Involvement of Fungal Pectin Methylesterase Activity in the Interaction Between *Fusarium graminearum* and Wheat

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The genome of *Fusarium graminearum*, the causal agent of *Fusarium* head blight of wheat, contains two putative pectin methylesterase (PME)-encoding genes. However, when grown in liquid culture containing pectin, *F. graminearum* produces only a single PME, which was purified and identified. Its encoding gene, expressed during wheat spike infection, was disrupted by targeted homologous recombination. Two Δpme mutant strains lacked PME activity but were still able to grow on highly methyl-esterified pectin even though their polygalacturonase (PG) activity showed a reduced capacity to depolymerize this substrate. The enzymatic assays performed with purified *F. graminearum* PG and PME demonstrated an increase in PG activity in the presence of PME on highly methyl-esterified pectin. The virulence of the mutant strains was tested on *Triticum aestivum* and *Triticum durum* spikes, and a significant reduction in the percentage of symptomatic spikelets was observed between 7 and 12 days postinfection compared with wild type, demonstrating that the *F. graminearum* PME contributes to fungal virulence on wheat by promoting spike colonization in the initial and middle stages of infection. In contrast, transgenic wheat plants with increased levels of pectin methyl esterification did not show any increase in resistance to the Δpme mutant, indicating that the infectivity of the fungus relies only to a certain degree on pectin degradation.

Fusarium graminearum is the causal agent of the *Fusarium* head blight (FHB) disease, which affects wheat, barley, and other small grain cereals. FHB alters the quality, weight, and composition of kernels, causing contamination by fungal toxins such as the trichothecene deoxynivalenol (Kikot et al. 2009; McMullen et al. 1997).

The fungus is known to enter the host through natural openings (Pritsch et al. 2000) or by penetrating the epidermal cell walls directly with short infection hyphae (Wanjiru et al. 2002). Anthers are reported as the primary infection site where spores of the fungus may land and then grow into the soft ovarian tissue and the inner surfaces of the lemma and palea

(Goswami and Kistler 2004) before spreading systemically through the spike. It is likely that *F. graminearum* penetrates and colonizes these host tissues via the activity of secreted cell wall–degrading enzymes (Kang and Buchenauer 2000a and b; Wanjiru et al. 2002).

Among cell wall–degrading enzymes, pectinolytic enzymes are the first degrading enzymes to be induced when *F. graminearum* is grown on plant cell walls and the first produced in infected tissues (Kikot et al. 2009). Pectins are mainly composed of two types of polymers: i) homogalacturonan, consisting of α1-4 linked D-galacturonic acid residues that may be variably methyl-esterified on the carboxylic group, and ii) rhamnogalacturonan, mainly consisting of an L-rhamnose and D-galacturonic acid repeat in which the rhamnose residues carry some neutral side chains of either arabinose, galactose, or both. Among the pectinases, endo-PGs (polygalacturonases) (EC 3.2.1.15), which break down the partially de-esterified homogalacturonan via a hydrolytic mechanism, and pectin methylesterases (PMEs) (EC 3.1.1.11), which catalyze the de-esterification of pectins by removing the methoxy esters, can act synergistically, causing plant tissue maceration and increasing the accessibility of cell-wall components for degradation by other enzymes. In particular, PGs are expressed in the early stages of host infection (Sella et al. 2005; Tomassini et al. 2009) and, by degrading the pectin component of the cell wall and middle lamella, have been shown to strongly contribute to disease symptoms caused by several fungi (Clay et al. 1997; Isshiki et al. 2001; Oeser et al. 2002; Shieh et al. 1997; ten Have et al. 1998, 2002), although other 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).

Fungal pectinases are also important during the infection of cereal plants (Oeser et al. 2002; Wanjiru et al. 2002), although *Poaceae* contain a low level of pectin in their cell wall (Vogel 2008). Indeed, homogalacturonan and methyl-esterified homo-galacturonan are abundant constituents of ovary cell walls in grasses such as rye (Tenberge et al. 1996), and methyl-esterified homogalacturonan has been found in the pericarp of wheat ovaries at the anthesis stage (Chateigner-Boutin et al. 2014). Additionally, at the initial stage of infection, *F. graminearum* producing PG activity affects the wheat ovary (Miller et al. 2004; Tomassini et al. 2009). As indirect evidence of the involvement of PG activity during *F. graminearum* wheat infection, the expression of a bean polygalacturonase-inhibiting protein (PvPGIP2) in transgenic wheat plants limits the disease

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symptom development caused by this pathogen (Ferrari et al. 2012). Since the PG hydrolyzes mostly de-esterified pectin (homogalacturonan), a PME activity that is able to demethylate wheat pectins is likely involved at the early stage of fungal infection.

PME is a virulence factor of the polyphagous necrotrophic fungus *Botrytis cinerea*, and the knockout of one of the two *pme* genes (*Bcpme1*), responsible for 75% of the fungal PME activity, caused a reduction in the fungal virulence on tissues of several host plants, such as apple fruits and leaves of grapevine and *Arabidopsis thaliana* (Valette-Collet et al. 2003). However, other authors (Kars et al. 2005) produced *B. cinerea* knockout mutants lacking both *Bcpme* genes (including *Bcpme1*) and their growth on highly methylated pectin was not affected, nor did they show a reduction in virulence. These conflicting results may be a consequence of different *B. cinerea* strains used in the gene disruption experiments (Kars et al. 2005).

Recently, transgenic durum wheat lines expressing high levels of the PME inhibitor of *Actinidia chinensis* (AcPMEI) showed an increased degree of methyl-esterification of cell-wall pectins and a significant reduction of disease symptoms caused by *F. graminearum* and *Bipolaris sorokiniana* (Volpi et al. 2011). Methyl-esterification levels of wheat cell-wall pectin appear, therefore, to influence plant resistance, because highly methyl-esterified pectin is less vulnerable to pectic enzyme degradation.

Using an in silico analysis of the *F. graminearum* sequenced genome, available online from the Institute of Bioinformatics and Systems Biology database and the *Fusarium* Comparative database, two putative PME-encoding genes have been identified. The gene *FGSG_03406* has a nucleotide sequence of 1,077 bp, which corresponds to a transcript of 981 bp and a deduced protein of 326 amino acids, including a secretion signal peptide. This PME has a typical single pectin esterase domain and shows a very high identity with several PMEs from other fungal species belonging to genera *Fusarium*, *Verticillium*, *Colletotrichum*, *Neonectria*, and *Diaporthe*. The gene FGSG_04439 is much more complex, with a nucleotide sequence of 7,767 bp, a hypothetical transcript of 7,557 bp, and a deduced protein of 2,518 amino acids, including the secretory signal peptide. Unlike the protein encoded by the *FGSG_03406* gene, the FGSG_04439 protein shows homology only with a few fungal putative PMEs of species belonging to the *Fusarium* genus. The size of this hypothetical protein is due to the presence of four putative pectinesterase domains.

Two recent transcriptomic studies (Güldener et al. 2006; Lysøe et al. 2011) seem to indicate that both genes are expressed by *F. graminearum* during barley and wheat spike infections, although the *FGSG_03406* transcript appears to be expressed earlier in the infected tissue (48 to 72 h postinfection [hpi]). However, in a comparative proteomic study, only the FGSG_03406 protein was detected in the secretome of *F. graminearum* grown on pectin or plant cell walls (Paper et al. 2007).

The main objective of the present work was to clarify the importance of the *F. graminearum* PME activity during FHB of wheat. Thus, we first characterized the PME activity produced by the fungus, and then, we produced a *F. graminearum* Δpme knockout mutant and tested its virulence on wheat spikes (*Triticum aestivum* and *Triticum durum*) and on transgenic AcPMEI wheat plants possessing an increased level of cell-wall pectin methyl-esterification.

The secondary objective was to determine if the contribution of the *F. graminearum* PME is to favor pectin degradation by the fungal PG activity. To this end, we verified the capability of the Δpme mutant to efficiently degrade pectins having different degrees of methyl-esterification, and we checked if the purified *F. graminearum* PME enhances the activity of the fungal PG (namely PG1) (Tomassini et al. 2009) on pectins having different esterification levels.

RESULTS

Production and purification of *F. graminearum* PME.

To obtain PME activity, *F. graminearum* was grown for 5 days in liquid culture medium containing pectin as the sole carbon source. The culture filtrate was then concentrated and analyzed by analytical isoelectric focusing (IEF) and, after a pectin gel blot assay, a single protein band with a basic isoelectric point (pI) of approximately 9.2 was visualized (data not shown).

The *F. graminearum* PME was purified by preparative IEF. Because the pI of this PME is very close to that of a *F. graminearum* PG, to avoid contamination, we purified the PME from the two most active fractions above pH 9.7, which were pooled and further purified by cation-exchange chromatography (MonoS-column). Two aliquots of the most active MonoS fraction were loaded onto a polyacrylamide (PAA) gel and were subjected to analytical IEF. After the run, a lane of the gel was stained with Coomassie brilliant blue and another lane was developed for PME activity, using a pectin gel blot assay. A unique protein band with pI 9.2 was evidenced in the PAA gel, and a broad PME band was detected on the pectin gel at the same pI (Fig. 1). After trypsin digestion, this protein band was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The five peptides obtained (Table 1) corresponded to the putative pectinesterase precursor protein deduced from the *FGSG_03406* gene.

FgPme1 gene expression in planta.

The expression of the gene encoding *FGSG_03406* PME (hereafter, *FgPme1*) was analyzed by real-time polymerase
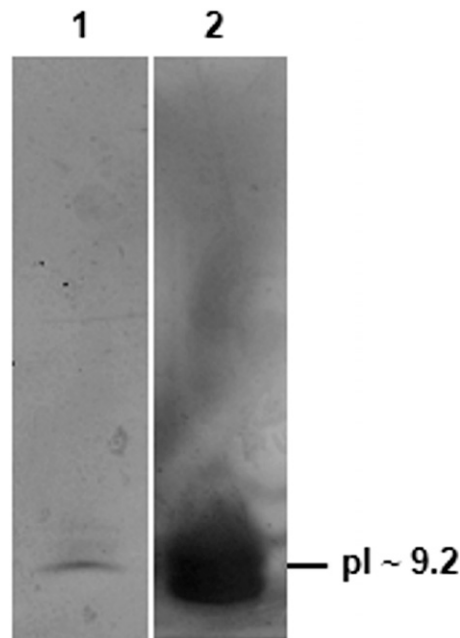

Fig. 1. Thin-layer isoelectric focusing (IEF) in the pH range 8.0 to 10.5 of the most active MonoS fraction. The sample was loaded onto two different lanes of a polyacrylamide gel. After focusing, one section of the gel (lane 1) was stained with Coomassie brilliant blue, the other (lane 2) was overlaid on an agarose gel containing pectin and was stained with ruthenium red. A single pectin methylesterase band with a basic isoelectric point (pI) of approximately 9.2 was visualized, was excised from the gel, and was subjected to digestion for identification by liquid chromatography-tandem mass spectrometry.

chain reaction (PCR) using RNA extracted from infected wheat spikelets at 0, 1, 2, 3, and 4 days postinfection (dpi) with *F. graminearum*. The fungal β-tubulin was the reference gene.

A relative expression analysis showed that the *FgPme1* transcript is already present in infected wheat spikelets at 1 dpi and accumulates at similar levels at 3 and 4 dpi (Fig. 2) but was not detected in mock-inoculated spikelets.

Knockout of the *F. graminearum FgPme1* gene.

The *F. graminearum* wild-type (WT) strain was transformed with the pGEM-ΔPME construct to obtain a targeted disruption of the *FgPme1* gene via integration of the hygromycin-resistance gene. Two independent transformation experiments were performed. In the first transformation, nine *F. graminearum* transformants were obtained, and after PCR screening, only three transformants did not produce the expected *Pme* amplicon (data not shown). After a single conidiation, these transformants were analyzed using a high-stringency Southern blot. Only one Δpme mutant strain (Δpme1.1) showed the absence of the 1.8-kb fragment corresponding to the *FgPme1* gene (Fig. 3, lane 1). Hybridization of this strain (Δpme1.1) with the hygromycin probe showed the expected 3.3-kb band corresponding to the homologous recombination of the disruption construct and a second band corresponding to the ectopic integration of the hygromycin cassette (Fig. 3, lane 7).

The second transformation produced 30 *F. graminearum* transformants resistant to hygromycin. A PCR analysis showed the lack of the *FgPme1* gene in only four transformants (data not shown). After a single conidiation, these four transformants were analyzed using a high-stringency Southern blot and all of the mutant strains showed the absence of the 1.8-kb band corresponding to the *FgPme1* gene (Fig. 3, lanes 2 to 5). However, only one of these (Δpme1.2) showed a single homologous integration of the hygromycin cassette (Fig. 3, lane 9).

In vitro characterization of the Δpme mutant strains.

To verify whether the *FgPme1* gene deletion impaired mycelial growth, the selected Δpme1.1 and Δpme1.2 strains were transferred to potato dextrose agar (PDA) and synthetic nutrient agar (SNA) plates. In both media, no difference in growth was observed between the WT and the mutant strains. Additionally, the conidial production of the mutant strains in carboxymethyl cellulose liquid culture or in SNA plates appeared similar to that of WT (data not shown).

A Δpme mutant and the WT strain were also grown on a liquid culture medium containing pectin (72% methyl esterification) as the sole carbon source. After 4 days, aliquots of these cultures were run in a PAA gel and, after IEF, the PME activity was detected using a pectin gel blot assay. The Δpme mutant culture did not show any PME bands, while the WT culture showed the expected basic PME band (with a deduced pI of approximately 9.2) (Fig. 4). Weak but clear bands corresponding to PG isoforms were also present in both samples. These results confirmed the success of the gene knockout and that the *FGSG_03406* gene encodes for the PME enzyme.

We also determined the amount of mycelium produced by WT and mutant strains by measuring the dry weight after 4 days of growth in liquid culture with pectin (72% methyl

esterification) as the sole carbon source. No significant difference was observed between the strains tested (Fig. 5), and the same result was obtained using a liquid culture containing 38% esterified pectin (data not shown).
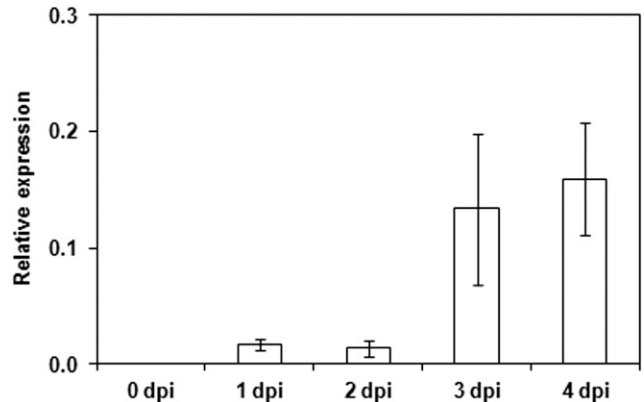

Fig. 2. Relative expression level of the *Fusarium graminearum FgPme1* gene at 0, 1, 2, 3, and 4 days after wheat spike infection (dpi). The *F. graminearum β-tubulin* gene was used as a reference gene. Data represent the average ± mean standard error of the relative expression of at least four independent quantitative polymerase chain reaction experiments.
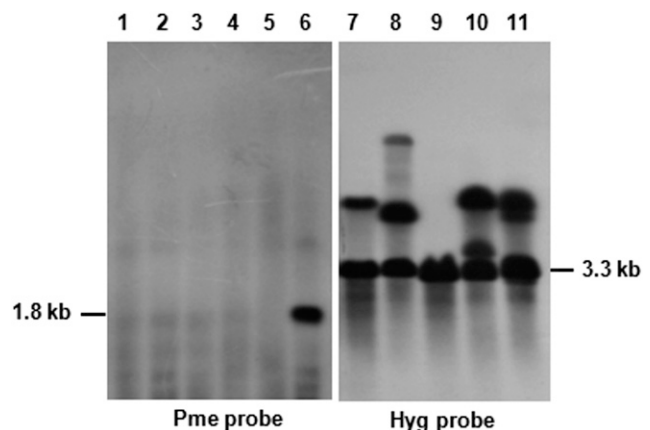

Fig. 3. High-stringency Southern blot analysis of *Fusarium graminearum* wild type (WT) and mutant strain genomic DNA digested with *Cla*I. Lanes 1 to 6, A fragment of the *FgPme1* gene was used as a specific probe. Only the WT strain (lane 6) showed a hybridization signal of approximately 1.8 kb, corresponding to the *FgPme1* gene. The Δpme mutant strains Δpme1.1 (lane 1) from the first fungal transformation and Δpme1.2, Δpme1.3, Δpme1.4, and Δpme1.5 (lanes 2 to 5, respectively) from the second fungal transformation did not show the hybridization signal corresponding to the *FgPme1* gene. Lanes 7 to 11, A fragment of the *hph* gene was used as a specific probe. The Δpme mutant strain from the first fungal transformation (Δpme1.1, lane 7) showed the expected 3.3-kb band corresponding to the homologous recombination of the disruption construct and a second ectopic integration. Among the Δpme mutant strains from the second fungal transformation, only Δpme1.2 (lane 9) showed a single homologous integration of the hygromycin cassette at 3.3 kb, while Δpme1.3, Δpme1.4, and Δpme1.5 (lanes 8, 10, and 11, respectively) showed one or two ectopic hybridization signals.

Table 1. Liquid chromatography-tandem mass spectrometry analysis of the excised protein band with pectin methylesterase (PME) activity

Observed	Mr(expt)	Mr(calc)	Ppm	Score	Peptide
1,393.80	1,392.79	1,392.74	42.2	57	R.VVSASVGYITANGR.D
1,532.79	1,531.78	1,531.72	38.6	57	K.SQADGLNNDGTATLR.V
1,602.79	1,601.78	1,601.73	32.1	93	K.CMIAGATDFIFGQR.A + Oxidation (M)
2,138.07	2,137.06	2,137.00	31.9	156	R.NAQLTIYGYTTDTSGYAGNK.V
2,410.16	2,409.15	2,409.06	37.6	65	K.GEENTGHVQFSEFGNTGAGSTGAR.A

To test the ability of WT and Δpme mutant strains to degrade highly methylated pectin, aliquots of the culture filtrates were adjusted to contain the same PG activity as determined on polygalacturonic acid (PGA) and were assayed on pectins with 38 and 72% degrees of methyl-esterification. After a prolonged incubation time (24 h), the culture filtrates of the two Δpme mutant strains had hydrolysis values comparable to those of the WT culture filtrate on PGA and 38% esterified pectin, while on the 72% esterified pectin, the hydrolysis percentage of the two Δpme mutant strains was approximately 2.5-fold lower than that of the WT (Fig. 6).

Complementation of the Δpme mutant.

The Δpme mutant was complemented by the introduction of the entire *pme* gene flanked by the native promoter and terminator sequences into the Δpme1.2 strain. The transformants obtained were screened by PCR (data not shown), and Southern blot analysis (Supplementary Fig. 1) confirmed the presence of the *pme* gene in three strains. An IEF analysis confirmed the restoration of the PME activity in the Pme2.8C strain (Fig. 4). The culture filtrate of this strain also showed an activity comparable to that of the WT culture filtrate on PGA and pectins (Fig. 6).

Infection of *T. aestivum* wheat plants.

Wheat spikes of *T. aestivum* were point-inoculated at anthesis with a spore suspension of WT, Δpme1.1, and Δpme1.2

strains, and symptom development was assessed up to 21 dpi. The knockout mutant strains maintained the capability to infect wheat plants. However, between 7 and 12 dpi, symptoms caused by the mutant strains were significantly reduced compared with those in the WT (Fig. 7). In particular, infection with the Δpme1.1 and Δpme1.2 strains showed a significant 20 to 25% reduction of symptomatic spikelets in comparison with the WT (Fig. 7). At later stages, the differences between the Δpme strains and the WT were not significant.

Infection experiments were also repeated with the complemented strain. The virulence of the Pme2.8C strain was comparable to that of the WT strain, and both had 25 to 30% more symptomatic spikelets between 10 and 14 dpi in comparison with the Δpme mutant (Fig. 8).

Infection of transgenic durum wheat plants having higher methyl-esterified cell walls.

The transgenic MJ15-69 line (69T) of *T. durum* constitutively expresses AcPMEI and has a higher level of methyl esterification of cell-wall pectin (about 50% esterification degree) (Volpi et al. 2011), in comparison with the WT untransformed cv. Svevo or the transformed null segregant line MJ15-69NS (69NS), which have pectins that are approximately 20% methyl esterified (Volpi et al. 2011; data not published). It was previously reported that the MJ15-69 line was more resistant to *F. graminearum* in comparison with the MJ15-69NS line (Volpi et al. 2011). To establish whether the contemporary presence of higher methyl-esterification levels of pectin cell walls in wheat and the absence of PME activity in *F. graminearum* can affect the outcome of the disease, plants of the MJ15-69NS and MJ15-69 lines were point-inoculated at anthesis with a suspension of spores of WT or Δpme1.1 strains and symptoms were assessed up to 18 dpi.

The Δpme1.1 mutant caused less severe symptoms compared with the WT strain on the *T. durum* MJ15-69NS line, with approximately 25 to 40% fewer symptomatic spikelets between 7 and 10 dpi (Fig. 9) while, at later stages, this difference decreased to only 10% and was not significant. On transgenic MJ15-69 plants, at every time point, the Δpme1.1 showed infection rates similar to those of the WT strain, with
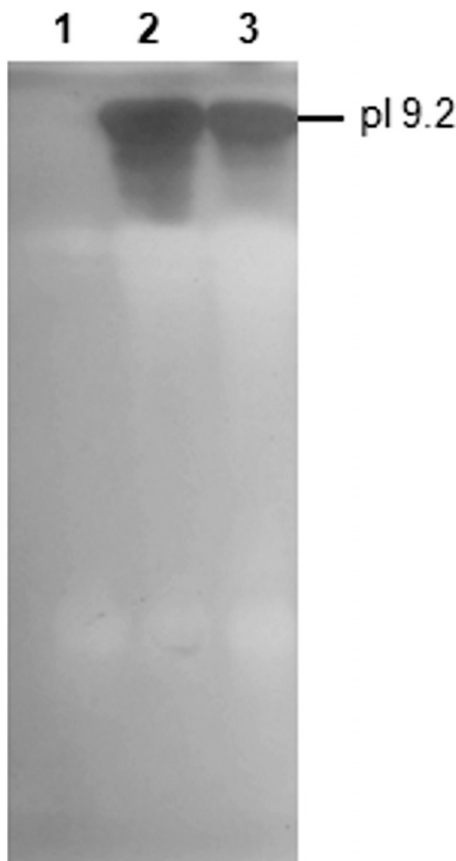

Fig. 4. Thin-layer isoelectric focusing (IEF) in the pH range 3 to 10 of the pectin methylesterase (PME) activity produced by *Fusarium graminearum* wild type (WT) (lane 2), Δpme1.2 mutant (lane 1), and Pme2.8C complemented strain (lane 3) in liquid culture containing pectin as the sole carbon source. An equal volume (20 μl) of 4-day-old liquid culture was loaded on the polyacrylamide (PAA) gel. After focusing, the PAA gel was blotted on an agarose overlay gel containing pectin (methylesterification degree 72%) buffered at pH 6.0, and the PME isoform was detected by ruthenium red staining. The isoelectric point of the *F. graminearum* PME (9.2) is reported on the right.
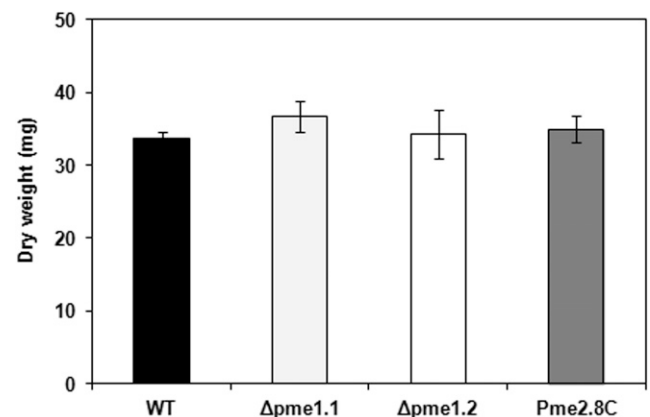

Fig. 5. Dry weight (mg) of wild type (WT), Δpme mutant, and complemented strains grown for 4 days in liquid culture containing pectin (methylesterification degree 72%) as the sole carbon source. Conidia of *Fusarium graminearum* WT, mutant, and complemented strains (1×10^4 conidia per milliliter) were grown in 25 ml of Szècsi liquid medium supplemented with 0.5% (wt/vol) pectin at 100 rpm and 25°C. Data represent the average ± standard error (indicated by bars) of two independent experiments. Data were statistically analyzed by applying the Tukey-Kramer's test. WT = *F. graminearum* wild-type strain; Δpme1.1 and Δpme1.2 = Δpme mutant strains; Pme2.8C = complemented strain.

about 35 to 45% fewer symptomatic spikelets between 7 and 10 dpi compared with null segregant plants (Fig. 9). At later stages, these differences decreased to only 10%.

PME affects PG activity on pectin.

To understand how much the *F. graminearum* PME affects the activity of the fungal PG on pectin, the purified PME was mixed with the main *F. graminearum* PG isoform (PG1) and the activity of the mixture was assayed on pectins with different degrees of methyl esterification (8, 38, and 72%). The PG activity was determined viscosimetrically. On 8% esterified pectin, the presence of the PME did not change the PG activity; however, on 38% esterified pectin, the presence of PME increased the PG activity slightly but significantly (approximately 8%) (Table 2). On 72% esterified pectin, the presence of PME was crucial for the PG viscosimetric activity because, in the absence of PME, the PG was unable to halve the initial relative viscosity of the substrate (Table 2). As expected, PME alone was unable to significantly decrease the viscosity of the three substrates.

DISCUSSION

The degree and pattern of methyl-esterification of plant cell-wall pectin components influences the plant's susceptibility to pathogens (Malinovsky et al. 2014). Fungal PMEs, by de-methyl esterifying pectin, are considered to favor pectin degradation by other pectinolytic enzymes, such as PGs and pectate lyases (PLs). Indeed, the activity levels of the latter enzymes decreases with an increase in the level of pectin methyl esterification (Kikot et al. 2009; Valette-Collet et al. 2003).

While several PG or PL enzymes of some pathogenic fungi are necessary for infection or for contributing to disease symptoms, to date, only the PME of the fungal pathogen *B. cinerea* has been investigated as a virulence factor (Kars et al. 2005; Valette-Collet et al. 2003).

Even though pectin is less abundant in the cell walls of wheat plants than in dicots or in noncommelinoid monocots (Vogel 2008), its methyl-esterification degree and pattern affect the resistance to rust infections (Wiethölter et al. 2003) and the increased level of methyl-esterification due to expression of a plant PME inhibitor (PMEI) reduces the development of disease symptoms caused by *F. graminearum* and *Bipolaris sorokiniana* (Volpi et al. 2011). These results suggest that fungal PME might play a role during wheat infection.

In pectin-containing medium, *F. graminearum* produces only a single basic PME, encoded by the *FgPme1* gene, which is expressed in infected spikelets mostly at 3 to 4 dpi, when the disease symptoms become visible.
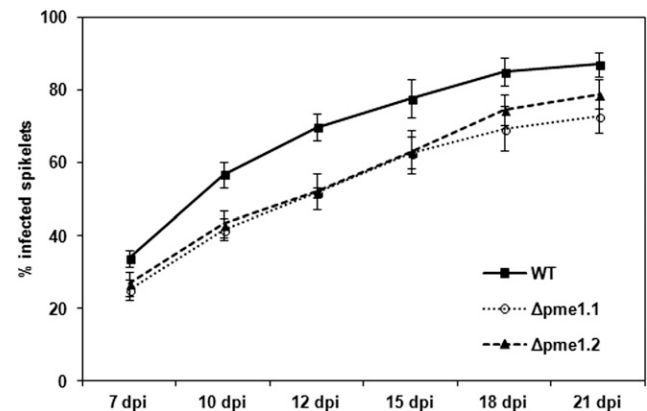

Fig. 7. Infection of *Triticum aestivum* (cv. Bobwhite) spikelets with *Fusarium graminearum* wild type (WT) and Δpme mutant strains. To infect two florets of two opposite spikelets, 10 μl of conidial suspensions (containing approximately 2,000 conidia) of WT and Δpme1.1 and Δpme1.2 mutant strains were used. Infected spikelets are expressed as the percentage of symptomatic spikelets from the total number of spikelets of the respective head. Symptoms were assessed up to 21 days postinfection (dpi). Data represent the average ± mean standard error (indicated by bars) of at least four independent infection experiments performed by inoculating at least 10 plants in each independent experiment. Average values of spikelets infected with Δpme1.1 and Δpme1.2 are significantly different from WT, according to Student's *t* test ($P \leq 0.01$ or 0.05) at 7, 10, and 12 dpi.
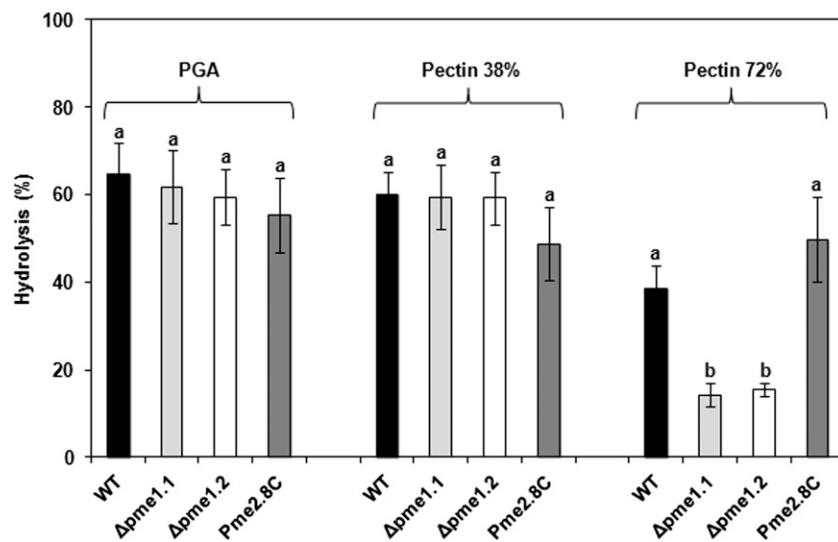

Fig. 6. Percentage of hydrolysis of polygalacturonic acid (PGA) and highly methyl-esterified apple pectins (Pectin 38% and Pectin 72%) determined after a prolonged incubation time (24 h) with aliquots of culture filtrates of wild type (WT), Δpme mutant, and complemented strains. Aliquots of culture filtrates containing an equal amount of PG activity (0.005 RU) were incubated with 0.1% (wt/vol) PGA or 0.1% (wt/vol) apple pectin 38% or pectin 72% at 30°C and pH 6.0. After 24 h, reducing-end groups were measured and the hydrolysis percentage of each substrate was calculated. Data represent the percentage of hydrolysis ± mean standard error (indicated by bars) of at least three independent experiments. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$. WT = *Fusarium graminearum* wild-type strain; Δpme1.1 and Δpme1.2 = Δpme mutant strains; Pme2.8C = complemented strain.

The *FgPme1* gene was successfully disrupted by homologous recombination, and two mutant strains were characterized. When the mutant strains were assayed for virulence on *T. aestivum* and *T. durum*, a significant reduction in symptomatic spikelets was observed between 7 and 12 dpi. Later, the difference with WT decreased, becoming non-significant. This result and the restoring of full virulence when a mutant strain was complemented with the *pme* gene confirmed that the *F. graminearum* PME is necessary to support the rate of wheat spike infection.
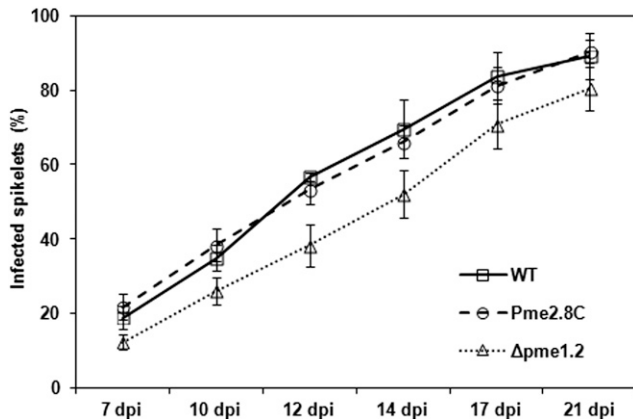

Fig. 8. Infection of *Triticum aestivum* (cv. Bobwhite) spikelets with *Fusarium graminearum* wild type (WT), Δpme1.2 mutant, and Pme2.8C complemented strain. To infect two florets of two opposite spikelets, 10 µl of conidial suspensions (containing approximately 2,000 conidia) of WT and Δpme1.2 and Pme2.8C strains were used. Infected spikelets are expressed as the percentage of symptomatic spikelets from the total number of spikelets of the respective head. Symptoms were assessed up to 21 days postinfection (dpi). Data represent the average ± mean standard error (indicated by bars) of at least four independent infection experiments performed by inoculating at least 10 plants in each independent experiment. Average values of infected spikelets of Δpme1.2 are significantly different from Pme2.8C and WT according to Student's *t* test ($P \leq 0.01$ or 0.05) at 10, 12, and 14 dpi.
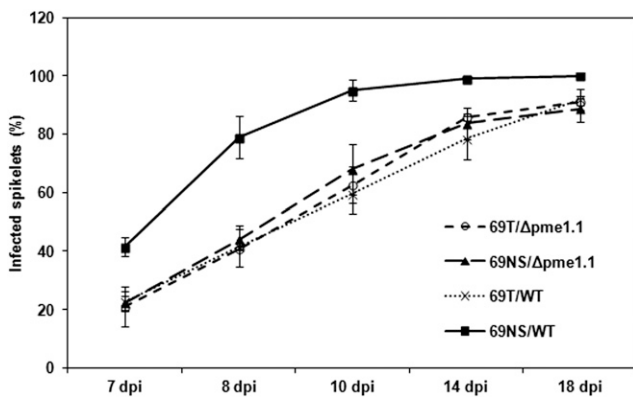

Fig. 9. Infection of spikelets of *Triticum durum* (cv. Svevo) MJ15-69 transgenic (69T) and MJ15-69NS null segregant (69NS) lines with *Fusarium graminearum* wild type (WT) and a Δpme mutant strain (Δpme1.1). To infect two florets of two opposite spikelets, 10 µl of conidial suspension (containing approximately 500 conidia) of WT and Δpme1.1 mutant strains were used. Infected spikelets are expressed as the percentage of symptomatic spikelets from the total number of spikelets of the respective head. Symptoms were assessed up to 18 days postinfection (dpi). Data represent the average ± mean standard error (indicated by bars) of three independent infection experiments performed by inoculating at least 10 plants in each independent experiment. Data were statistically analyzed by applying the Tukey-Kramer's test. Average values of MJ15-69NS spikelets infected with WT (69NS/WT) are significantly different from 69T/WT, 69NS/Δpme1.1, and 69T/Δpme1.1 at 7, 8, and 10 dpi ($P < 0.05$).

Whether fungal PME supports infection by de-esterifying pectin and increasing the activity of fungal PGs or PLs is not obvious from our results. In fact, the enhancing effect of PME on PG activity is clearly visible in in vitro assays with highly esterified pectin and is less or not at all evident on pectin with lower degrees of methylesterification (i.e., 38 and 8%). The average degree of pectin methyl esterification measured on leaf wheat cell walls (approximately 20%) (Volpi et al. 2011) is not very high and was confirmed on cell walls extracted from wheat glumes (data not published). With this level of methyl esterification, the question of how the fungal PME could play, in planta, a significant role in favoring pectin degradation and, as a consequence, fungal infection remains open. However, the wheat cell wall is much more complex than the pectic substrates used in the in vitro assays and the level of methyl esterification may be different within a plant tissue depending on the developmental stage. For example, in a recent study using immunolabeling experiments, Chateigner-Boutin et al. (2014) showed that the methylation level of homogalacturonan present in the pericarp of *T. aestivum* varies during grain formation.

The behavior observed on the transgenic AcPMEI durum wheat plants is also worth mentioning. In these plants, the inhibition of the endogenous PME activity increases the average level of pectin methyl esterification to approximately 50% (Volpi et al. 2011) and the symptom severity caused by inoculation with the WT or Δpme mutant strain was the same or similar to that produced by Δpme on untransformed control plants. Thus, the combined actions of inhibiting the endogenous plant PME activity by AcPMEI expression and of eliminating fungal PME activity by gene disruption did not provide any appreciable additive effect on the reduction of symptoms. Although fungal and plant PMEs may have different modes of action, with plant PMEs suggested to remove methyl groups in a processive, block-wise fashion and fungal PMEs in a more random fashion (Jolie et al. 2010), it seems that the impairment of both these activities contributes only to a certain extent to a delay in infection. It cannot, however, be ruled out that pectin lyase (PNL) activity, which acts on esterified pectins, can partially substitute for the combined action of PME and PG or PL. Indeed, three *pnl* genes are present in the *F. graminearum* genome and at least one of them is expressed during wheat spike infection (Lysøe et al. 2011; Paper et al. 2007; Tomassini et al. 2009). Additionally, we observed that the growth of the Δpme mutant strains in an artificial medium containing highly methyl-esterified pectin was not impaired. This result could be explained by the presence of other carbon sources that are usually contained in commercial pectin preparations but also by the PNL activity that we detected at a low level in the artificial medium (data not shown).

In conclusion, the *F. graminearum* PME contributes to fungal virulence on wheat by promoting wheat spike infection. Nevertheless, its role seems limited to the initial and middle stages

Table 2. Viscosimetric assays of polygalacturonase (PG) activity on pectins (PECT) having different degrees of methyl-esterification (8, 38, and 72%) in the presence or absence of pectin methylesterase (PME)

Enzyme	PECT 8%	PECT 38%	PECT 72%
PG	$3,894 \pm 40$[a]	$5,626 \pm 238$	∞[b]
PG + PME	$3,864 \pm 82$	$5,167 \pm 190$	$9,078 \pm 1515$
PME	∞	∞	∞

[a] Viscosimetric activity (T_{50}) is expressed as the time (in seconds) required by PG to decrease the initial relative viscosity of the reaction mixture by 50%. Each value is the mean of at least two replicates ± standard error. Average values of PG and PG + PME on pectin 38% are significantly different, according to Student's *t* test ($P \leq 0.05$).

[b] ∞ indicates the T_{50} value was not measurable (not able to decrease the initial relative viscosity by 50%).

of infection because symptoms development is only delayed. Later, other virulence factors, such as the mycotoxin deoxynivalenol (Bai et al. 2002), may become the predominant factor favoring pathogen spread.

MATERIALS AND METHODS

Fungal strains and growth conditions.

Fusarium graminearum WT strain 3827, provided by G. Vannacci (University of Pisa, Italy), and the mutant strains were cultured at 24°C on PDA (Difco Laboratories, Detroit).

To induce the production of PME activity, mycelial discs (5 mm diameter) were taken from the edge of actively growing colonies and were placed in 250-ml Erlenmeyer flasks (1 disc to 10 ml of medium) containing 50 ml of Szécsi medium (Szécsi 1990) supplemented with 1% (wt/vol) apple pectin (72% esterified) (Sigma-Aldrich, Milano, Italy). The cultures were grown for 4 days at 24°C on an orbital shaker (at 100 rpm). The fungal liquid cultures obtained (150 ml) were then filtered for an analysis of PME and PG activities.

Fungal growth was determined on 25-ml Szécsi medium containing 0.5% apple pectin (approximately 38% esterification [Carl Roth KG, Karlsruhe, Germany]; approximately 72% esterification [Sigma-Aldrich]) inoculated with 1×10^4 conidia per milliliter. After 4 days of culturing at 25°C on an orbital shaker at 100 rpm, the mycelia were filtered through a Wilson sieve (40 μm), were washed twice with deionized water, were dried in an oven at 80°C for 3 days, and were weighed.

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

Conidia of *F. graminearum* WT and mutant strains were obtained by culturing five mycelium discs (5 mm diameter) in 50 ml of carboxymethyl cellulose liquid medium (Cappellini and Peterson 1965). After 5 to 7 days, the liquid cultures were filtered through a sterile gauze, and conidia were collected and were diluted to approximately 2×10^5 conidia per milliliter. Alternatively, *F. graminearum* WT and mutant strains were cultured on SNA agar plates (Urban et al. 2002) to induce conidiation. Conidia were recovered by scraping agar plates with a sterile glass rod, were diluted in sterile water, and were counted using a Thoma chamber.

Purification of the *F. graminearum* PME.

A *F. graminearum* culture filtrate was concentrated to a final volume of 50 ml using the Vivaflow 200 (10,000 MWC PES) system (Sartorius, Milano, Italy) and was then diluted threefold and again concentrated. This operation was repeated twice. This material was used to purify the fungal PME by preparative IEF and MonoS ion exchange chromatography.

Preparative IEF was performed in a LKB 8100-1 column using a mixture of 1 ml each of pH 7 to 9 (Amersham Biosciences, Uppsala, Sweden) and 9 to 11 (Serva, Heidelberg, Germany) carrier ampholytes, and a sorbitol gradient. A 0.01 M acetic acid solution was used at the anode and a 0.2 M sodium hydroxide solution at the cathode. The IEF was performed at a constant 5 W for 48 h. Fractions of 2 ml were collected and assayed for pH and PME activity using analytical IEF. PME activity was detected in the fractions from pH 8.4 to 11.7; however, fractions up to pH 9.7 also contained *F. graminearum* PG2, with a pI approximately 9.1 (Tomassini et al. 2009). To avoid the presence of this PG, the PME was purified only from the two most active fractions with pH levels greater than 9.7. These were pooled, were dialyzed against 50 mM sodium

acetate buffer (pH 5.0), and were loaded on a MonoS HR 5/5 column (GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM sodium acetate buffer at pH 5.0. The MonoS column was eluted with 50 ml of a 0 to 0.5 M NaCl gradient. Fractions of 0.75 ml were collected and assayed for PME activity. The most active MonoS fraction was analyzed by analytical IEF on a 0.8 mm thick PAA gel containing 1.6% (vol/vol) of carrier ampholytes covering the pH ranges 3 to 10 (GE Healthcare) or 8.0 to 10.5 (Sigma-Aldrich). After staining with Coomassie blue, the only visible protein band was excised from the gel and was subjected to tryptic digestion prior to LC-MS/MS identification. Protein digestion and identification were performed by S. Tolin at the Centre for Research on Proteomics of Padua University.

PME activity assay.

The PME activity was analyzed using a cup-plate agar diffusion assay. A gel was prepared with 0.1% (wt/vol) apple pectin (approximately 72% esterified) and 1% (wt/vol) agarose in McIlvaine's buffer (25 mM citric acid and 115 mM Na_2HPO_4, pH 6.0). The gel was poured into petri dishes (12 ml per plate) and 6 mm diameter wells were made in each plate. The samples were then loaded into each well and plates were incubated at 30°C for 16 h. Plates were stained with 0.02% (wt/vol) ruthenium red for 45 min and were then washed with water to remove unbound dye. The formation of a red halo around the wells indicated the de-esterification of pectin and the presence of PME activity. A 0.5-cm radius halo was defined as one agarose diffusion unit.

PME activity was also analyzed after an analytical IEF run by blotting the PAA gel, containing 1.6% (vol/vol) carrier ampholytes, covering the pH range 3 to 10 (GE Healthcare) or 8.0 to 10.5 (Sigma-Aldrich), on a 15-ml overlay gel containing McIlvaine's buffer, 0.05% (wt/vol) pectin from apple (72% esterification) and 1% (wt/vol) agarose at pH 6.0 (pectin gel blot assay). After incubating 45 min at 30°C, the blotted agarose gel was stained with 0.05% (wt/vol) ruthenium red.

PG activity assays on highly methylated pectin.

The culture filtrates of *F. graminearum* WT and Δpme mutant strains grown on Szécsi medium supplemented with 1% (wt/vol) apple pectin (approximately 72% of methyl esterification) were assayed for PG activity by the reducing-end groups method described by Milner and Avigad (1967) with some modifications (Sella et al. 2004). Briefly, 4 μl of the culture filtrates were incubated for 30 min at 30°C in a 200-μl reaction mixture containing PGA (85% titration) (Sigma-Aldrich) dissolved at a concentration of 0.5% (wt/vol) in 50 mM sodium acetate buffer at pH 6.0, which was determined to be the optimal pH for *F. graminearum* PG activity (Tomassini et al. 2009).

One reducing unit (RU) was defined as the amount of enzyme required to release 1 μmol min^{-1} of reducing groups, using D-galacturonic acid as the standard. An equal amount of PG activity (0.005 RU) from each culture was then assayed on 0.1% (wt/vol) PGA or 0.1% (wt/vol) apple pectin (approximately 38 or 72% esterification) buffered at pH 6.0. After 24 h of incubation at 30°C, reducing-end groups were measured as reported above and the hydrolysis percentage of each substrate was calculated.

The activity of the main *F. graminearum* PG, named PG1 and purified as previously reported, (Tomassini et al. 2009), was determined on apple pectin having different esterification degrees (approximately 8, 38, and 72%) using the viscosimetric method in the presence or absence of the purified fungal PME. Viscosimetric activity was assayed by measuring the decrease in relative viscosity at 30°C of a 2-ml reaction mixture

containing 0.5% (wt/vol) pectin substrate dissolved in 50 mM sodium acetate buffer (pH 6.0) supplemented with 0.1 mg of bovine serum albumin per milliliter, and 40 viscosimetric units (VU) of PG1 activity with or without 1.4 agarose diffusion units of PME activity. One VU, determined on 0.5% (wt/vol) PGA, was defined as the amount of enzyme causing a 50% decrease in the initial viscosity of the reaction mixture at pH 6.0 in 1,000 minutes. Micro-Ostwald capillary viscosimeters (i.d. = 0.70 nm) connected to an AVS 310 system (Schott Geräte, Mainz, Germany) were used. The time employed by the enzyme to decrease the initial relative viscosity of the reaction mixture by 50% was defined as T_{50} (expressed in seconds).

Growth and infection of wheat plants.
Wheat seeds of *T. aestivum* cv. Bobwhite and *T. durum* cv. Svevo were surface-sterilized by immersion in sodium hypochlorite (0.5% vol/vol) for 10 min, were rinsed thoroughly in sterile water, and were incubated for 3 days in the dark on wet filter paper for germination. Seedlings were then vernalized at 4°C for 7 to 10 days before transplanting in soil. Plants were grown in a climatic chamber with a 14-h photoperiod and 19°C day and 17°C night temperatures for approximately one month, with a 14-h photoperiod and 22°C day and 20°C night temperatures until anthesis.

Wheat spike infections were performed by inoculating single spikelets at anthesis (Zadoks stage 65-67) (Zadoks et al. 1974) with *F. graminearum* WT or mutant strains. Spikes were inoculated by pipetting 10 µl of a fresh conidial suspension containing approximately 2,000 conidia for cv. Bobwhite or 500 conidia for cv. Svevo between the glumes of two florets of two opposite central spikelets. At least three independent experiments were performed by inoculating at least 10 plants with each strain. After inoculation, spikes were covered for 3 days with a plastic bag to maintain a moist environment. Plants were then moved to a growth chamber with 85% relative humidity under a 14-h photoperiod and at 22°C day and 20°C night temperatures. Disease symptoms were assessed by counting the number of visually diseased spikelets at different days and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets. Data were statistically analyzed by applying the two-tailed Student's *t* test or Tukey-Kramer's test.

Nucleic acid extraction.
Genomic DNAs of *F. graminearum* WT and mutant strains were extracted from 200 to 400 mg of mycelium obtained in complete medium liquid culture as reported by Henrion et al. (1994).

RNA was extracted from 100 mg of frozen wheat spikes infected with *F. graminearum* at 0, 1, 2, 3, and 4 dpi, using the RNeasy plant mini kit (Qiagen GmbH, Milano, Italy) following the manufacturer's instructions. RNA was then treated with DNaseI (Promega, Milano, Italy) following the manufacturer's instructions and was quantified both spectrophotometrically and by a denaturing 1% (wt/vol) agarose gel.

Construction of the gene replacement vector and fungal transformation-mediated gene disruption.
The disruption of the *F. graminearum Pme1* gene (Munich Information Center for Protein Sequences database entry *FGSG_03406*) was obtained by targeted homologous recombination. To generate the disruption construct, a fragment of 1,701 bp containing 798 bp of the coding region and 903 bp of a downstream region was obtained by PCR, using specific oligonucleotides fg03406-5int (5′ACAGCGAAGAAGAGCCAAGCAGAT-3′) and fg03406-3downnested (5′-TATGTTGATGGGCGATTAT TGTC-3′). All of the primers were designed using the DNAMAN

(Lynnon Biosoft) program. The amplification was performed using the REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich) in a 50-µl volume. The PCR conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 52.5°C for 30 s, and 72°C for 1 min. The amplicon obtained was cut from an agarose gel and was purified and cloned into the pGEM-T Easy vector (Promega), following the manufacturer's instructions. An *Escherichia coli* colony correctly transformed with the recombinant vector was grown in a lysogeny broth high-salt culture (Sigma-Aldrich). After an overnight incubation at 37°C, the plasmid DNA was extracted and purified according to the MIDI Nucleobond Ax kit (Macherey-Nagel GmbH & Co., Milano, Italy) manual. The recombinant plasmid was then cleaved with *Eco*47III and *Eco*NI (Promega) to remove 210 bp of the *FgPme1* coding sequence. The DNA fragment containing the vector and the two flanking regions was then separated from the 210-bp fragment on a 1% (wt/vol) agarose gel and was cut and purified using the Wizard SV Gel and PCR cleanup system (Promega). The hygromycin-resistance gene *hph* (hygromycin B phosphotransferase), used as a selective marker (Punt et al. 1987), was then amplified using specific oligonucleotides containing adaptors with *Eco*47III and *Eco*NI recognition site sequences (Hyg-Eco47IIIUP: 5′-GAGCGCTGACAGCTCTTGTTCGGTCG-3′ and Hyg-EcoNIDOWN: 5′-GCCTTCGACAGGAGCGAGGTGGGT GATGTAG-3′) and the pAN7-1 vector (Voigt et al. 2005) containing the *hph* gene with regulatory sequences as the template. The amplification was performed with the REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich) in a 50-µl volume. The PCR conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The amplicon of the expected size (approximately 1,800 bp) was digested with *Eco47*III and *Eco*NI and was used in a 20-µl ligase reaction containing the previously cleaved recombinant plasmid, which contained the homologous flanking regions of the *pme* gene, 1× ligation buffer and 1 µl of T4 DNA ligase (Promega). The final recombinant plasmid obtained after ligation, named pGEM-ΔPME, contained the hygromycin-resistance gene between the upstream and downstream flanking sequences, approximately 600 and 900 bp, respectively, of the *F. graminearum Fgpme1* gene.

To obtain enough of the construct for transformation, an *E. coli* colony transformed with the pGEM-ΔPME vector was grown in a lysogeny broth high-salt culture (Sigma-Aldrich). After an overnight incubation at 37°C, the plasmid DNA was extracted according to the MIDI Nucleobond Ax kit (Macherey-Nagel GmbH & Co.) manual and the construct (approximately 3.2 kbp) was cleaved from the pGEM-T vector using *Apa*I and *Sal*I (Promega) and was used to transform protoplasts of the *F. graminearum* WT strain. Protoplastation and transformation were performed as reported by Nguyen et al. (2012).

The hygromycin-resistant mutants obtained underwent single conidiation and were screened using PCR with the specific primers Fg3406-INTF1 and Fg3406-INTR1 (Table 3), the REDTaq ReadyMix PCR reaction mix and the following PCR conditions: 94°C for 3 min, then 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Transformants having a deleted fragment of the *FgPme1* gene were then tested by Southern blot hybridization.

Complementation of the Δpme mutant.
Complementation of the Δpme mutant was performed by cotransforming protoplasts of the Δpme1.2 mutant strain with the entire *pme* gene, which was flanked by the native promoter and terminator sequences, and with the selective marker *nptII*, which encodes geneticin resistance under control of the *gpdA* promoter (Ilgen et al. 2009), excised from the pII99 vector with

*Bgl*II and *Hin*dIII (Promega). The *pme* gene with promoter and terminator regions (3,174 bp) was amplified using PCR from the *F. graminearum* WT strain 3827. The amplification was performed in a 50-µl volume using the REDTaq ReadyMix PCR reaction mix and the primers 03406-5nest (5′-CGCTCTG CTCTGTTAGTATTTC-3′) and 03406-3nest (5′-AGCGGGA CAGCTATCAGTTA-3′). PCR conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 4 min.

The amplicon was cloned into pGEM-T Easy, was excised with *Sal*I and *Sac*II (Promega), and was then used for cotransformation. The transformants obtained were screened for the presence of *pme* and *nptII* genes by PCR and Southern blot analysis (Table 3).

Southern blot analysis.

For the Southern blot analysis, approximately 5 to 7 µg of genomic DNA was digested with *Cla*I (for screening knockout mutants) (Promega) or with *Eco*RV and *Pst*I (for screening complemented strains) (Thermo Scientific, Milano, Italy), was separated on a 1.0% (wt/vol) agarose gel, and was blotted onto a Hybond NX membrane (Amersham Biosciences, Milano, Italy). Digoxygenin (DIG)-labeled (Roche, Mannheim, Germany) specific probes were generated, using PCR with specific primers and genomic or plasmid DNA as the template (Table 3), and were used for overnight hybridizations at 65°C. The PCR reaction was performed in a 50-µl volume using DIG-11-dUTP (Roche). PCR conditions consisted of an initial step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, a suitable annealing temperature (Table 3) for 1 min, and 72°C for 2 min.

Southern hybridization and detection of the DIG-labeled probes were performed according to manufacturer's instructions. The membranes were exposed to X-ray film (X-Omat AR, Kodak, Rochester, NY, U.S.A.) for approximately 3 h.

Digestion with *Cla*I produced a 1.8-kb fragment in the WT that hybridized with the *Fgpme1* probe and a fragment of 3.3 kb in the mutant strains that hybridized with the hygromycin probe in cases of homologous construct integration. Digestion with *Eco*RV and *Pst*I produced an 8.4-kb fragment in the WT that hybridized with the *Fgpme1* probe.

FgPme1 expression in planta.

For the reverse transcription (RT) reaction, 0.5 µg of an oligo-dT (15/18 thymine) reverse primer was mixed with approximately 1 µg of the target RNA in a total volume of 10 µl. After incubation at 70°C for 5 min and then at 4°C for 5 min, the mixture was added to a solution containing 1× ImPromII buffer (Promega), 1 mM of each dNTP, MgCl$_2$ to a final concentration of 3 mM, 1 µl ImPromII RT (Promega), and water to a final volume of 30 µl. The RT was carried out in a thermal cycler with the following cycle: 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C.

The amplification of *FgPme1* and the *F. graminearum β-tubulin* reference gene (accession number FGSG_06611) were performed by real time PCR (Rotor-Gene Q 2plex; Qiagen GmbH) using

specific primers (fg03406-5int: 5′-ACAGCGAAGAAGAGCCA AGCAGAT-3′; fg03406-3int: 5′-CTCCAGGGTCGGCCAAGG TAGTAG-3′; BetatubF: 5′-CCCAACAACGTCCAAACTTCC-3′; BetatubR: 5′-CCTCGGTGAATTCCATCTCGTC-3′). The 20-µl reaction mixture contained 10 µl of 2× Brilliant III Ultra-Fast SYBR green QPCR mastermix (Agilent Technologies, Milano, Italy), 0.4 µM of each specific primer and 3 µl of cDNA as the template.

The real time quantitative (q)PCR was performed using 40 cycles of the following: 15 s at 95°C, 15 s at 56°C, and 40 s at 72°C. Relative expression levels were analyzed using the Rotor-Gene 2.0.3.2 Software version (Qiagen GmbH). Three independent qPCR experiments were performed.

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Table 3. Nucleotide sequences of primers used to screen knockout and complemented mutants and to amplify the labeled probes for Southern blot analysis

Primer name	Sequence	T_a; product size[a]
Fg3406-INTF1	CCAGAAGACCAGCATGAG	
Fg3406-INTR1	CCTCCTTCGACAGGTGAT	50°C; 250 bp
HygPRBf	AAAGTGCCGATAAACATAAC	
HygPRBr	AGAGTGATGCTTTGAGAGAG	55°C; 475 bp
GenPRBf	AGGATCTCCTGTCATCTCA	
GenPRBr	CCAAGCTCTTCAGCAATA	55°C; 400 bp

[a] T_a = annealing temperature.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Fusarium* Comparative database:
http://www.broadinstitute.org/annotation/genome/fusarium_graminearum

Institute of Bioinformatics and Systems Biology Fungal and Microbial Genomics database: http://mips.helmholtz-muenchen.de/genre/proj/FGDB