-2$), respectively, and possibly other unknown virulence genes. A seedling assay, successfully developed to compare virulence of strains collected from the field and to assess fungicidal treatments (10), was used to compare the mutants.

The first apparent result was that behavior of the mutants observed in soybean mirrored that observed in wheat. In fact, the mutant strains that show reduced virulence (i.e., $\Delta tri5$, $\Delta Fgl1$, and $\Delta FgOS-2$), or avirulence (i.e., $\Delta gpmk1$) (17,18,29,39) compared with the WT in wheat spike infection, were correspondingly less virulent ($\Delta tri5$, $\Delta Fgl1$, and $\Delta FgOS-2$) or avirulent ($\Delta gpmk1$) in soybean seedlings. Accordingly, the $\Delta xyl03624$ mutant, which is virulent in wheat (36), was also fully virulent in soybean. However, there was a different ranking of symptom severity in the two hosts. In wheat the $\Delta tri5$, $\Delta Fgl1$, and $\Delta FgOS-2$ mutants give similar reductions of symptom severity compared with the WT (17,29,39), while in soybean $\Delta FgOS-2$ was clearly less virulent than the $\Delta tri5$ and $\Delta Fgl1$ mutants. In fact, in wheat, following inoculation of a single floret, the $\Delta tri5$, $\Delta Fgl1$, and $\Delta FgOS-2$ mutants colonize only the inoculated spikelet but rarely proceed further along the spike (17,29,39). In soybean the $\Delta tri5$ and $\Delta Fgl1$ strains produced lesions quite similar in appearance to those produced by the WT, although reduced in length; while the $\Delta FgOS-2$ mutant produced dark brown lesions and more restricted symptoms. The anatomy of the infected organs in wheat and soybean may at least partially explain the different symptoms caused by the mutants. In wheat the rachis node represents a histological barrier that prevents the $\Delta tri5$ and $\Delta Fgl1$ mutants from further colonizing the spike, and DON or lipase have been demonstrated to be necessary to overcome this barrier (17,39). Even the $\Delta FgOS-2$ mutant had down-regulated production of DON (29), as also measured during soybean infection, which may explain its inability to infect the entire spike. However, in soybean the absence of discontinuity between root and hypocotyl tissues allows the progress of infection of the $\Delta tri5$ and $\Delta Fgl1$ mutants and, therefore, the lack of DON and lipase seemed to only slow the colonization of seedlings. Since, in wheat, lipase and DON are thought to suppress different plant defense reactions, possibly they also play a similar role in soybean, even if the type of plant defense responses may differ. However, the reduced DON production of the $\Delta FgOS-2$ mutant in soybean cannot explain alone the reduced virulence of this mutant. In fact, the $\Delta FgOS-2$ showed much less extended lesions than $\Delta tri5$, which is completely unable to produce DON. Looking for alternative explanations, we

checked whether the mutant strains allowed a different accumulation in infected tissues of glyceollin I, the major phytoalexin in etiolated soybean seedlings, which has long been regarded and also recently supported as a candidate resistance factor against some oomycetes and fungi (2,9,23,25). However, there were no macroscopic differences in glyceollin I level in infected seedlings when disease symptoms caused by the fungal strains became appreciable. Only in seedlings inoculated with the $\Delta gpmk1$ strain was glyceollin present in traces; however, at the time of tissue sampling (3 dpi), disease symptoms were not detectable because this mutant is strongly impaired in tissue colonization. Instead, we observed that, compared with WT and other mutants, the $\Delta FgOS-2$ was several-fold more sensitive to glyceollin. Since the levels of glyceollin measured in soybean seedlings were about 10-fold those demonstrated as inhibitory in vitro against the $\Delta FgOS-2$ mutant, a role of this phytoalexin in contrasting the development of this strain in infected tissue is likely. Therefore, the *F. graminearum* FgOS-2 protein kinase, an ortholog of yeast HOG1 stress-related MAPKs (15), seems necessary for inducing tolerance to this plant phytoalexin, and the increased sensitivity to glyceollin of the $\Delta FgOS-2$ mutant may explain some of the impaired virulence of this strain in soybean. Recently, it was reported that Hog1-type MAPKs mutants of *Alternaria brassicicola* are hypersensitive to the Arabidopsis camalexin (20). Camalexin and glyceollin are produced through an indole and pterocarpan pathway, respectively (1). Although these substances are chemically different, a target on the fungal membrane surface may activate the HOG pathway which seems to regulate a common mechanism responsible for tolerance to host phytoalexins. It is possible that Hog1-type MAPKs mutants cannot metabolize the phytoalexin or are impaired in its rapid transport out of the cell. In fact, ABC transporters are involved in resistance to phytoalexins as shown for *F. sambucinum* infecting potato (13).

The avirulence of the $\Delta gpmk1$ strain in wheat was also confirmed in soybean, where very small and rare necrotic lesions were observed only with the higher conidia dose. This strain did not show an increase in sensitivity to the soybean phytoalexin but was reported by Ramamoorthy et al. (34) as more sensitive to the plant defensin Msdef1; additionally, this mutant is severely impaired in production of extracellular enzymes including lipase (19). Therefore, Gpmk1, a key component of the renamed Kss1/Fus3-Type MAPKs cascade (15), has pleiotropic effects on the fungal physiology related to plant infection which could explain the drastic reduction of virulence both in wheat and soybean. This result is in agreement with an increasing amount of evidence showing that orthologs of *gpmk1/Kss1* gene play an essential role for fungal pathogenicity in different pathosystems (15).

In conclusion, although wheat spike and soybean seedlings are two very different experimental systems, this study showed that *F. graminearum* DON and lipase, which are responsible for full virulence of the fungus in wheat, also contribute to symptom development in soybean. However, the role of DON and FGL1 lipase in the disease development seems to be reduced in soybean in comparison to that observed in wheat spike, where these fungal factors are essential for the spreading of the disease along the spike. Instead, as shown in wheat, *F. graminearum* OS-2 and Gpmk1 MAPKs are essential factors for the progress of infection and symptom development in soybean where OS-2 MAPK also plays an important role providing resistance to the soybean phytoalexins. It should therefore be interesting to investigate whether some antifungal mechanisms raised in soybean against *F. graminearum*, such as phytoalexin accumulation, have similar mechanisms in wheat.

ACKNOWLEDGMENTS

We thank S. Odorizzi for glyceollin determinations and G. Fusco (Syngenta, Italy) for providing the soybean seeds. Research was sup-

ported by “ProgrammaVigoni” (2009 to 2011) funded by the Italian Ministry of University and Research and the Deutscher Akademischer Austauschdienst.

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