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e-Xtra*

Synergistic Effect of Different Plant Cell Wall–Degrading Enzymes Is Important for Virulence of *Fusarium graminearum*

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Endo-polygalacturonases (PGs) and xylanases have been shown to play an important role during pathogenesis of some fungal pathogens of dicot plants, while their role in monocot pathogens is less defined. *Pg1* and *xyr1* genes of the wheat pathogen *Fusarium graminearum* encode the main PG and the major regulator of xylanase production, respectively. Single- and double-disrupted mutants for these genes were obtained to assess their contribution to fungal infection. Compared with wild-type strain, the Δpg mutant showed a nearly abolished PG activity, slight reduced virulence on soybean seedlings, but no significant difference in disease symptoms on wheat spikes; the Δxyr mutant was strongly reduced in xylanase activity and moderately reduced in cellulase activity but was as virulent as wild type on both soybean and wheat plants. Consequently, the ΔpgΔxyr double mutant was impaired in xylanase, PG, and cellulase activities but, differently from single mutants, was significantly reduced in virulence on both plants. These findings demonstrate that the concurrent presence of PG, xylanase, and cellulase activities is necessary for full virulence. The observation that the uronides released from wheat cell wall after a *F. graminearum* PG treatment were largely increased by the fungal xylanases suggests that these enzymes act synergistically in deconstructing the plant cell wall.

For most necrotrophic fungi, an important role in pathogenesis is played by enzymes degrading the plant cell wall (Cooper et al. 1988), a physical barrier that pathogens have to overcome to penetrate and colonize the host tissue and obtain nutrients. Among cell wall–degrading enzymes (CWDEs), endo-polygalacturonases (PGs) (EC 3.2.1.15) hydrolyze the homogalacturonan pectic polymers of the cell wall by cleaving the internal α-1,4-D-galacturonic acid backbone and are expressed in the early stages of host infection (Reignault et al. 2008). Although several authors have ruled out an involvement of pectinolytic enzymes in pathogenicity (Di Pietro and Roncero, 1998; Gao et al. 1996; Scott-Craig et al. 1998), pectic enzymes were demonstrated as important virulence factors of some fungal pathogens, mostly infecting dicotyledonous plants (Isshiki et al. 2001; Kars et al. 2005; Shieh et al. 1997; ten Have et al. 1998) that have a cell wall rich in pectin (Carpita and Gibeaut 1993). In grasses that possess a cell wall with a small amount of pectin (Meineke et al. 2014; Vogel 2008), only the PG activity of *Claviceps purpurea*, an ovary-infecting pathogen causing ergot disease in rye, was shown as a pathogenicity factor (Oeser et al. 2002).

Endo-1,4-β-D-xylanase (EC 3.2.1.8) (further referred to as endo-xylanase) is another class of CWDEs involved in the hydrolysis of xylan. Xylan is a major component of hemicelluloses and is particularly abundant in the primary cell wall of monocotyledonous plants (Meineke et al. 2014; Vogel 2008), in which it forms a complex structure composed of a D-xylose backbone linked by β-1,4 bridges. Endo-xylanases break down the xylan backbone by catalyzing the hydrolysis of the β-1,4 linkages and can play an important role during plant infection, not only by degrading the cell wall xylan but, also, by inducing necrosis in the host tissues independently from their enzymatic activity (Brito et al. 2006; Noda et al. 2010; Sella et al. 2013). However, only the xylanases of some fungi have been shown as virulence factors (Brito et al. 2006; Yu et al. 2016), while others were demonstrated to be dispensable in pathogenesis (Apel et al. 1993; Apel-Birkhold and Walton 1996; Gómez-Gómez et al. 2002; Wu et al. 1997).

F. graminearum is the main causal agent of *Fusarium* head blight (FHB), a devastating disease that commonly affects the ear of cereals such as wheat, barley, and other small grains (Goswami and Kistler 2004) and is also responsible for root and collar rot of soybean seedlings (Pioli et al. 2004). The infection of wheat spikes occurs at flowering, when sexual or asexual spores of the fungus arrive on spikelets carried by wind and rain. Spores germinate and penetrate into the host tissue by exploiting the natural openings of the ovary and at the bottom of the lemma and palea (Bushnell et al. 2003; Pritsch et al. 2001) or by actively penetrating the epidermal cells through hyphae, infection cushions, and lobate appressoria (Boenisch and Schäfer 2011). After floral invasion, intercellular hyphae spread throughout the spikelet down into the rachis node and, subsequently, systemically through the spike (Brown et al. 2010). A histological study of the *F. graminearum* infection process showed that, in the first stages of the infection process, the fungus seems to establish a biotrophic interaction with the host plant, switching to necrotrophy at later infection stages (Brown et al. 2012). In fact, *F. graminearum* secretes in the

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infected grains toxic secondary metabolites such as the trichotecenes, among which is deoxinivalenol (DON), a mycotoxin dangerous for human and animal health (Goswami and Kistler 2004). In particular, DON induces cell death (Desmond et al. 2008) and is a *F. graminearum* virulence factor, since it has been shown to be crucial for the spread of the fungus within the wheat spike (Bai et al. 2001).

The characterization of *F. graminearum* genes involved in virulence or pathogenicity is an essential step for better understanding the mechanisms of fungal pathogenesis. In particular, this fungus is known to produce high amounts of pectinases and xylanases during the infection process (Kikot et al. 2009; Wanyoike et al. 2002).

In an analysis of the *F. graminearum* genome database, two genes (Munich Information Center for Protein Sequences [MIPS] database entries FGSG_11011 and FGSG_03194) encoding for endo-PGs have been previously identified and have been investigated for their expression during wheat spike infection (Tomassini et al. 2009). These analyses showed that transcription of both *Fgpg* genes occurs within the first 12 h after spike inoculation and peaks at 24 h (Tomassini et al. 2009). The PG isoforms encoded by these two genes (named PG1 and PG2) are secreted both in vitro and in vivo in the early stages of wheat infection, with the activity of PG1 largely exceeding that of PG2 (Tomassini et al. 2009). In particular, the secretion of a high amount of PG1 in the wheat ovary (Tomassini et al. 2009) seems consistent with the characteristic composition of this tissue. In fact, *F. graminearum* appears to colonize the ovary within 12 h (Miller et al. 2004) and homogalacturonans and methyl-esterified homogalacturonans have been shown to be abundant constituents of the ovary cell wall in grasses (Chateigner-Boutin et al. 2014; Tenberge et al. 1996). Thus, the degradation of the spikelet soft tissue may be achieved with the contribution of PG activity. An indirect demonstration of the possible role of *F. graminearum* PG activity during wheat infection is represented by the reduction of disease symptoms caused by the fungus on transgenic wheat plants expressing a PG-inhibiting protein (PGIP) effective against PG1 (Ferrari et al. 2012). Moreover, a high degree of pectin methyl esterification can reduce PG activity and transgenic wheat plants with an increased level of pectin methyl esterification showed reduced FHB symptoms (Volpi et al. 2011). Consistently, *F. graminearum* pectin methyl esterase activity contributes to fungal virulence on wheat spike, likely favoring PG activity (Sella et al. 2016a).

The first aim of the present work was to evaluate the possible contribution to pathogenicity of the *F. graminearum* PG1. Disrupted mutant strains of the corresponding encoding gene (*Fgpg1*) were obtained by targeted homologous recombination and were characterized both in vitro and by infection experiments on wheat spikes and soybean seedlings.

The second aim of this work was to verify a possible cooperative effect of *F. graminearum* PG and xylanase activities in the degradation of the plant cell wall during plant infection. Previously, *F. graminearum* strains disrupted in the *xyr1* gene, a transcriptional regulator of xylanase genes, showed a dramatic reduction of xylanase activity and a partial reduction of cellulase activity but an unmodified virulence, compared with the wild type (WT), on *Triticum aestivum*, *T. durum*, and *Glycine max* (Sella et al. 2016b). Therefore, a *F. graminearum* double knock-out mutant of *pg1* and *xyr1* genes was produced and the obtained ΔpgΔxyr strains were tested by infection experiments of soybean and wheat, in order to verify the effect of the simultaneous disruption of these two genes on fungal virulence. To confirm the synergistic effect between different types of CWDE, the activity of the purified *F. graminearum* PG1 was tested on wheat cell walls in the presence of two purified fungal xylanases.

RESULTS

Production of *F. graminearum* Δpg disruption mutant.

The *F. graminearum* WT (strain 3827) was transformed with a specific construct to obtain the targeted integration of the hygromycin resistance gene replacing the PG1 coding sequence. After a preliminary check by polymerase chain reaction (PCR), the 35 transformants obtained were subjected to single conidiation. Gene disruption was confirmed by PCR (Supplementary Fig. S1) and, on three of them, by high-stringency Southern blot analysis (Supplementary Fig. S2). In particular, two strains, labeled Δpg 2.8 and Δpg 2.13, showed a single homologous integration of the disruption construct, while the Δpg 2.3 strain showed an additional ectopic integration of the hygromycin resistance gene.

To check whether this mutation impair mycelium growth, mutant strains were transferred to potato dextrose agar (PDA) plates and to complete medium (CM) liquid medium, but no difference in growth was observed, compared with WT strain (data not shown).

In vitro characterization of the Δpg mutant strains.

The PG activity secreted by the three Δpg mutant strains in liquid culture containing pectin as the sole carbon source was tested by a viscosimetric assay, after 4 days of growth. Compared with WT, the three Δpg mutant strains showed a reduction of total PG activity by about 90% (Fig. 1).

A polygalacturonic acid sodium salt substrate (PGA) agarose overlay assay, performed after an isoelectric focusing (IEF) analysis of aliquots of concentrated liquid cultures, confirmed that PG1 is strongly produced by the WT strain, while PG2 is almost undetectable. A faint band close to PG1 was also visible in the WT culture sample. Instead, the *F. graminearum* Δpg 2.13 mutant showed the faint PG2 isoform and the absence of the PG1 band, including the minor isoforms near PG1 (Fig. 2).

The presence of the PG1 isoform was further investigated in infected wheat ovaries by a gel activity assay performed by loading ground ovaries collected 4 days after wheat spikelet inoculation. The PG1 isoform was clearly present in the WT strain (Supplementary Fig. S3) but was undetectable in ovary tissue infected with the Δpg 2.13 mutant. No PG band was visible when loading ground healthy ovaries.

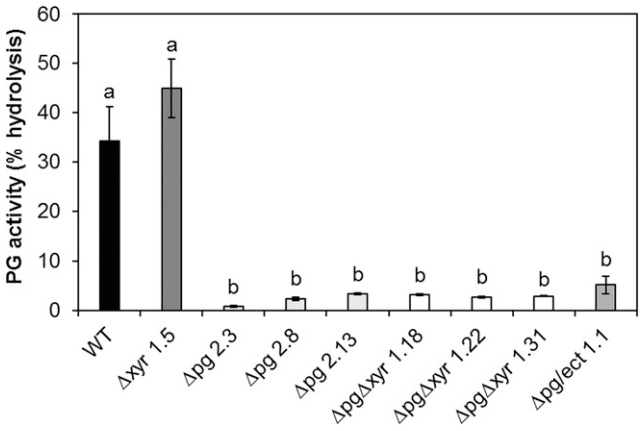

Fig. 1. Polygalacturonase (PG) activity produced by *Fusarium graminearum* wild type (WT) and mutants in liquid culture with 0.5% (wt/vol) pectin as sole carbon source. PG activity was measured by the viscosimetric assay at pH 6 and was expressed as percentage of hydrolysis of the substrate (0.25% wt/vol pectin) after 30 min of incubation at 30°C. Each datapoint represents the mean ± standard error of at least two biological replicates. Average values were significantly different according to the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

We also evaluated the dry weight obtained by growing WT and Δpg mutant strains in liquid culture with pectin as the sole carbon source. After 7 days of culture, no significant difference between WT and mutant strains was observed (data not shown and Figure 3A).

Plant infection experiments with the Δpg mutant strains.

To determine whether the *F. graminearum pg1* gene is involved in virulence, infection experiments of soybean seedlings and wheat spikes were performed (Fig. 4A and B). While, on soybean, the virulence of the mutant strains was significantly reduced compared with the WT strain by about 30% (Fig. 4A), on wheat spikes, the virulence of WT and the three Δpg strains was comparable at 21 days postinoculation (dpi) (Fig. 4B) and, also, at 10 dpi (data not shown). Since no significant difference in virulence between the three Δpg strains was observed, one of them, the Δpg 2.13 strain, was selected to produce the double mutant ΔpgΔxyr.

Production of the *F. graminearum xyr1* disruption mutant on the 3827 strain background.

A *Fgxyr1* deletion mutant was produced on the WT 3827 strain with the same construct used by Sella et al. (2016b) for obtaining the Δxyr mutant on the PH1 strain background. Several mutant strains were obtained and were screened by PCR for the absence of the *Fgxyr1* gene, and the disruption was confirmed in three of them (Supplementary Fig. S4). Enzymatic assays and infection experiments with the Δxyr 1.5 strain confirmed the results previously obtained with the Δxyr mutant in the PH1 strain background (Sella et al. 2016a), with about 75% reduction of xylanase activity (Fig. 5) and 90% reduction of dry weight on xylan containing medium (Fig. 3B) and a virulence comparable with WT on both soybean and wheat (Fig. 4A and B).

Fungal transformation to obtain the ΔpgΔxyr mutant.

To obtain the *Fgxyr1* gene disruption on the Δpg background, protoplasts of the *F. graminearum* Δpg 2.13 strain were transformed with a construct containing a geneticin resistance gene (*Gen*) as a selectable marker to replace the *Fgxyr1* gene. In total, 40 geneticin-resistant colonies were obtained and three were selected by PCR for the disruption of the gene of interest (Supplementary Fig. S5). After single conidium isolation of these transformants, Southern blot analysis was performed, using a probe specific for the *Gen* gene (Supplementary Fig. S6). The geneticin probe gave no hybridization signal for the WT and Δpg strains, while the double knock-out mutant strains tested (ΔpgΔxyr 1.18, ΔpgΔxyr 1.22, and ΔpgΔxyr 1.31) showed a single hybridization signal of the expected size of 8.3 kb. A Δpg strain transformed with the *Fgxyr1* replacement construct showed a *Gen* hybridization signal higher than that of the expected size, indicating an ectopic integration of the construct. This strain was labeled Δpg/ect 1.1 and was used as a negative Δxyr control of ΔpgΔxyr strains.

In vitro characterization of the ΔpgΔxyr strains.

The double mutant strains confirmed by PCR and Southern blot analysis were grown on PDA and CM agar plates; no difference was observed compared with WT and single mutant strains (data not shown). To confirm the effective disruption of the *Fgxyr1* transcriptional regulator gene in the double mutant strains, total xylanase activity produced after 3 days of culture in a liquid medium containing xylan as sole carbon source was determined on xylan substrate, according to the dinitrosalycilic (DNS) method. As expected, the xylanase activity produced by the ΔpgΔxyr strains as well as by the Δxyr strain was about 75% less than that produced by the WT and Δpg strains (Fig. 5).

PG activity produced by the double mutant strains in liquid cultures with pectin as sole carbon source was first determined by radial gel diffusion assay. While the WT and the Δxyr mutant

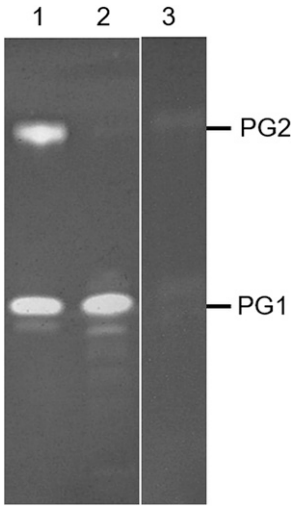

Fig. 2. Thin-layer isoelectric focusing in the pH range of 6.0 to 10.5 of the polygalacturonase (PG) activity produced by *Fusarium graminearum* wild type (WT) and mutant strains in liquid culture containing pectin as the sole carbon source. Four-day-old liquid cultures were filtrated, concentrated, and dialyzed. When compared with the WT strain (lane 2), the Δpg 2.13 mutant (lane 3) produced only PG2 isoform, barely visible in both strains. Purified PG1 and PG2 standards were loaded on lane 1.

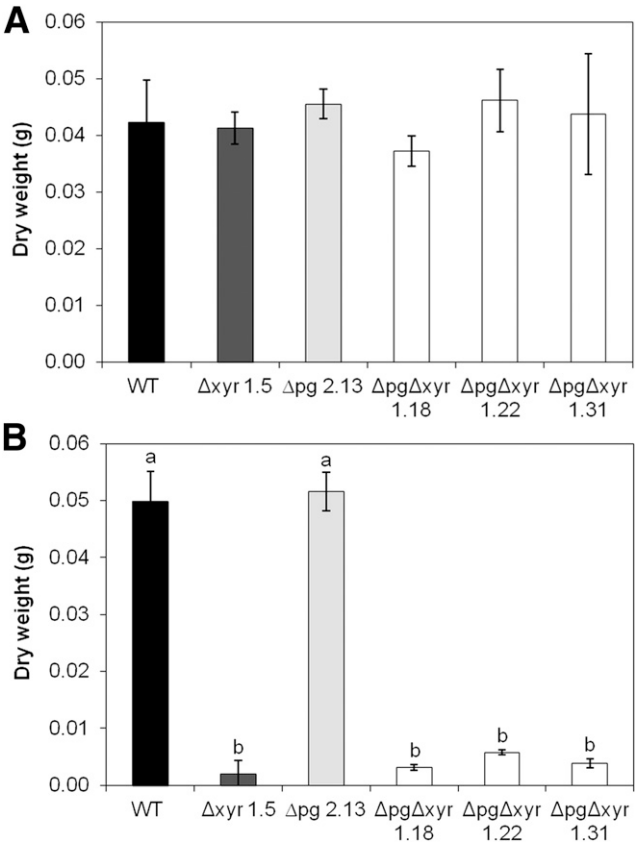

Fig. 3. Dry weight of wild type (WT) and mutant strains grown for 7 days in a liquid culture containing **A,** pectin or **B,** xylan as the sole carbon source. Data represent the average ± standard error (indicated by bars) of three independent experiments, each one performed by weighing the mycelium from three flasks per strain. Average values were significantly different only on xylan medium, according to the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

strain produced a halo of PG activity corresponding to about 1.3 agarose diffusion units (U), Δpg and ΔpgΔxyr strains did not produce any visible halo (Supplementary Fig. S7). The loss of PG activity in the double mutant was confirmed by the viscosimetric assay, with a reduction of PG activity of about 90% compared with WT (Fig. 1).

Since it was previously shown that the Δxyr mutant is also partially impaired in cellulase activity (Sella et al. 2016b), the ΔpgΔxyr mutant strains were grown for 3 days in a liquid medium containing carboxymethyl cellulose as sole carbon source. According to the DNS method, the Δxyr mutant and the double mutant strains produced about 50% less cellulase activity compared with WT (Fig. 6).

Growth experiments were also performed by inoculating 1×10^4 conidia per milliliter of WT and mutant strains in 20 ml of Szécsi medium (Szécsi 1990) supplemented with pectin or xylan as sole carbon source. In the medium containing pectin, no significant difference in dry weight between WT and mutant strains was observed (Fig. 3A), while there was a significant dry weight reduction of about 90% when the ΔpgΔxyr mutant strains were grown on xylan (Fig. 3B), similarly to what was observed with the Δxyr mutant.

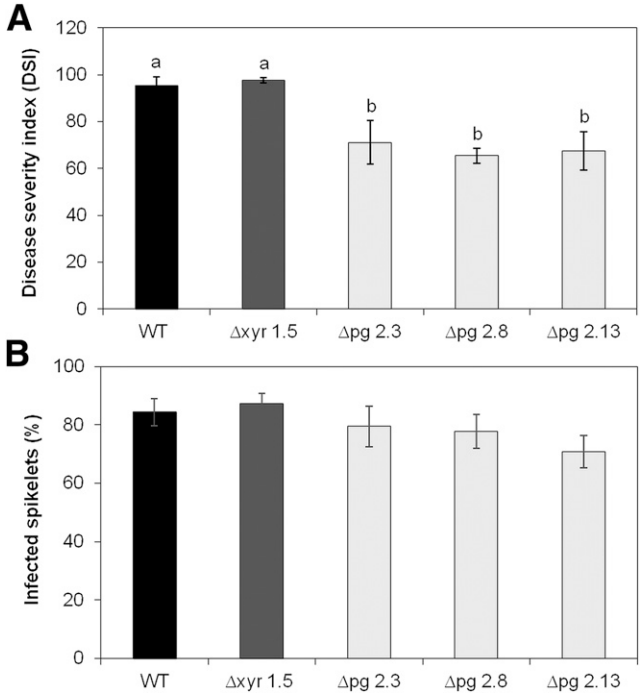

Fig. 4. Infection of soybean seedlings (cv. Demetra) and wheat spikes (cv. Bobwhite) with *Fusarium graminearum* wild type (WT) and single mutant strains. **A,** Spore suspension (200 µl) containing 2×10^4 conidia of the *F. graminearum* WT or mutant strains were dropped on each soybean seed, and disease symptoms, expressed as disease severity index, were assessed on seedlings at 6 days postinoculation (dpi). Bars represent the mean ± standard error of at least two independent infection experiments performed with the rolled towel method. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$. **B,** Spore suspension (10 µl) containing 2,000 conidia of the *F. graminearum* WT or mutant strains were pipetted between the glumes of two florets of two opposite central spikelets. Disease symptoms were assessed at 21 dpi by counting the number of visually diseased spikelets. Infected spikelets are expressed as percentage of symptomatic spikelets on the total number of spikelets of the respective head. Data represent the average ± mean standard error (indicated by bars) of at least four independent experiments performed inoculating at least eight plants with each strain. Data were statistically analyzed by applying the Tukey-Kramer's test.

Mutant strains retain the ability to form infection structures.

The *F. graminearum* WT, Δpg 2.13, and ΔpgΔxyr 1.22 mutant strains were transformed to constitutively express the green fluorescent protein (GFP), in order to localize the fungus in the wheat tissue. Transformants were selected by PCR and were analyzed by fluorescence microscopy to confirm the GFP expression (data not shown). The GFP mutants were then used in wheat infection experiments; in particular, paleas were inoculated with 250 conidia and were incubated in petri dishes at 23°C. After 5 days, the mutant strains were analyzed by fluorescence microscopy and still retained the ability to produce infection cushions and lobate appressoria like the WT strain (Supplementary Fig. S8).

Plant infection experiments with the ΔpgΔxyr mutant.

Wheat spikes of *T. aestivum* were inoculated at anthesis with a spore suspension (200 conidia) of WT, ΔpgΔxyr, Δpg, and Δpg/ect 1.1 strains. The Δxyr was not included in these experiments because it behaves as the WT (Fig. 4B). At 21 dpi, the ΔpgΔxyr strains (ΔpgΔxyr 1.18, ΔpgΔxyr 1.22, ΔpgΔxyr 1.31) showed a percentage of infected spikelets significantly lower by about 60% than that of the WT and about 50% lower than that of the Δpg strain. The magnitude of the disease symptoms caused by the Δpg mutant was not significantly different from that of the WT (Fig. 7; Supplementary Fig. S9). As expected, the virulence of the Δpg containing ectopically the *Fgxyr1* replacement construct (Δpg/ect 1.1) was similar to that of the Δpg mutant.

Infection experiments were also performed by inoculating soybean seeds with 2×10^4 conidia of the different strains. Symptoms were measured on seedlings at 6 dpi and were expressed as the disease severity index (DSI). The DSI caused by the ΔpgΔxyr strains was significantly lower, about 65%, than that of the WT and about 40% lower than that of the Δpg and Δpg/ect 1.1 mutants (Fig. 8).

Synergistic effect of *F. graminearum* PG and xylanase activities.

To verify if the *F. graminearum* PG and xylanase activities have a synergistic effect in degrading the plant cell wall, the

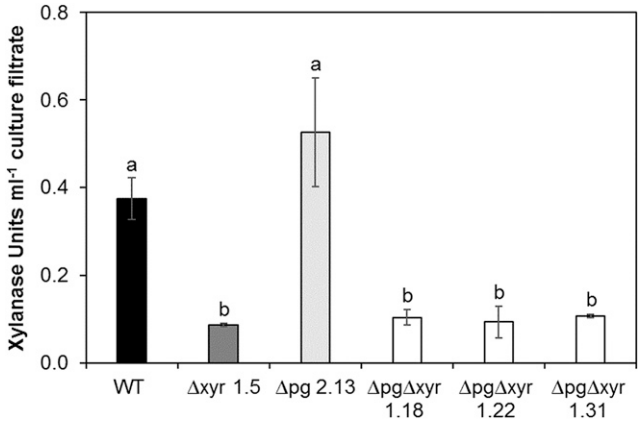

Fig. 5. Xylanase activity produced by *Fusarium graminearum* wild type (WT) and mutant strains. Culture filtrates (50 µl), collected after 3 days of culture in Szécsi medium (Szécsi 1990) with 0.5% xylan as the sole carbon source, were incubated in 1 ml of reaction mixture containing 0.5% (wt/vol) beechwood xylan. Xylanase activity, measured by dinitrosalycilic assay, was expressed as xylanase units per milliliter of culture filtrate, defining one xylanase unit as the amount of enzyme required to release 1 µmol of xylose in 1 min. Data represent the average ± standard error (indicated by bars) of two independent experiments, each one performed using two flasks per strain. Average values were significantly different according to the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

main PG isoform (PG1) and the two most expressed xylanases (FGSG_10999 and FGSG_03624) of *F. graminearum* were incubated separately and together in the presence of wheat cell wall. The amount of uronides released by mixing together the two enzymatic activities was about 35% higher than the sum of the uronides released by the two enzymatic activities tested separately (Fig. 9).

DISCUSSION

Gene disruption is a genetic technique useful to investigate the role of specific factors produced by pathogenic organisms during plant infection and to determine their contribution to the development of disease symptoms. By using this approach, we assessed the contribution to plant infection of the CWDEs endo-PGs and endo-xylanases produced by the fungal pathogen *F. graminearum.*

In culture supplemented with pectin and in infected wheat ovaries, this fungus secreted high levels of PG activity, in particular of an endo-PG isoform named PG1 (Tomassini et al. 2009). In order to directly establish the role of the *F. graminearum* PG1 activity during plant infection, three Δpg mutant strains were obtained and characterized. The mutants showed a strongly impaired PG activity both in vitro and in vivo on infected wheat ovaries, but they were able to grow like the WT on pectin-containing medium. Infection experiments showed a different behavior of the mutant according to the host used in the infection assay. In fact, although these mutant strains were drastically reduced in their capacity to produce PG activity compared with the WT, their virulence was slightly reduced only in soybean seedlings. The dispensability of the *F. graminearum* PG1 activity for fungal virulence on wheat and its significant contribution to soybean symptoms is in agreement with the different content in pectin of these tissues; in fact, pectin is known to be more abundant in the cell wall of dicots, such as soybean, than in graminaceous monocots, such as wheat (Vogel 2008). The results obtained in wheat with the Δpg mutant apparently contrast with the improvement of resistance to FHB obtained with the transgenic PGIP plants (Ferrari et al. 2012). Therefore, other factors such as an alteration of the cell-wall composition could contribute to the reduction of FHB symptoms in the PGIP transgenic plants (Joubert et al. 2006; Tundo et al. 2016).

During infection of wheat spikes, *F. graminearum* expresses, at high levels also, several endo-xylanase–encoding genes (Sella et al. 2016b), and wheat transgenic plants constitutively expressing TAXI-III, a *T. aestivum* xylanase inhibitor, show a delay of FHB symptoms (Moscetti et al. 2013). These observations suggest that the *F. graminearum* xylanase activity could be involved in fungal virulence on wheat spikes. Indeed, a recent paper showed that the deletion of the *F. graminearum* FGSG_10999 xylanase, one of the most expressed during wheat spike infection (Sella et al. 2016b), produced a mutant strongly reduced in virulence (Sperschneider et al. 2016). In contrast, the targeted gene replacement of a *F. graminearum* transcriptional regulator of the xylanase genes (XYR1) in the PH1 strain of *F. graminearum* produced a mutant strongly impaired in xylanase activity but with a virulence comparable to the WT strain on both soybean seedlings and wheat spikes (Sella et al. 2016b). This result was unexpected in wheat because the cell wall of this plant is particularly rich in glucuronoarabinoxylans (Meineke et al. 2014; Vogel 2008). The unaltered virulence of the *Fgxyr1* gene disruption mutant is confirmed here also with a Δxyr mutant of the *F. graminearum* 3827 strain, i.e., the same isolate used for the targeted disruption of the *Fgpg1* gene. Our result is in agreement with those obtained by disrupting the *Xlr1* gene of *Magnaporthe oryzae*, a fungal pathogen of the monocot species rice, and the *XlnR* gene of *F. oxysporum* (Battaglia et al. 2013; Calero-Nieto et al. 2007) and seems to rule out a role of *F. graminearum* xylanases during plant infection, although we cannot exclude that the residual xylanase activity of the Δxyr mutant may be sufficient to support fungal infection.

Overall, our findings indicate that the lack of PG or xylanase activity does not affect *F. graminearum* virulence on wheat spike, while PG1 activity is required for full virulence on soybean. The result obtained in wheat is in accordance with the unaffected virulence observed in several fungal mutants with single CWDE-encoding genes disrupted (Apel et al. 1993; Di Pietro and Roncero 1998; Gao et al. 1996; Gómez-Gómez et al. 2002; Scott-Craig et al. 1998; Wu et al. 1997). However, PG and xylanase may have an overlapping role, whereby one or the other

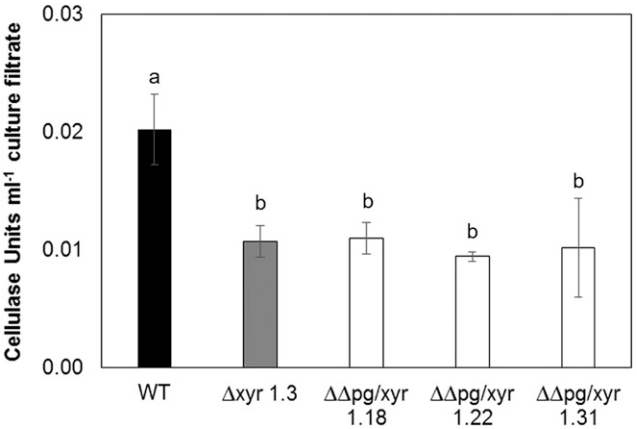

Fig. 6. Cellulase activity produced by *Fusarium graminearum* wild type (WT) and mutant strains. Culture filtrates (100 µl), collected after 3 days of culture in Szécsi medium (Szécsi 1990) with 0.5% carboxymethyl cellulose (CMC) as the sole carbon source, were incubated in 1 ml of reaction mixture containing 0.5% (wt/vol) CMC. Cellulase activity, measured by dinitrosalycilic assay, was expressed as cellulase units per milliliter of culture filtrate, defining one cellulase unit as the amount of enzyme required to release 1 µmol of glucose in 1 min. Data represent the average ± standard error (indicated by bars) of two independent experiments. Average values were significantly different according to the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

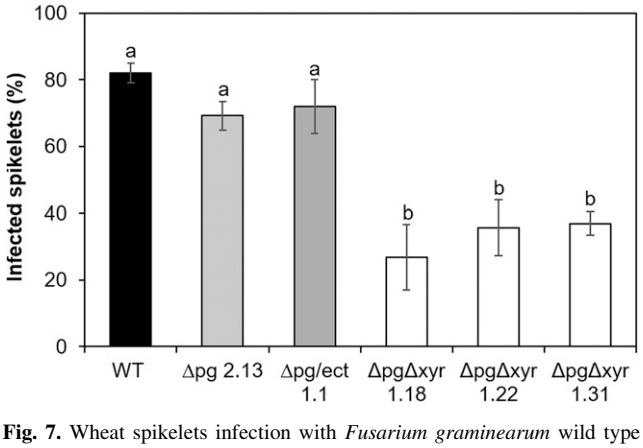

Fig. 7. Wheat spikelets infection with *Fusarium graminearum* wild type (WT) and mutant strains. Spore suspension (10 µl) containing 2,000 conidia of the *F. graminearum* WT or mutant strains were pipetted between the glumes of two florets of two opposite central spikelets. Disease symptoms were assessed at 21 days postinoculation by counting the number of visually diseased spikelets. Infected spikelets are expressed as percentage of symptomatic spikelets on the total number of spikelets of the respective head. Data represent the average ± standard error (indicated by bars) of at least six independent experiments performed infecting cv. Bobwhite wheat spikes. Similar results were obtained by infecting cv. Nandu with 200 conidia per floret. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

enzymatic activity may be sufficient for loosening the cell wall, allowing the advancement of fungal hyphae into the plant tissue. In fact, pectin and xylan are polymers strictly interwoven in the primary plant cell wall (Kikot et al. 2009), and PG and xylanase activities could work synergistically to break down the cell-wall structure. Assuming that the simultaneous reduction of both PG and xylanase activity could be detrimental to the progress of infection, in this work, the ΔpgΔxyr double mutant was obtained and characterized both in vitro and in planta. As expected, the three ΔpgΔxyr mutant strains obtained showed a dramatic reduction of both xylanase and PG activities in liquid media with xylan or pectin as sole carbon sources. Growth experiments performed on the same media showed a significant reduction of growth of the ΔpgΔxyr mutant strains only in xylan-containing medium, similarly to what was observed with the Δxyr deletion mutant (Sella et al. 2016b; this paper). The observation that the lack of PG activity does not affect the ability of the Δpg and ΔpgΔxyr mutants to grow in pectin-containing medium could depend on the contribution to fungal growth of other pectinase activities, such as pectate lyase or pectin lyase. However, the presence in the medium of other carbon sources, which are usually contained in the commercial pectins used for our growth experiments, could have also affected this result (Sella et al. 2016a).

Infection experiments with the ΔpgΔxyr mutant strains showed symptoms significantly reduced in wheat and soybean compared with WT and Δpg mutant, thus indicating that the concurrent lack of PG and xylanase activities affects the virulence of *F. graminearum*; this result is particularly interesting in wheat spikes, in which, as described above, the single Δxyr and Δpg mutants were as virulent as the WT. However, since the double mutant strains were also partially impaired in cellulase activity although retaining about 50% of the activity, compared with WT, we cannot rule out a possible contribution of the cellulase activity to explain the reduced virulence of the ΔpgΔxyr mutant. The synergistic effect of xylanase and PG activities was confirmed by incubating together the PG1 and two *F. graminearum* endo-xylanases (FGSG_10999 and FGSG_03624) (Sella et al. 2013; Tundo et al. 2015) with wheat cell walls. The uronides released by PG1 and xylanases together were about 35% more abundant than the sum of uronides released by the two enzymes separately incubated with the cell walls.

Finally, we demonstrated that the Δpg and ΔpgΔxyr mutants expressing GFP produced infection cushions and lobate appressoria like the WT. This indicates that the reduced virulence of the double mutant is not related to a different ability to produce infection structures but is most likely due to a different ability to progress inside the infected tissues, probably for its altered capacity to degrade the cell-wall polysaccharides.

In conclusion, our results showed that the *F. graminearum* PG1 activity is involved in fungal virulence on soybean, while is dispensable in wheat infection. In this host, the combined presence of *F. graminearum* PG- and Xyr1-regulated enzymatic activities is necessary to break down cell-wall components and facilitate host infection, thus indicating that full fungal virulence depends on the synergistic effect of different enzymatic activities. This result is in accordance with the further improvement of wheat resistance to FHB when bean PGIP and wheat TAXI were pyramided together in the same wheat line (Tundo et al. 2016). Our results confirm that this effect is likely due to a combined inhibitory effect against the *F. graminearum* PG and endo-xylanase activities.

MATERIALS AND METHODS

Fungal culture and plant growth conditions.

F. graminearum WT (strain 3827) and mutants were cultured at 24°C on PDA (Difco Laboratories, Detroit).

Conidia of *F. graminearum* WT and mutants were produced by culturing five PDA discs (5 mm diameter) containing actively growing mycelium in 50 ml of carboxymethyl cellulose (CMC) (Sigma-Aldrich, Milano, Italy) liquid medium (Cappellini and Peterson 1965) in an orbital shaker at 150 rpm and 28°C for 7 days. Liquid cultures were then filtrated with a sterile gauze, were washed with sterile water, and conidia were counted with the Thoma chamber. Alternatively, conidia were obtained by inoculation of synthetic nutrient agar plates (Urban et al. 2002) with a mycelium plug and incubation at 25°C for 20 days. Conidia were recovered by scraping agar plates with a sterile glass rod, were diluted in sterile water, and were counted.

For assaying PG and for dry weight experiments, WT and mutants were cultured by inoculating 1×10^4 conidia ml^{-1} in 20 ml of Szécsi medium (Szécsi 1990) supplemented with 0.5% (wt/vol) apple pectin (70 to 75% esterification) (Sigma-Aldrich) or beechwood xylan (Sigma-Aldrich) at 25°C and 100 rpm. PG activity was measured by assaying cultural filtrate aliquots (filtered through a 100 µm Wilson sieve) obtained after 4 and 7 days of growth. Fungal growth was determined, after 7 days of growth, by

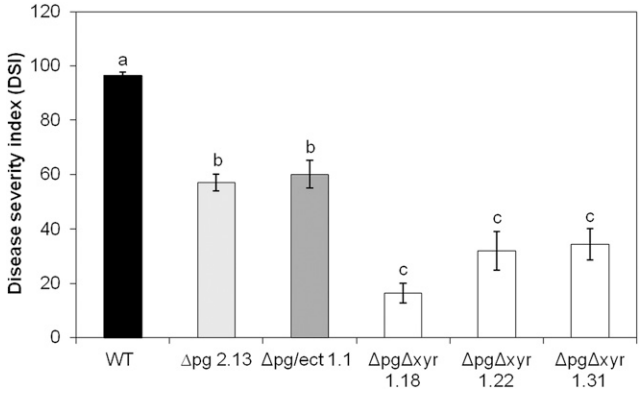

Fig. 8. Infection of soybean seedlings with *Fusarium graminearum* wild type (WT) and mutant strains. Spore suspension (200 µl) containing 2×10^4 conidia of the *F. graminearum* WT or mutant strains were dropped on each soybean seed (cv. Demetra) and disease symptoms (disease severity index) were assessed at 6 days postinoculation. Data represent the mean ± standard error of at least six independent infection experiments performed with the rolled towel method. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$.

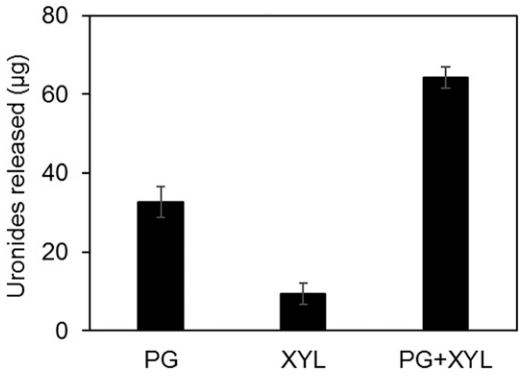

Fig. 9. Uronides released incubating *Fusarium graminearum* polygalacturonase and xylanase activities with *Triticum aestivum* cell walls. The PG1 polygalacturonase and the FGSG_10999 and FGSG_03624 xylanases were purified and used, alone or in combination, in the presence of 1% (wt/vol) wheat cell walls. Data, expressed as microgram equivalents of uronides released from a 300-µl reaction mixture, were obtained with the uronic acid assay and represent the mean ± standard error (indicated by bars) of two independent experiments.

weighing the mycelium previously filtered through a Wilson sieve (40 µm), washing twice with deionized water and oven drying at 80°C for 3 days. For assaying xylanase or cellulase activities, 2×10^5 conidia of WT and mutants per milliliter were first inoculated in 20 ml of Szécsi medium containing 0.5% (wt/vol) glucose at 25°C; after 3 days, mycelia were collected, were washed thoroughly with water, and were incubated in 20 ml of Szécsi medium containing 0.5% (wt/vol) beechwood xylan or CMC at 25°C and 100 rpm. Xylanase and cellulase activities were measured by assaying cultural filtrate aliquots obtained after 3 days of growth.

To obtain mycelia for DNA extraction, WT and mutant strains were grown in 50 ml of CM (1% [wt/vol] glucose, 0.05% [wt/vol] yeast extract, 0.5% [wt/vol] yeast nitrogen base without aminoacids and ammonium sulfate) for 3 days at 150 rpm and at 24°C.

Wheat seeds of (*Triticum aestivum* L.) 'Bobwhite' and 'Nandu' were surface-sterilized with sodium hypochlorite (0.5% vol/vol) for 10 min and were incubated for 3 days in the dark on wet filter paper for germination. Seedlings were vernalized at 4°C for 7 to 10 days and were then transplanted in soil. Wheat spikes were grown in a greenhouse at 18 to 20°C, 60% humidity, and a photoperiod of 14 h.

Construction and preparation of the cassettes for gene replacement.

To generate the construct for disrupting the *F. graminearum pg1* gene (MIPS database entry FGSG_11011), its flanking homologous regions were amplified by PCR, using *F. graminearum* genomic DNA, extracted as described by Henrion et al. (1994), as the template. Specific oligonucleotides were designed to amplify the upstream (primers 1 and 2) and downstream (primers 3 and 4) flanking regions (Supplementary Table S1; Supplementary Fig. S10). The amplification of the flanking regions was performed with REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich) in a 50-µl volume. The PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 53°C for 30 s, and 72°C for 1 min. The amplicons obtained were cut from agarose gel, were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Milano, Italy), and were used in a second PCR to fuse the homologous flanking regions, with the hygromycin resistance gene (*Hyg*, hygromycin B phosphotransferase) used as selection marker. The fusion PCR reaction was performed with REDTaq ReadyMix PCR reaction mix in a 50-µl volume, using 400 ng of the *Hyg* gene, cut with *Sma*I (Fermentas, Milano, Italy) from the pAN7-1 vector (Voigt et al. 2005), and 200 ng of the purified flanking regions containing tails homologous to the 5 and 3′ region of the *Hyg* gene. The fusion PCR conditions were as follows: 94°C for 3 min, followed by 20 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 4 min. The fusion PCR product was then used as template in a nested PCR reaction with specific primers to obtain the full construct (Supplementary Table S1). The nested PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 53°C for 30 s, and 72°C for 4 min. The amplicons obtained were cut from agarose gels, were purified, and were then cloned into the pGEM-T Easy vector (Promega, Milano, Italy), following the manufacturer's instructions. The construct was then cleaved with *Apa*I and *Sal*I from the recombinant vector, obtained from a positively transformed *Escherichia coli* colony, and was used to transform *F. graminearum* protoplasts.

To generate the construct for disrupting the *F. graminearum xyr1* gene (MIPS database entry FGSG_17662) in the Δpg background (strain 3827), its flanking homologous regions were amplified by PCR with specific primer pairs under the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min. The fragments of the expected sizes were cut and purified from agarose gel and were used in a fusion PCR with the geneticin resistance gene (*Gen*) used as selection marker. For the PCR reaction, 500 ng of the *Gen* gene, cut with *Bgl*II and *Hin*dIII from the pII99 vector (Jansen et al. 2005), and 100 ng of each flanking region containing tails homologous to the 5 and 3′ region of the *Gen* gene were used. The fusion PCR was performed with Pfu polymerase in a final volume of 50 µl under the following conditions: 94°C for 3 min, 20 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 5 min. The fusion product was used as template in a nested PCR performed with specific primers under these conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 10 min. The nested fragment was purified as reported above and was cloned into the pGEM-T vector. The construct was then cleaved with *Apa*I and *Not*I from the recombinant vector obtained from a positively transformed *E. coli* colony and was used to transform *F. graminearum* Δpg protoplasts.

The construct for the *Fgxyr1* gene replacement vector for transformation of *F. graminearum* WT 3827 strain was performed as reported by Sella et al. (2016b).

The GFP-PNR1 plasmid (Martinez-Rocha et al. 2016) was cut with *Hin*dIII, precipitated with isopropanol and resuspended in water. About 20 µg of the digested construct was used for each fungal transformation.

Fungal transformation.

Protoplast formation and fungal transformation were performed according to Nguyen et al. (2012) and Sella et al. (2016a).

Hygromycin- or geneticin-resistant colonies were selected and transferred to 3-mm CM plates (Leslie and Summerell 2006) supplemented with 200 µg ml^{-1} of hygromycin or geneticin. Resistant mutants were screened by PCR, using primer pairs internal to the *Hyg*, *Gen*, *Fgpg1*, and *Fgxyr1* genes, under the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Selected mutants were then single-conidiated, first in water agar (1.6%) plate for 3 h at 28°C and, then, on CM plates supplemented with the selection antibiotic. Transformant colonies without the *Fgpg1* and *Fgxyr1* genes were tested by Southern blot hybridization.

Transformation of the *F. graminearum* WT, Δpg 2.13, and ΔpgΔxyr 1.22 mutant strains for the constitutive expression of the GFP produced about 20 transformants from each strain. After selection on CM plates supplemented with 200 µg ml^{-1} of nourseothricin, colonies were single-conidiated and were analyzed by PCR and fluorescence microscopy (Leica stereo microscope) to confirm GFP expression (data not shown).

Southern blot analysis.

For Southern blot analysis, approximately 5 to 15 µg of genomic DNA, extracted as previously reported, were digested with specific restriction enzymes, were separated on a 1% (wt/vol) agarose and Tris-acetate-EDTA gel and were blotted onto a Hybond NX membrane (Amersham Biosciences, Milano, Italy). Digoxygenin (DIG)-labeled specific probes were generated by PCR with specific primers, using genomic or plasmid DNA as template, and were used for overnight hybridization at 65°C. The PCR reactions were performed in a volume of 25 µl, using DIG-11-dUTP (Roche, Mannheim, Germany), and consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Southern hybridization and detection of the DIG-labeled probes were performed according to manufacturer's instruction. Membranes were exposed to X-ray film (X-Omat AR, Kodak, Rochester, NY, U.S.A.) for approximately 1 to 3 h.

Xylanase, PG, and cellulase activity assays.

Xylanase and cellulase activities secreted by *F. graminearum* WT and mutants grown in liquid cultures containing xylan or CMC as sole carbon source was measured through the DNS assay (Bailey et al. 1992), using D-xylose (Merck, Milano, Italy)

or D-(+)-glucose (Sigma-Aldrich) as standard. Reducing sugars released were measured after incubating 50 or 100 µl of each fungal culture filtrate in a total reaction mixture of 500 µl, containing 0.5% (wt/vol) beechwood xylan (Sigma-Aldrich) or CMC dissolved in 50 mM sodium citrate buffer (pH 5). The incubation was performed at 37°C for 30 min for xylanase activity or 14 h for cellulase activity. One unit of xylanase or cellulase activity was defined as the amount of enzyme required to release 1 µmol of xylose or glucose in 1 min, under the assay conditions.

PG activity was determined by viscosimetric and radial gel diffusion assays. The enzymatic activity of culture filtrates was assayed by measuring the decrease in relative viscosity at 30°C of a 2-ml reaction mixture containing 100-µl aliquots of culture filtrates and the PGA (85% titration) (Sigma-Aldrich) dissolved at a final concentration of 0.25% (wt/vol) in 50 mM sodium acetate buffer at pH 6.0. Since the Δpg and ΔpgΔxyr mutant strains were able to lower the initial relative viscosity by 50% only when the analysis was considerably prolonged, the percentage of substrate hydrolysis was calculated after 30 min of incubation. Micro-Ostwald capillary viscosimeters (i.d. = 0.70 nm), connected to the AVS 310 system (Schott Geräte, Mainz, Germany), were used.

For the radial gel diffusion assay (Ferrari et al. 2003), plates containing 0.8% (wt/vol) agarose and 1% (wt/vol) PGA dissolved in 50 mM sodium acetate buffer, pH 6, were prepared and 30 µl of each fungal culture, produced by *F. graminearum* WT and mutant strains grown in liquid cultures containing pectin as sole carbon source, were loaded in 0.5 cm diameter wells. After 20 h of incubation at 30°C, agarose plates were treated with 6 M HCl to detect PG activity, were visualized by the appearance of a halo. One agarose diffusion unit was defined as the amount of enzyme that produced a halo of a 0.5-cm radius (external to the inoculation well).

Analysis of the PG pattern produced by mutants.

The fungal liquid cultures (150 ml) obtained in Szécsi medium supplemented with pectin were filtered through Sartorius MGA membranes and successively through cellulose acetate membranes with pore sizes of 0.8, 0.45, and 0.2 µm (Sartorius, Göttingen, Germany). A solution of $(NH_4)_2SO_4$ with 20% saturation was added to the culture filtrates. After 3 h, a centrifugation was performed for 30 min at 4°C at 10,000 × *g*. The supernatants were salted out with $(NH_4)_2SO_4$ to 75% saturation, overnight at 4°C, and mixtures were centrifuged at 25,000 × *g* for 40 min at 4°C; the resulting pellets were resuspended in 1 ml of Milli-Q quality water (Millipore, Bedford, MA, U.S.A.) and were dialyzed overnight at 4°C against deionized water. An equal volume of each sample (30 µl) was analyzed by analytical IEF, using a 0.8-mm-thick polyacrylamide (PAA) gel containing 1.6% (vol/vol) carrier ampholytes, obtained by mixing equal volumes of ampholytes covering the pH ranges 6.0 to 8.0 (Sigma-Aldrich) and 8.0 to 10.5 (Amersham Biosciences, Uppsala, Sweden). The PG isoforms were detected on a pectate agarose overlay gel buffered at pH 5.0, according to Ried and Collmer (1985).

The PG pattern produced by the Δpg mutant on wheat spikes was also analyzed by grinding 12 infected ovaries with floret glumes collected 4 days after spikelet inoculation in 50 µl of water; the poured tissue was loaded on a PAA gel as described above.

Enzymatic treatment of wheat cell walls.

The 300-µl reaction mixture contained 1% (wt/vol) *T. aestivum* cell walls, dissolved in 50 mM sodium acetate buffer, pH 6.0, supplemented with, per milliliter, 0.2 mg of streptomycin and 0.1 mg of bovine serum albumin, 200 U of the *F. graminearum* PG1 isoform, and 0.1 U of each of the two more-expressed *F. graminearum* xylanases (FGSG_03624 and FGSG_10999). The *F. graminearum* PG1 isoform was purified as described by Tomassini et al. (2009); 1 unit of PG activity was defined as the amount of enzyme required to release 1 µmol of reducing groups per minute, using D-galacturonic acid as standard. The *F. graminearum* FGSG_03624 and FGSG_10999 xylanases were purified as previously reported (Sella et al. 2013; Tundo et al. 2015).

After the addition of the enzyme samples, tested separately or mixed together, the mixtures were incubated at 30°C for 20 h and micrograms of uronides released were measured, using the method described by Blumenkrantz and Asboe-Hansen (1973) and D-galacturonic acid as standard.

Plant inoculation.

Soybean seeds were inoculated with 200 µl of a suspension containing 1×10^5 conidia ml^{-1}, diluted with sterile water and preincubated at room temperature (22 to 24°C) for 16 h, according to the 'rolled towel' protocol (Sella et al. 2014). Symptoms were evaluated, at 6 days postinfection, as the percentage of the ratio between lesion length and total seedling length (DSI).

T. aestivum spikelets wheat cultivars Bobwhite and Nandu were inoculated by WT and mutant strains at anthesis (Zadoks stage 65 to 67) (Zadoks et al. 1974), pipetting 10 µl of a fresh conidial suspension containing approximately 2×10^5 conidia ml^{-1} for 'Bobwhite' or 2×10^4 conidia ml^{-1} for 'Nandu', between the glumes of two florets of two opposite central spikelets. After inoculation, spikes were covered for 3 days with a plastic bag to keep a moist environment. Symptoms were estimated at 21 days postinfection, dividing the number of infected spikelets by total number of spikelets per spike. Independent experiments were performed by inoculating at least 10 plants with each strain. Data were statistically analyzed by applying the Tukey-Kramer's test.

For histological analysis by fluorescence microscopy, paleas of *T. aestivum* cv. Nandu were detached from the floret with a blade, were washed with 0.01% (vol/vol) Tween 20 for 20 min, were rinsed with sterile water, and were placed in petri dishes on 1.6% (wt/vol) granulated agar (Difco Laboratories). Inoculation was performed, dropping 5 µl of a conidial suspension containing approximately 250 conidia on the adaxial side of paleas. After inoculation, petri dishes were sealed with parafilm and were incubated in a growth chamber at conditions described above.

Fluorescence microscopy.

Infection structures of WT and mutants were investigated by fluorescence microscopy, as reported by Quarantin et al. (2016), by exciting GFP at 488 nm and detecting the fluorescence at 500 to 509 nm. Autofluorescence of the plant was excited at 405 nm and detected at 410 to 490 nm.

ACKNOWLEDGMENTS

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Technical University of Munich *Fusarium graminearum* genome database: http://mips.gsf.de/genre/proj/fusarium