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Looking for a role of polygalacturonase of *Fusaria* during cereal infection

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

31.01.2008

Alessia Tomassini

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Riassunto

Per penetrare attivamente nei tessuti dell'ospite i patogeni fungini producono enzimi in grado di degradare i componenti della cuticola e dalla parete cellulare. Tra questi le pectinasi, e in particolare le *endo*-poligalatturonasi (PG), sono espresse nelle fasi precoci dell'infezione e degradano la componente pectica della parete cellulare e della lamella mediana contribuendo alla patogenicità o virulenza di diversi funghi fitopatogeni. Le pectinasi sono considerate tra i principali responsabili della macerazione della parete cellulare di piante dicotiledoni, caratterizzata da un elevato contenuto di pectina. Recentemente è stata dimostrata l'importanza di questi enzimi anche nella patogenesi di alcune graminacee, sebbene queste piante abbiano una parete cellulare con un basso contenuto di pectina.

Fusarium graminearum e *Fusarium verticillioides* sono due importanti patogeni dei cereali e producono PG in coltura liquida. La dimostrazione che le PG di questi funghi sono fattori di virulenza potrebbe consentire di sviluppare strategie mirate ad aumentare la resistenza delle piante ospiti all'infezione da parte di questi patogeni. Per chiarire l'importanza di questi enzimi durante il processo infettivo, per prima cosa sono state purificate e caratterizzate le due *endo*-PG secrete in coltura da *F. graminearum*. Queste PG presentano differenti caratteristiche biochimiche, quali l'optimum di pH e il meccanismo di attacco del substrato. L'espressione dei corrispettivi geni codificanti è stata inoltre monitorata durante l'infezione di piante di frumento confrontandola con l'espressione dei geni codificanti pectin liasi e xilanasi: poiché i geni codificanti le pectinasi risultano espressi prima di quelli codificanti la xilanasi, è possibile che le pectinasi, ed in particolare le PG, abbiano un ruolo nelle prime fasi del processo infettivo.

Al fine di chiarire l'importanza nella patogenesi delle due PG purificate e caratterizzate di *F. graminearum*, e di una PG di *F. verticillioides*, precedentemente caratterizzata (Sella et al., 2004; Raiola et al., in press), sono stati prodotti mutanti con delezione dei geni *pg* mediante ricombinazione omologa sito specifica. La patogenicità di ciascun mutante è stata valutata eseguendo delle prove di infezione. I mutanti singoli di *F. graminearum* mantengono la capacità di infettare le piante di frumento. Tuttavia, poiché la perdita di attività dovuta al "knock-out" di una PG potrebbe essere compensata dall'attività della rimanente, risulta necessario ottenere il doppio mutante *pg* e valutarne la virulenza. Anche

il mutante di *F. verticillioides* mantiene la capacità di infettare le piante di mais, ma in questo caso la delezione del gene *pg* determina una riduzione della virulenza. In particolare, il sintomo di necrosi osservato durante l'infezione con il fungo wild-type sembrerebbe associato alla presenza della PG nel tessuto infetto.

Summary

To penetrate and colonize host tissue most pathogenic fungi produce enzymes degrading the cuticle and the plant cell wall. Among these, pectinases and in particular *endo*-polygalacturonases (PGs), are expressed in the early stages of host infection and contribute to the virulence or the pathogenicity of several phytopathogenic fungi by degrading the pectin component of the cell wall and middle lamella. Pectic enzymes are considered the main factors responsible for the maceration of the pectin rich cell wall of dicotyledonous plants. Recently, the importance of these enzymes has been shown also in the pathogenesis of some Graminaceous, although these plants have a cell wall consisting of small amount of pectin.

Fusarium graminearum and *Fusarium verticillioides* are two relevant pathogens of cereal species and are know to produce PG activity in liquid culture. The demonstration that the PGs of these fungi are virulence factors might contribute to develop strategies aimed to increase the resistance of host plants to infection by these pathogens. To clarify the importance of these enzymes during pathogenesis, firstly the two *endo*-PGs secreted in vitro by *F. graminearum* were purified and characterized. These PGs showed different biochemical properties, like optimum pH and substrate cleavage mechanism. The expression of their encoding genes was analysed also during wheat infection compared to the expression of pectin lyase and xylanase encoding genes: since pectinases genes were expressed earlier than xylanase gene, pectinases, and in particular PGs, might play a role during the early stage of infection.

To establish the importance in pathogenesis of the two purified and characterized *F. graminearum* PGs, and of *F. verticillioides* PG, previously characterized (Sella et al., 2004; Raiola et al., in press), their encoding genes were disrupted by targeted homologous recombination. The pathogenicity of each mutant was tested by inoculating host plants. Single *F. graminearum* mutants maintained the capability to infect wheat plants. However, since the loss of PG activity due to the knock-out of a single *pg* gene could be compensated by the activity of the remaining PG, a double knock-out mutant should be obtained and tested in infection experiments. Also the *F. verticillioides* mutant maintained the capability to infect maize plants, but in this case the *pg* gene disruption caused a reduction of

virulence. In particular, the necrotic symptom observed during infection with the wild-type strain might be related to the presence of the PG in the infected tissue.

Introduction

Plant cell wall functions as a physical barrier to biotic and abiotic agents (ten Have et al., 2002), but some pathogenic fungi are able to degrade it by producing specific cell wall degrading enzymes (CWDEs) during plant infection. Among CWDEs, pectinolytic enzymes, which cleave plant cell wall pectins, are the earliest to be secreted (Collmer and Keen, 1986; Alghisi and Favaron, 1995). These enzymes have different mode of action on pectic substrate and, among them, polygalacturonases (PGs) cleave the α-1,4 galacturonosyl bonds of unesterified or partially methylesterified homogalacturonan regions of the middle lamella and primary cell wall, thus facilitating the growth of hyphae within the plant tissue. PGs can be divided in *endo-* and *eso-* acting enzymes based on their cleavage mode of action: *endo*-PGs cleave the polysaccharide randomly while *eso*-PGs cleave only the terminal residue.

Fungi produce PG isoforms with different amino acid sequence, specific activity, pH optimum and substrate preference: this multiplicity of isoforms can enable the pathogen to infect various hosts at different environmental conditions (Wubben et al., 2000; Markovic et al., 2001; De Lorenzo et al., 2001). To favour the activity of PGs and other CWDEs many fungal pathogen modulate the ambient pH during the attack. Thus, in order to facilitate PG and xylanase activity some fungi lower the pH by secreting organic acids, while others secrete ammonium to alkalinize the plant tissue thus favouring the activity of pectin lyases (Prusky and Yakoby, 2003; Aleandri et al., 2007).

The role of PGs as determinant of pathogenicity and/or virulence was demonstrated in *Aspergillus flavus*, *Alternaria alternata* and *Botrytis cinerea* (Shieh et al., 1997; Isshiki et al., 2001; ten Have et al., 1998). In other cases, fungal mutants with the *pg* genes disrupted did not show any reduction in pathogenicity or virulence (Di Pietro and Roncero, 1998; Scott-Craig et al., 1998). This could be due to the polygenic nature of *pg* encoding genes, and therefore the disruption of a single gene might be complemented by the production and secretion of other isoforms exhibiting similar activities (Hammer and Holden, 1997). Acting on middle lamella of parenchyma plant cells, pectinolytic enzymes are considered factors of maceration in soft rot diseases. However, PGs produced by some necrotrophic fungi can cause programmed cell death in their host, and this effect is unrelated to the enzymatic activity (Poinssot et al., 2003; Zuppini et al., 2005).

The importance of PGs in fungal infection is also demonstrated by the presence in plant tissues of specific inhibitors of this enzyme, the polygalacturonase inhibiting proteins (PGIPs). PGIPs likely evolved to hinder pectin degradation by inhibiting PGs and therefore contrast the progression of the fungal infection (De Lorenzo et al., 2001). *Pgip* genes are organized in multigene families encoding isoforms with LRR domains and possess different recognition specificities similarly to those expressed by many resistance genes (D'Ovidio et al., 2004; De Lorenzo et al., 2001).

The pectin poor cell wall of monocotyledonous plants seems less susceptible to action of pectinolytic enzymes (Carpita and Gibeaut, 1993). However, recent experimental evidences suggest a role of PGs in some diseases of monocot plants: pectinases are thought to be necessary for *Fusarium culmorum* to break down the major cell wall components during infection and spreading in wheat host tissues (Kang and Buchenauer, 2000); pectin degradation has an essential role in successful colonization of rye tissue by *Claviceps purpurea* (Oeser et al., 2002); PG is the earliest CWDE secreted in culture by the fungal pathogen *Mycosphaerella graminicola* (Douaiher et al., 2007). Recently, the demonstration that wheat plants expressing a bean PGIP show reduced extent of foliar symptoms by *Bipolaris sorokiniana* supports a role for fungal PGs as virulence factors in wheat (Janni et al., 2008).

Fusarium graminearum and *Fusarium verticillioides* are two important pathogens of monocot species, causing yield and quality losses. *F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is the common causal agent of *Fusarium* head blight (FBH), a serious disease of wheat and barley. The diseased grains have poor quality and are contaminated with mycotoxins, such as deoxinivalenol (DON), resulting not suitable for consumption by both humans and animals (McMullen et al., 1997). *F. verticillioides* (Saccardo) Nirenberg (synonym *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland) is considered as the primary pathogen of corn, but has also been isolated from other crops such as wheat, rice, oat, sorghum and asparagus (Bacon et al., 1994; Lori et al., 1998). This fungus produces mycotoxins and, among these, fumonisins are the most important (Pitt et al., 1997; CAST, Task Force Report No. 139, 2003).

F. graminearum and *F. verticillioides* are known to produce PG activity during liquid culture (Szécsi, 1990; Sella et al., 2004; Raiola et al., in press), but the role played by these enzymes during plant infection has not been ascertained yet.

An involvement of *F. graminearum* PGs during wheat infection is possible: Wanjiru et al. (2002) showed by immuno-gold labelling a degradation of the pectin of the middle lamella and the primary cell wall of parenchyma cells and the junction areas between cells of the ovary and lemma in the infected tissue. In fact, *F. graminearum* is thought to penetrate host tissue through these regions (Wanjiru et al., 2002; Goswami and Kistler, 2004); in particular Miller et al. (2004) observed that pollen and anthers are the major targets of the fungal growth during the initial stages of infection, with the fungus quickly progressing towards the soft tissue of the ovary.

Since the whole genome of *F. graminearum* is sequenced (http://mips.gsf.de/genre/proj/fusarium/) and contains only two putative *endo-pg* genes, in the first chapter of this thesis I present the purification and characterization of the two *endo*-PGs secreted in vitro by *F. graminearum* and encoded by these two *pg* genes. To investigate the possible involvement of these PGs in the infection process, I also analysed the expression of *pg* genes during wheat infection compared to the expression of pectin lyase and xylanase encoding genes.

The *endo*-PG produced *in vitro* by *F. verticillioides* (named PGIII) and its encoding gene have been intensively studied (Sella et al., 2004; Raiola et al., in press). PGIII is able to escape inhibition by bean, soybean, leek and asparagus PGIP, but its role during plant infection is unknown.

With the aim to establish the importance of the PG activity produced by *F. verticillioides* and *F. graminearum* in fungal pathogenesis, I tried to knock-out the *pgIII* gene of *F. verticillioides* and the two *pg* genes of *F. graminearum* by targeted homologous recombination. The results of these experiments are presented in the second chapter together with some preliminary experiments of infection of wheat and maize with these mutants.

Understanding the role played by *F. graminearum* and *F. verticillioides* PGs during pathogenesis might also indicated whether a PGIP-based strategy may be exploited to increase resistance of crops to diseases caused by these fungi.

Chapter I

Purification, characterization and expression of *Fusarium graminearum* polygalacturonases *in vitro* and during wheat infection

1.1. Introduction

Fusarium graminearum produces PG activity *in vitro* and the analysis of pectic enzyme zymograms showing the presence of different *eso-* and *endo-*PG isoforms (Szécsi, 1990). However, these enzymes have not been purified and characterized yet, as well as the expression of these isoenzymes during host infection.

Since the *F. graminearum* genome has been completely sequenced and is available at the web site http://mips.gsf.de/genre/proj/fusarium/, I looked for the *endo-pg* encoding sequences annotated in the database. In this work I succeeded to identify, purify and characterize the two *endo-*PG proteins encoded by *F. graminearum*. Since the fungus may modify the pH of the tissue and the pH affects enzymatic activity (ten Have et al., 1998), I paid particular attention to pH changes in wheat spike after fungal infection and I determined the activity of the two purified PGs at different pH values. Since the cleavage pattern of the PG substrate could influence the rate and the degree of host tissue degradation during the infection process, I analysed also the type and amount of uronides released *in vitro* from polygalacturonic acid, the preferred PG substrate.

Moreover, I analysed the expression of the two *pg* encoding genes during wheat infection and I compared their expression with those of the genes encoding three putatively secreted pectin lyases (*pnl*) and one xylanase gene (*xyl A*) (All sequences were identified in the MIPS database). The timing of gene expression of pectinase genes (*pg* and *pnl* genes) compared to that of xylanase gene has clarified the possible role of these two types of enzymes during wheat infection and in particular their involvement in the different phases of fungal attack.

1.2. Materials and methods

1.2.1. Fungal cultures

F. graminearum (strain 3824) was cultured at 23°C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). Mycelium discs (5 mm diameter) were taken from the edge of actively growing colonies and placed in 250-ml Erlenmeyer flasks (1 disc/10 ml of medium) containing 50 ml of medium consisting of $NH_4H_2PO_4$ 0.09 % (w/v), $(NH_4)_2HPO_4$ 0.2 % (w/v), $MgSO_4 \cdot 7H_2O$ 0.01 % (w/v), KCl 0.05 % (w/v), citrus pectin (ICN) 1% (w/v) (Szécsi, 1990). The cultures were grown for 6 days at 23°C on an orbital shaker (110 rpm). Aliquots of 1 ml were taken daily from each of three flasks and after centrifugation, the supernatant was frozen at -20°C until assayed for enzyme activity.

For plant inoculation *F. graminearum* was grown on SNA agar plates (Urban et al., 2002). After 21 days plates were gently scraped with sterile water and diluted to obtain approx. $1x10^5$ conidia ml^{-1}.

1.2.2. Enzyme assays

PG activity was determined at different pH by viscosimetric and reducing-end groups assays. Viscosimetric activity was assayed by measuring the decrease in relative viscosity at 30°C of a 2 ml reaction mixture containing 1 ml of 1% (w/v) polygalacturonic acid sodium salt substrate (PGA, 85% titration; Sigma-Aldrich, Milano, Italy) dissolved in 50 mM sodium acetate buffer (at pH 4.0, 4.6, 5.0 and 6.0), and in 50 mM Tris HCl buffer (at pH 7.0 and 8.0), and 1 ml of the enzyme suitably diluted in the same buffer. The buffers contained also BSA at 0.1 mg/ml. Micro-Ostwald capillary viscosimeters (i.d. = 0.70 nm), connected to AVS 310 system (Schott Geräte, Mainz, Germany), were used. One enzyme unit was defined as the amount of enzyme causing a 50% decrease of the initial relative viscosity of the reaction mixture in 1,000 min. At 50% decrease of the initial relative viscosity aliquots of 100 μl of the reaction mixture were assayed for reducing end-groups by the method described by Milner and Avigad (1967), as reported in Sella et al. (2004). One enzyme unit was defined as the amount of enzyme required to release 1 nmol/s of reducing groups (nkat).

1.2.3. Analysis of the PG pattern and purification of PG isoforms

To analyse the isoform pattern of the PG activity produced during the fungal growth, *F. graminearum* was grown for different times (from 6 to 72 hours) as above reported. At each time-point the content of two flasks was pooled, filtered through Sartorius MGA membranes, and then, in succession, through cellulose acetate membranes with pore size of 0.8 µm, 0.45 µm and 0.2 µm (Sartorius, Milano, Italy). The mycelium collected by filtration was frozen in liquid nitrogen and stored a -80°C for successive RNA analysis. The culture filtrates were brought with $(NH_4)_2SO_4$ to 20% of the saturation value and, after a rest interval of 3 h at 4°C, it was centrifuged for 30 min at 4°C at 10,000 g. The supernatant was brought to 75% saturation with $(NH_4)_2SO_4$ and conserved overnight at 4°C. The mixture was then centrifuged for 40 min at 4°C at 25,000 g, the pellet was resuspended in 1 ml Milli-Q quality water (Millipore, USA) and dialyzed. An equal volume of each sample (30 µl) was analysed by analytical IEF using a 0.8 mm thick polyacrylamide (PAA) gel containing 1.6 % (v/v) carrier ampholytes, covering the pH range 6.0-8.0 (Sigma-Aldrich, Milano, Italy) or the pH range 8.0-10.5 (Amersham Biosciences, Uppsala, Sweden). PG isoforms were detected by the agarose overlay technique described by Ried and Collmer (1985). IEF standard (Broad pI pH 3.0-10.0) was from Pharmacia.

For PG isoforms purification the contents of 20 flasks of 4-days-old culture were pooled and filtered as above described. The filtrate was concentrated and diluted three times with 150 ml of Milli-Q quality water using the membrane Vivaflow 200 (10,000 MWC PES) (Sartorius, Milano, Italy) to a final volume of 50 ml. This sample was isoelectrofocused by preparative IEF using ampholytes pH range 3.0-10.0 (Fluka, Sigma-Aldrich, Milano, Italy) and PG active fractions were analyzed for the isoforms content by analytical IEF. Selected fractions were dialyzed overnight against 50 mM sodium acetate buffer pH 5 and loaded on a cation-exchange Mono-S column (Pharmacia, Uppsala, Sweden), as previously reported (Favaron et al., 1988). Eluted fractions were assayed for PG activity by the reducing end-groups method, and the fraction with the highest activity from each PG peak was analysed for isoform pattern. These fractions were also analysed by SDS-PAGE according to Laemmli (1970) and stained with Coomassie Brilliant Blue R (Sigma-Aldrich, Milano, Italy). PG proteins (approximately 10 µg each) of these fractions were also electroblotted on Immobilon-P membrane (Millipore, USA), stained with Coomassie Brilliant Blue R

(Sigma-Aldrich, Milano, Italy) and subjected to N-terminal sequence analysis at the Molecular Structure Facility of the University of California, Davis. SDS-PAGE molecular weight standards (low range) were from Bio-Rad Laboratories (Hercules, CA, USA).

1.2.4. Substrate fragmentation pattern

The purified PGs (0.33 nkat) were incubated for 1 h at 30°C with 1 ml of 0.5% (w/v) PGA dissolved in 50 mM sodium acetate buffer at pH 5.0 or in 50 mM Tris HCl buffer at pH 7.0 or 8.0. After incubation, the mixture was boiled for 10 min to stop the enzyme reaction, it was diluted 1:3 with 20 mM Tris HCl buffer at pH 7.9 and the hydrolysis products were separated by a Mono-Q HR5/5 anion–exchange chromathographic column with a linear gradient of NaCl (from 0 to 1 M) as previously reported (Favaron et al., 1992; Sella et al., 2004). Uronides content was determined in each fraction as reported by Blumenkrantz et al. (1973).

1.2.5. Inoculation of wheat plants and sampling

Wheat seeds (cv. Bobwhite) were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 20 min, and then rinsed thoroughly in sterile water. Plants were grown in climatic chamber with a 14 h photoperiod and 20/16°C day/night temperature. At anthesis (Zadoks stage 65-67; Zadoks et al., 1974), spikes were inoculated by dropping between the bracts of two florets of two opposite spikelets 10 µl of a fresh conidial suspension, containing approximately 1,000 conidia. The inoculated plants were then covered for 3 days with a plastic bag to maintain high moisture, and the temperature of the climatic chamber was increased to 22/18°C. The inoculated spikelets were harvested at different times starting from 6 h until 12 days. The spikelets were frozen in liquid nitrogen and stored at –80°C for successive RNA analysis. Mock inoculated spikes served as controls.

1.2.6. Determination of pH

Two infected spikelets, weighing approx 100 mg f.w., were collected at 7 days after inoculation with *F. graminearum*, and homogenised in 0.6 ml of distilled water using a

potter. After a brief centrifugation (5 min) at 10,000 g, the pH level of the supernatant was measured. Mock inoculated spikelets were used as control.

1.2.7. Extraction of RNA and primers design

Total RNA was extracted from samples of 6 infected spikelets and from 100 mg of frozen mycelium obtained from liquid culture with the RNeasy Plant Mini Kit (Qiagen, Milano, Italy), following the manufacturer's instruction. Contaminating DNA was removed using DNA-free TM (Ambion, TX, USA).

Forward and reverse primers specific for the *endo-pg* genes (*Fgpgu1* and *Fgpg*; MIPS database entry fg11011 and fg03194), the *pnl* genes (*Fgpnl*; MIPS database entry fg01607, fg03483 and fg03121), the *xylanase A* gene (*Fgxyl A*; MIPS database entry fg10999) and the rRNA 18S gene (*Fg18S*; NCBI accession number AB250414) were designed using the Primer 3 software (Rozen and Skaletsky, 2000) and have the following sequences: FgPGU1F   CATCAAGACCGTTTCTGGAG,   FgPGU1R TAGGGGTACCAGTGGGAGAG, FgPGF GCGTCAAGGCTTTCAAGAAC, FgPGR TAGGGTCTCCGTGGAGGTCA, FGPLF CATCCAGAACGTTCACATCAC, FGPLR GACGGCCGATGAGAGAGGT, FgXYLF GGATGGAACCCTGGTACTGG, FgXYLR GTCGATCGAAGGCTGTTGG, Fg18SFA CACCAGGTCCAGACACAATG, Fg18SRA CAAACTTCCATCGGCTTGA.

1.2.8. Real time RT-PCR (qRT-PCR)

qRT-PCR experiments were performed using the *iCycler iQ* (Bio-Rad Laboratories, Hercules, CA, USA) and the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Milano, Italy) containing the fluorogenic dye SYBR® Green I. Two sets of experiments were performed: one using total RNA extracted from mycelium obtained in liquid culture (6, 12, 24, 48, 72 h) as template, the other using total RNA obtained from infected spikelets at different times (6, 12, 24, 48, 72, 96, 144, 192, 288 hpi). The experimental conditions were the same as reported in Sella et al. (2005).

Relative expression analysis was determined by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Applied Biosystems User Bulletin No. 2-P/N 4303859). Calculation and

statistical analysis were performed by Gene Expression Macro™ Version 1.1 (Bio-Rad Laboratories, Hercules, CA, USA). The efficiencies of target and endogenous genes were determined by performing qRT-PCR on serial dilutions of RNA template over a 100-fold range (Livak and Schmittgen, 2001), and resulted similar. Real time RT-PCR experiments were carried out on RNA extracted from two separate experiments of plant inoculation and liquid culture. Results of a single test per set of experiment were reported.

1.3. Results

1.3.1. PG activity in liquid culture

F. graminearum was grown on a mineral medium with pectin as the sole carbon source to monitor the activity of PGs produced by the fungus in liquid culture. The PG activity, determined both viscosimetrically and by reducing end-groups assay, reached a maximum 4 days after inoculation and then maintained a steady value in the next two days (Fig. 1). The isoenzyme pattern secreted during the first 4 days of culture showed the prevalence of a band with isoelectric point (pI) at 8.15. This isoform was already present after 24 hours of culture and its activity increased then after (Fig. 2). Few weaker PG bands appeared after 48 hours of culture and most of them were localized near the prevalent PG isoform. At 96 hours another isoform with pI 9.1 was barely visible (Fig. 2).
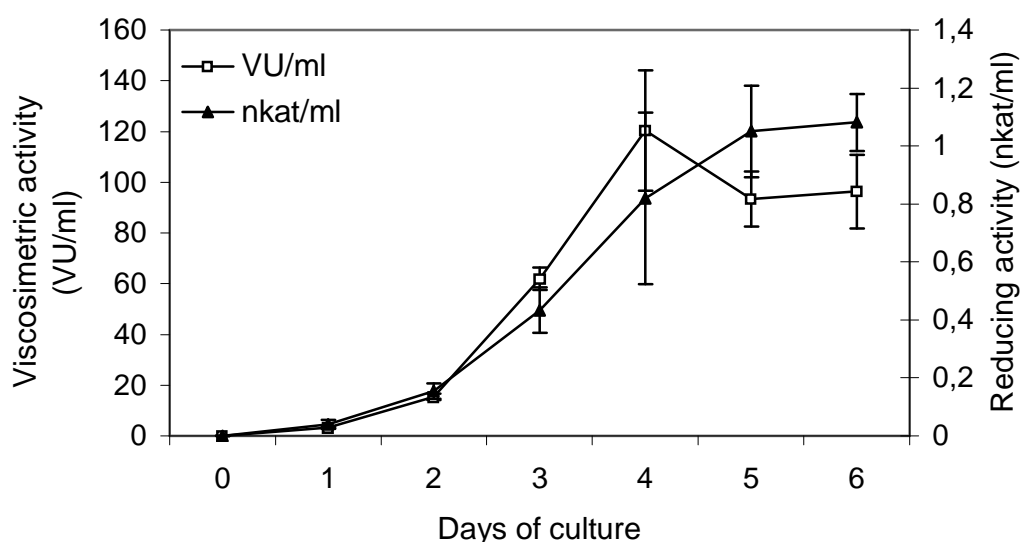

Figure 1 – PG activity produced by *F. graminearum* in liquid culture measured at pH 4.6 by reducing-end groups (—▲—) and viscosimetric (—□—) assays expressed in nkat and in viscosimetric units (VU), respectively. Each data point represents the mean of three determinations±SD carried out on three different flasks.
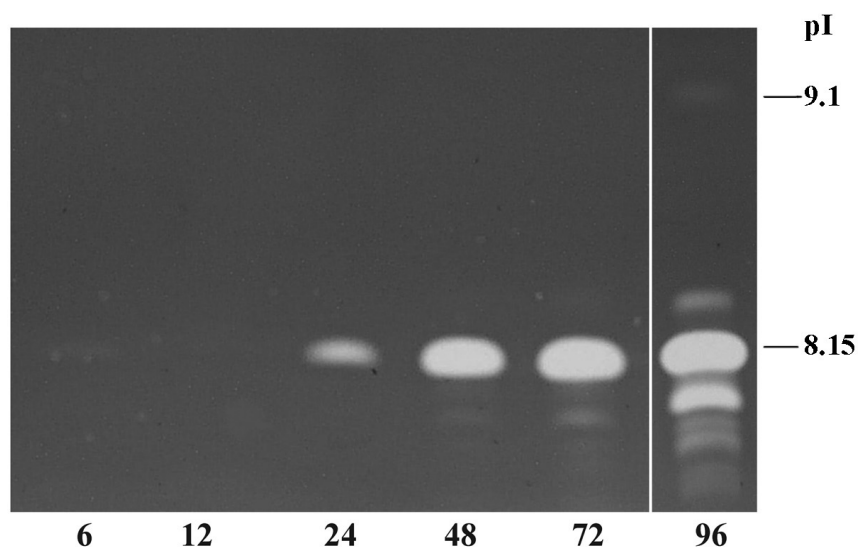
Figure 2 – Thin layer IEF in the pH range 6.0-10.5 of crude PG activity produced by *F. graminearum* in liquid culture at different times (6, 12, 24, 48, 72 and 96 h of culture). PG activity contained into two flasks was precipitated by $(NH_4)_2SO_4$, dialyzed and resuspended in 1 ml of water. Thirty μl of each sample were loaded. pI values are indicated on the right.

1.3.2. Purification and characterization of the *endo*-PG isoforms

As above reported several PG isoforms were present in the crude PG extract of *F. graminearum* (Fig. 2). However, only two putative genes encoding *endo*-PGs have been annotated in the genome of *F. graminearum* (database entry fg11011 and fg03194 in the MIPS database) with theoretical pI of 7.0 and 9.1, respectively. In order to characterize these two PGs, the PG isoform with pI 9.1 and the more abundant with pI 8.15 were purified from the culture filtrate of *F. graminearum*. After separation by preparative IEF, several active fractions were obtained containing the PG with pI 8.15 and one of them was selected for the successive analysis (Fig. 3A, lane 1). The few fractions containing both the isoforms with pI 8.15 and 9.1 were pooled (Fig. 3A, lane 2) and loaded upon a Mono-S column. This step allowed the separation of the two isoforms labelled PG1 and PG2, respectively (Fig. 3B, lanes 3 and 4). The purified PGs, when submitted to SDS-PAGE analysis, presented a single protein band with estimated molecular mass of about 42 KDa for PG1 and 43 KDa for PG2 (Fig. 4). N-terminal sequence analysis showed that PG1 and

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PG2 corresponded respectively to the gene sequences fg11011 and fg03194 in the MIPS database (Table 1), consistently with theoretical and measured pIs.

Table 1 – N-terminal sequences of *F. graminearum* PG1 and PG2 and corresponding MIPS database entry of their encoding genes.

Protein	N-term	MIPS entry
PG1	ASCTF	fg11011
PG2	ATSC	fg03194

The influence of pH on the activity of the two purified PG isoforms was assessed in the pH range 4.0-8.0. The results showed that PG1 was active at pH values ranging from 4.0 to 7.0, with a pH optimum at 5.0 or 7.0 depending if the reducing-end groups or viscosimetric method were used, respectively. This PG was inactive at pH 8.0 (Fig. 5). PG2, instead, was poorly active at pH below 6.0 and showed pH optima of 7.0 and 8.0 when assayed with the two different methods, respectively (Fig. 5).

The cleavage patterns of the two PGs after incubation at different pH optima with the PGA substrate were analyzed by chromatographically separating the oligomeric products. At pH 5.0, PG1 produced relatively larger amount of monogalacturonic acid (47 μg) and mainly oligogalacturonides from dimer to pentamer; instead, at pH 7.0, this PG produced smaller amount of monogalacturonic acid (17 μg) and higher amount of oligomers longer than trimer (Fig. 6). PG2 presented similar profiles at the two pHs, but the oligomers with higher degree of polymerization were more represented at pH 7.0 than at pH 8.0 (Fig. 6). It is worth noting that the first peak, corresponding to the dimer, was apparently absent in the digestion mixture obtained with PG2.

1.3.3. pH values in wheat infected spikelets

The pHs of health and infected spikelets were measured 7 days after inoculation with *F. graminearum*. The pH of the mock inoculated spikelets was 6.1, while that of the inoculated spikelets was 7.1.
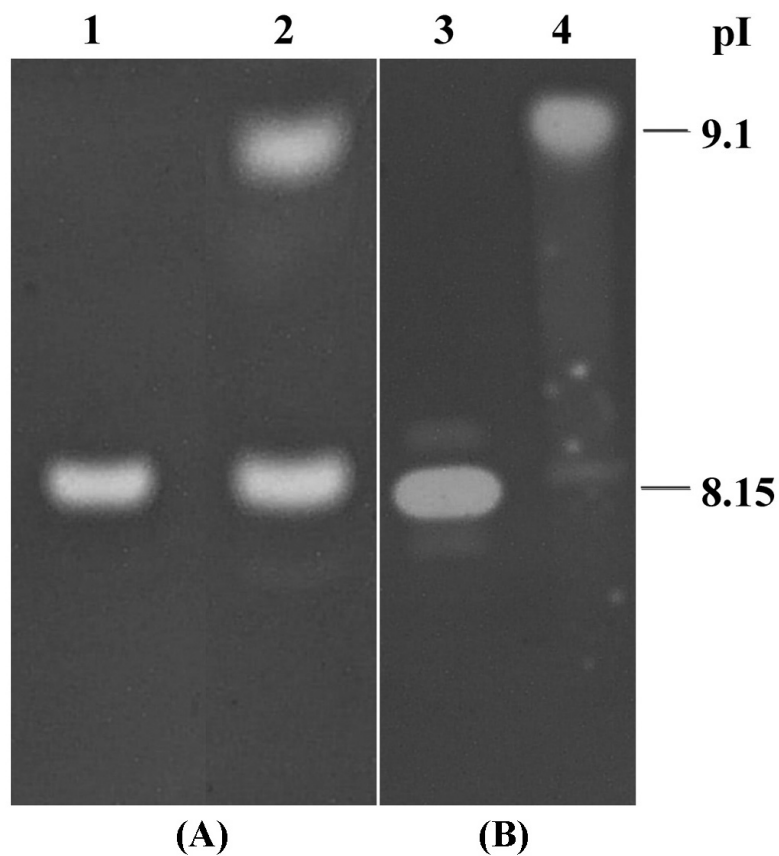
Figure 3 – Thin layer IEF in the pH range 6.0-10.5 of *F. graminearum* PG isoforms during purification. **(A)** After preparative IEF (pH range 3.0-10.0) fractions were obtained containing only the PG1 isoform (lane 1, as an example), or both PG1 and PG2 isoforms (lane 2, as an example). **(B)** The fractions containing both PG isoforms were pooled and loaded upon a Mono-S column to separate PG1 (pI 8.15, lane 3) from PG2 (pI 9.1, lane 4). On each lane about 0.83 nkat of PG activity, as determined at their optimum pH (pH 5.0 or pH 7.0 for PG1 and PG2, respectively), were loaded on the gel. Estimated pIs are reported on the right.
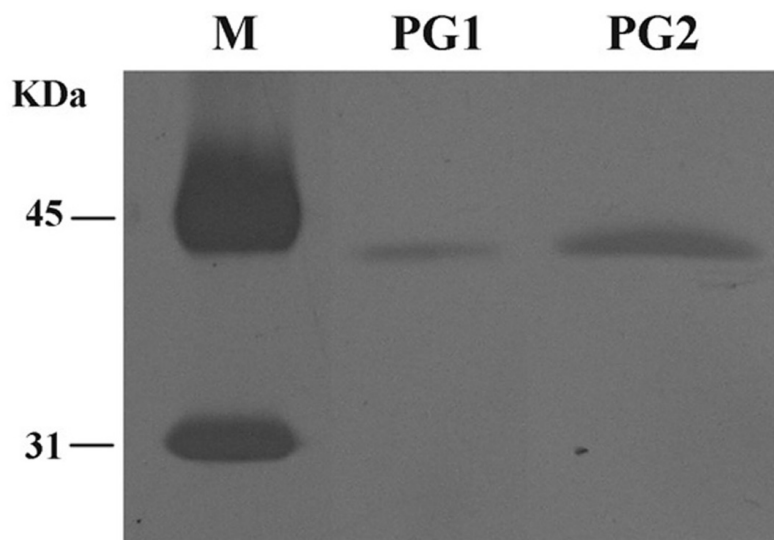
Figure 4 – SDS-PAGE analysis of the purified PG1 and PG2 isoforms. Protein (about 0.67 nkat of PG1 and 0.61 nkat of PG2) was stained with Coomassie Brilliant Blue R. M: molecular weight standard (low range; Bio-Rad Laboratories); molecular masses are shown on the left.

Figure 5 – Effect of pH on PG1 and PG2 activity. Activity was determined viscosimetrically (—□—) and by the reducing end-groups assay (—▲—). The PGA substrate (0.5% w/v) was dissolved in 50 mM sodium acetate buffer for pH values from 4.0 to 6.0, and in 50 mM Tris HCl for pH 7.0 and 8.0. Each data point represents the mean of three determinations±SD.

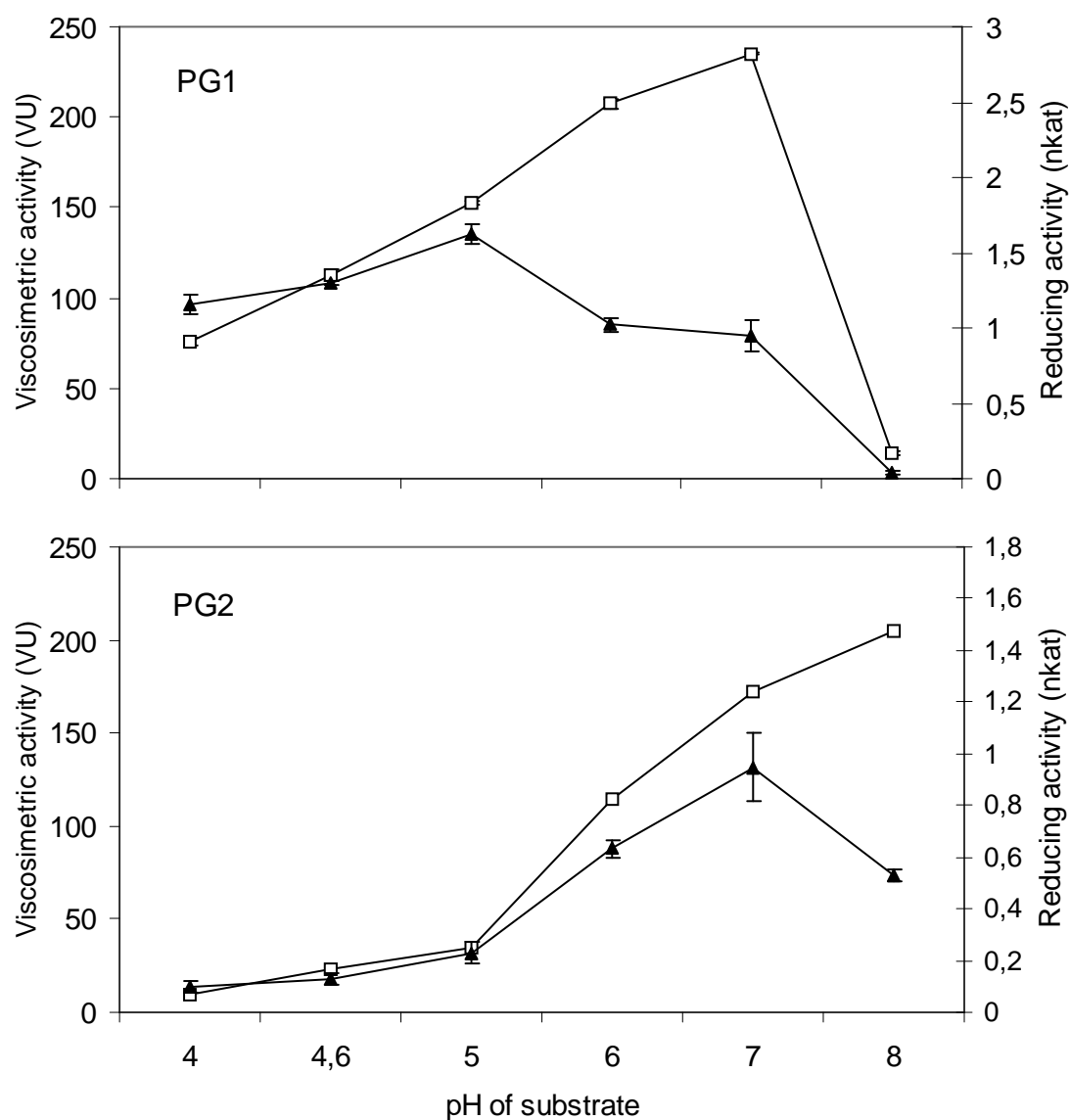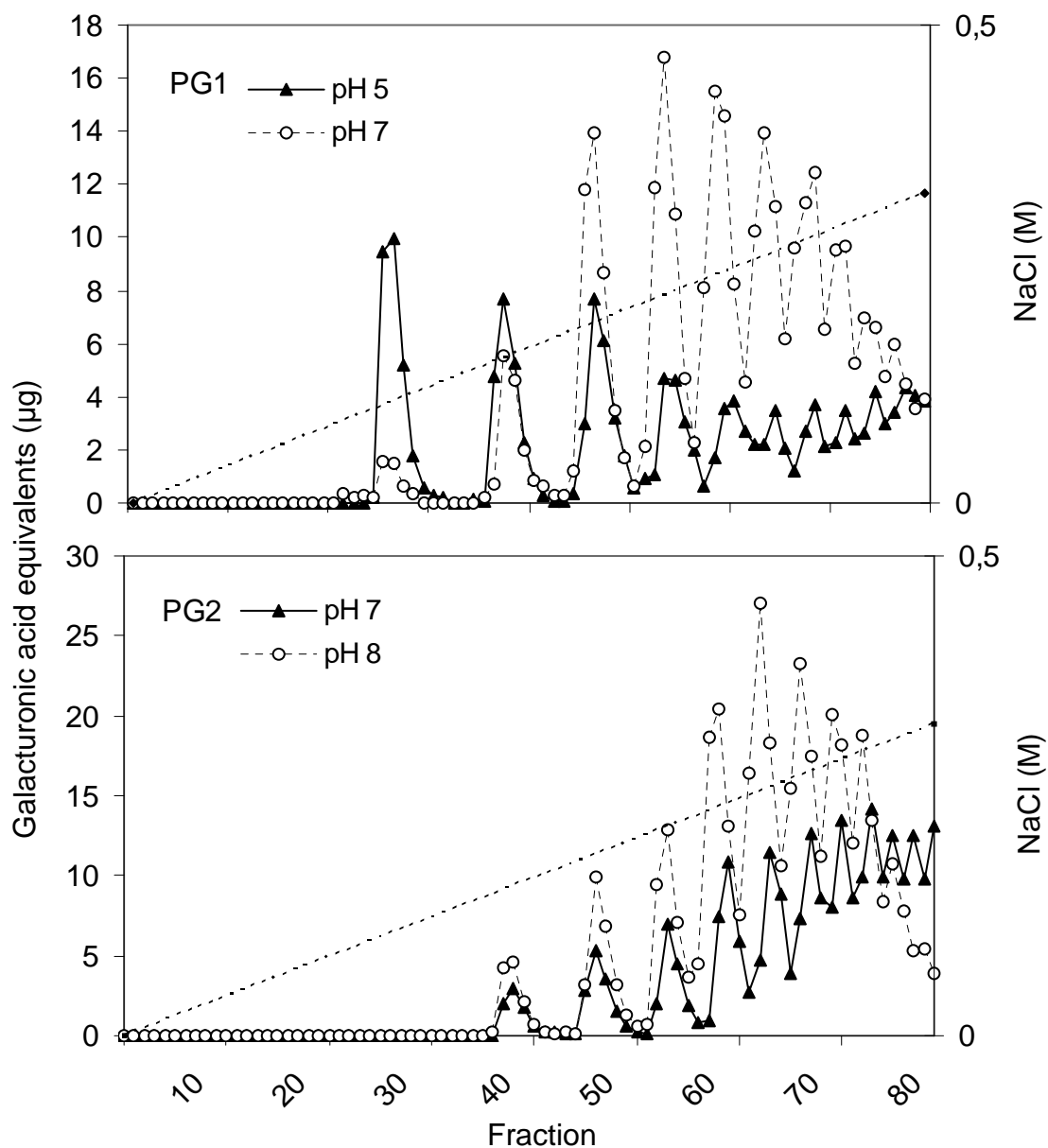**Figure 6** – MonoQ-column separation of oligogalacturonides released from the PGA substrate (0.5% w/v) after PG1 and PG2 digestion at different pH values. 0.35 ml of the incubation mixture was diluted 1:3 with buffer A (20 mM Tris HCl pH 7.9) and loaded onto a Mono-Q HR5/5 column. Ten fractions of 1 ml were collected before the start of the gradient (····). Eighty fractions (0.25 ml) were collected after the start of the gradient (buffer A + 1 M NaCl). Aliquots of 100 or 25 μl of the two groups of fractions, respectively, were assayed for uronides content. Monogalacturonate, which elutes before the start of the gradient, was determined to be about 47 and 17 μg in the digestion mixture of PG1 at pH 5.0 and pH 7.0, respectively, and 5.5 and 3.3 μg in the digestion of PG2 at pH 7.0 and pH 8.0, respectively. The first peak eluting with the gradient after PG1 and PG2 digestion are the digalacturonate and the trigalacturonate, respectively. Oligomers with increasing degree of polymerisation follow in succession (Favaron et al., 1992).

1.3.4. Analysis of gene expression in liquid culture and during wheat infection

qRT-PCR experiments were performed to quantify and compare the temporal expression of the two *pg* genes (fg11011 and fg03194) in liquid culture and during wheat infection. For the relative quantification *F. graminearum* rRNA 18S was used as housekeeping gene.

In liquid culture, using total RNA extracted from mycelium, the transcripts of both *pg* genes were already detectable at 6 h, the first data point analyzed (Fig. 7). *Pg1* reached the maximum after 24 h from inoculation with a 10 folds increase of the transcript level, while *pg2* gene reached a maximum of expression earlier, at 12 h, but the transcription level increased only 3 folds (Fig. 7).

The expression analysis was also performed during *F. graminearum* infection of wheat using total RNA extracted from infected spikelets (Fig. 8). In this analysis the expression of *xyl A* gene, encoding a *F. graminearum* xylanase, and the transcripts of the three *pnl* genes were also considered. For the *pnl* genes, a couple of primers was designed in order to amplify all the three genes. The results displayed that *pg2* gene was expressed at 6 hpi, earlier than *pg1* and *pnl*. Moreover, all the genes encoding pectic enzymes reached the maximum of expression 24 hpi (about 4 fold increase for *pg1* gene, 10 fold increase for *pg2* and 1.3 fold increase for *pnl*). These genes were expressed earlier than the *xyl A* gene, which was strongly induced only 48 hpi reaching the maximum at 96 hpi with a relevant increase in the transcript level (110 fold) (Fig. 9).
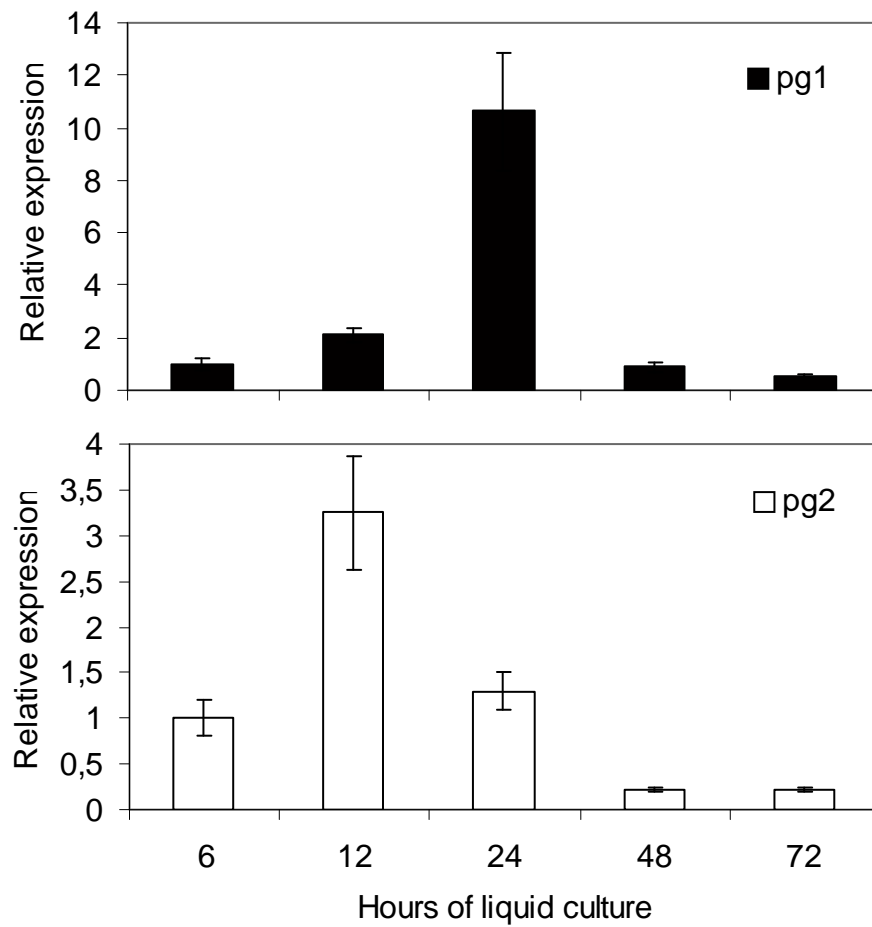
Figure 7 – Quantification of *F. graminearum pg1* and *pg2* transcript accumulation during liquid culture. Each transcript was normalized with the *F. graminearum* rRNA 18S gene as housekeeping. A single qRT-PCR test of two different experiments is reported. Error bars represent standard errors.
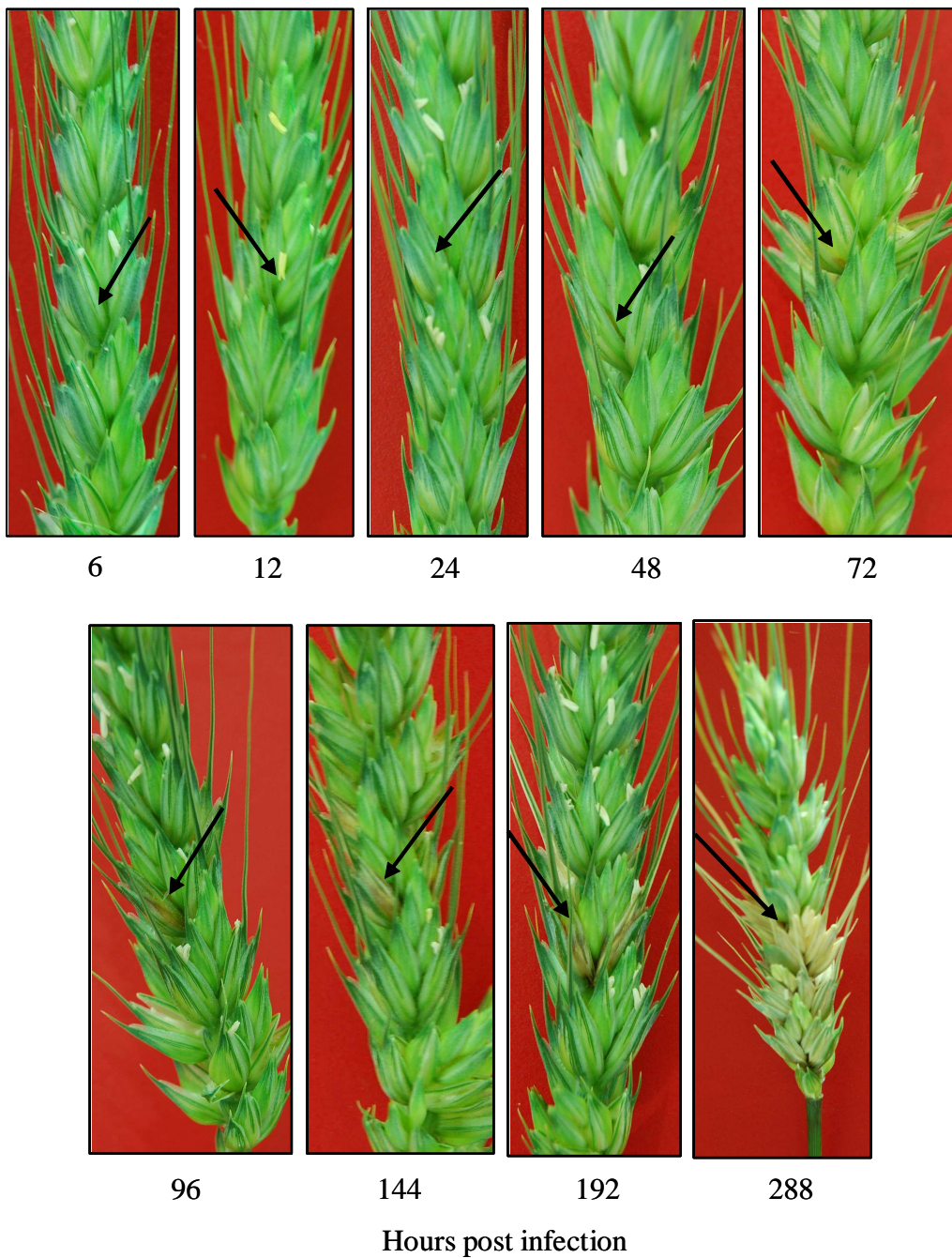
Figure 8 – Wheat spikes infected with *F. graminearum* and collected at different time points post inoculation. Two florets of two opposite spikelets per spike were inoculated with 10 µl of conidial suspension containing approximately 1,000 conidia. Arrows indicate inoculation site.

Figure 9 – Quantification of *F. graminearum pg1*, *pg2*, *pnl* and *xyl A* transcript accumulation during wheat spikelets infection. Each transcript was normalized with the *F. graminearum* rRNA 18S gene as housekeeping. A single qRT-PCR test of two different experiments was reported. Error bars represent standard errors.
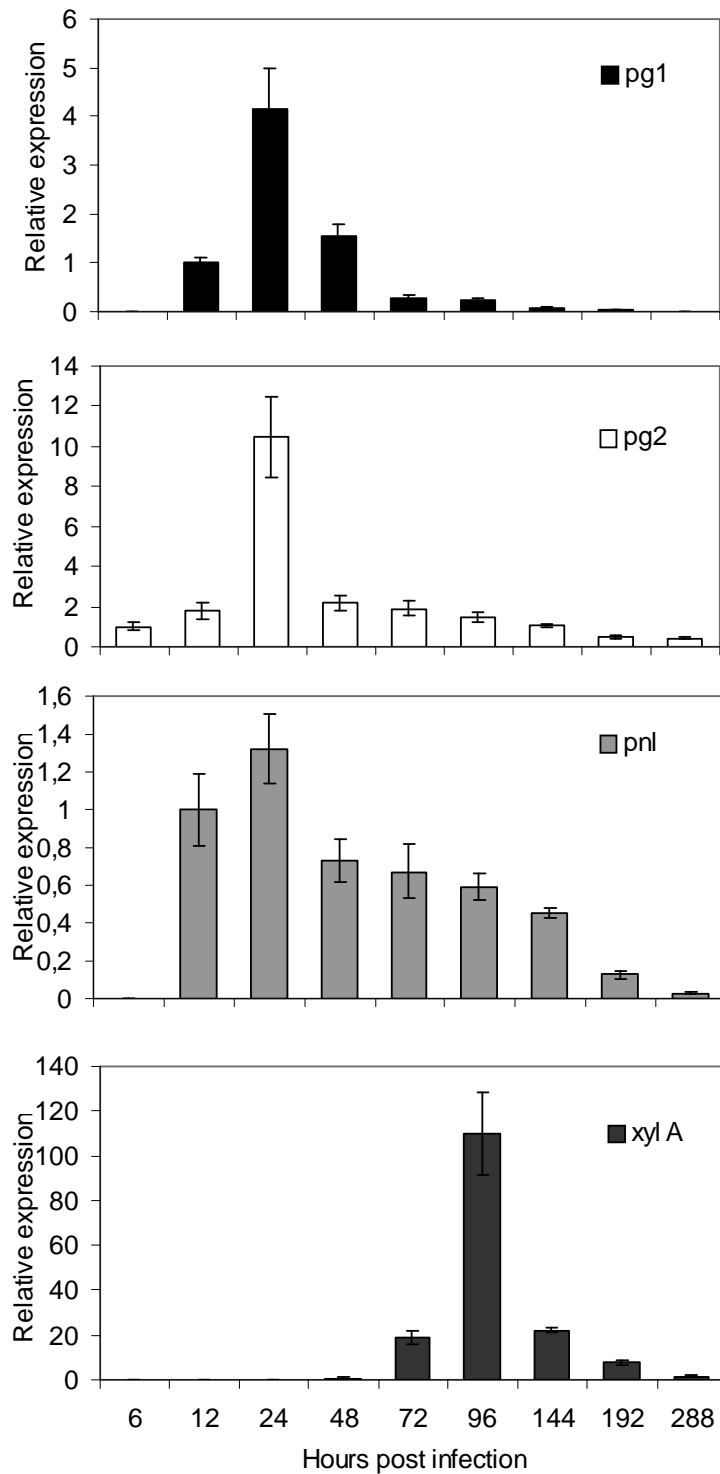
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1.4. Discussion

F. graminearum colonizes different organs during infection of wheat plants. The infection starts from anthers and ovary, proceeds to spikelet bracts and finally reaches the vascular and parenchyma tissue of the rachis (Miller et al., 2004; Wanjiru et al., 2002). As shown by immunocytological labelling of plant cell wall components, the infection process is likely assisted by the activity of different types of CWDE (Wanjiru et al., 2002). To address the involvement of pectic enzymes in the infection process I first characterized the two *endo-*PG proteins produced by *F. graminearum* in liquid culture, and then I monitored the expression of these two PGs during wheat spike infection. Expression analysis was also extended to some pectin lyase and one xylanase genes.

As revealed by N-terminal Edman sequencing, the two PG isoforms purified from in vitro culture correspond to the two *pg* genes annotated in the *F. graminearum* genome database. In culture the major difference between the two PGs regards their expression and activity. As observed by gel activity assay, PG2 (the product of the fg03194 gene) appeared largely overcome by PG1 (the product of the fg11011 gene), and this feature seems explained by a lower expression of the PG2 gene as revealed by qRT-PCR analysis. Besides, the gel activity assay was performed at pH 4.6 and could have underestimated the activity of PG2 which has a pH optimum near 7.0. A neutral pH optimum is rather unusual for fungal PGs that mainly prefer acidic or sub-acidic values.

As shown by viscosimetric and enzyme product analyses, the pH modifies also the pattern of substrate degradation. This was particularly evident with PG1 where the rise of pH from the optimum value of 5 to 7 increases the ratio of longer oligomers upon the shorter ones and reduces the release of monogalacturonate indicating a shifting of the enzyme hydrolysis cleavage towards more internal linkages. Thus at pH 7.0 both PG1 and PG2 showed a similar pattern of substrate degradation. A more internal cleavage of the pectin network is usually associated to a greater macerating activity of the enzyme. Interestingly, the pH values measured in healthy and infected wheat spikelets indicate that the fungus increases the pH of the tissue during the infection process from about 6.0 to 7.0. Alkalinization of tissue might also promote the activity of other pectinolytic enzymes such as pectate lyase and pectin lyase (Szécsi, 1990; Guo et al., 1995; Di Pietro and Roncero, 1996). The observed increase of pH in the infected tissue is in agreement to the raise of pH of

apoplastic fluids measured by Aleandri et al. (2007) after infection of wheat seedlings with the closely related species *F. culmorum*. Therefore, *F. graminearum* by secreting PG1 and PG2 would guarantee the PG activity over a broad pH range (from 4 to 8) and the rising of pH during infection could increase the macerating activity of PG1.

Expression analysis during *F. graminearum-T. aestivum* interaction shows that transcription of both *pg* genes occurs within the first 12 h after spike inoculation and peaked at 24 h. However, the *pg2* transcript showed an earlier appearance, a greater fold increase and a more prolonged expression in comparison with the *pg1* transcript. This finding partially subvert the expression pattern observed in liquid culture where PG2 is less represented than PG1, and suggest that PG2 could play a more important role during wheat spikelet infection. The timing of expression of the *pg* genes seems consistent with the characteristics of the tissue initially infected after spikelet inoculation. In fact, Miller et al. (2004) observed that the fungus affects the ovary within 12 h and homogalacturonans and methyl-esterified homogalacturonans have been shown to be abundant constituents of ovary cell wall in grasses as rye (Tenberge et al., 1996). Thus the degradation of the spikelet soft tissue may be achieved with the contribution of the two PGs here characterized. Pectin and pectate lyase activity may also contribute to this process and indeed an expression pattern similar to that of the *pg1* gene was observed when the bulk of 3 *pnl* genes was monitored, although a more steady-state expression of these genes occurred from 12 h until 6 days after inoculation.

Xylanase activity has been strongly suggested to play a prominent role in fungal pathogenesis of cereal plants because the cell wall of these plants are particularly enriched in the arabinoxylan component (Carpita and Gibeaut, 1993; Giesbert et al., 1998; Kang and Buchenauer, 2000; Lalaoui et al., 2000; Oeser et al., 2002). Indeed 12 xylanase or putative xylanase gene sequences are listed in the *F. graminearum* database. Recently, the X*yl A* gene (MIPS database entry fg10999) has been reported as one of the few *F. graminearum* xylanase genes early expressed during barley infection and with the highest transcript level (Güldener et al., 2006). The expression analysis of this gene shows that *XylA* transcript appears later than the *pg* and *pnl* transcripts, in fact it become detectable only 2 days after spike inoculation reaching the maximum level at 3 days. Thus the timing of xylanase expression seems more consistent with hyphal colonization of the floral bracts, a process

slower than ovary infection and accomplished between 3 and 5 days post inoculation (Miller et al., 2004).

Recently a proteomic analysis of wheat spike harvested from 3 to 14 days after infection with *F. graminearum* revealed the presence of the products of five xylanase genes (including that of the *XylA* gene), one pectin lyase gene and none polygalacturonase gene (Paper et al., 2007). This finding further supports the importance of xylanase genes whilst the lacking of detection of PG and most pectin lyase products may be due to the low titre of these proteins, expressed mainly at the earliest phase of plant infection (1-3 days).

Taken together, these results suggest a possible role of pectinases, and mainly PGs, in the initial establishment of the fungus on soft host tissues, whilst xylanases seem involved later in fungal proliferation on harder and more lignified floral tissue. The expression results obtained with *F. graminearum* are consistent with those obtained with other fungal wheat pathogens: Kang and Buchenauer (2000) showed that in the cell wall of wheat tissues infected with *F. culmorum* the degradation of pectin was greater than that of xylan and cellulose; Douaiher et al. (2007) growing *M. graminicola* in vitro, observed that the secretion of pectinases was earlier than that of xylanases and suggested that a similar behaviour may be retained during wheat leaf penetration and colonization.

The characterization of the functional properties of *F. graminearum* PGs and the expression analysis of their encoding genes during wheat infection shed new light upon the possible role of these enzymes in the infection process. However, to fully clarify the role of the two PGs during the infection process, I knocked-out their encoding genes by targeted homologous recombination and the virulence of the mutants was determined by wheat infection experiments. These results are reported in the second chapter of this thesis.

Chapter II

Gene disruption approach to investigate the role of *Fusarium graminearum* and *Fusarium verticillioides* polygalacturonases during plant infection

2.1. Introduction

The characterization of fungal genes involved in pathogenesis is an essential step for the production of plants more resistant to diseases caused by these pathogens. The targeted disruption of genes by transformation-mediated homologous integration is a valuable method to define the function of a gene of interest (Schäfer, 1994; Struhl, 1983). Moreover, the availability of a highly efficient procedure for transformation is a key point in exploring genomes with a high throughput. This strategy has been established only in some plant pathogenic fungi like *F. graminearum* (Voigt et al., 2005) and *Cochliobolus heterostrophus* (Degani et al., 2004).

The main strategy to disrupt genes in fungi (May, 1992) is based on a vector cassette where the marker gene for clone selection, usually the hygromycin resistance gene *hph* (Punt et al., 1987), is flanked by DNA sequences homologous to the flanking regions of the target gene. In this case, a double crossover is necessary for gene replacement (Aronson et al., 1994; Royer et al., 1999). A different approach, named split marker technology, uses PCR reactions to fuse the regions flanking the target gene with overlapping parts of the selectable marker gene (Catlett, 2005). In this case, disruption of the target gene occurs by a triple crossover event.

In general, the efficiency of the gene targeting depends on the length of the homologous regions, as well as the transcriptional status of the target loci (Maier and Schäfer, 1999). Furthermore, gene targeting efficiency is also strongly species-dependent and is correlated with the dominant pathway of DNA double-strand break repair (Schäfer, 2001). For example, in the haploid fungus *F. graminearum* the predominant mechanism to repair double strand breaks most probably occurs by homologous recombination (Maier et al., 2005).

Aim of the present work was to evaluate the importance of *F. graminearum* and *F. verticillioides* PGs during the infection process of some host species through knock-out of their *pg* encoding genes by targeted homologous recombination.

In particular, I disrupted each single *pg* gene of *F. graminearum*, encoding the *endo*-PGs purified and characterized in the previous chapter and showing different functional properties, and the *F. verticillioides pgIII* gene encoding an *endo*-PG highly secreted in liquid culture and previously characterized (Sella et al., 2004; Raiola et al., in press).

The virulence of knock-out mutants has been evaluated on host plants like maize in the case of *F. verticillioides* and wheat for *F. graminearum.*

2.2. Materials and methods

2.2.1. Fungal strains and culture conditions

F. graminearum strain 8/1, kindly provided by Prof. Dr. W. Schäfer (Voigt et al., 2005), and *F. verticillioides* strain PD were cultured at 23°C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). To obtain mycelium for DNA extraction, wild-type and mutant strain were grown in 50 ml complete medium (CM; 1% (w/v) glucose, 0.05% (w/v) yeast extract, 0.5% (w/v) yeast nitrogen base) for 3 days at 24°C.

F. graminearum wild-type and mutant strains were cultured on SNA agar plates (Urban et al., 2002) to induce conidiation. Conidia were recovered by scraping the agar plates with sterile water and a sterile glass rod.

2.2.2. Nucleic acid extraction and Southern blot analysis

Genomic DNA from *F. graminearum* and *F. verticillioides* wild-type and mutant strains was extracted from the mycelium obtained in liquid culture. After addition of 1 ml of 2X CTAB (2% (w/v) CTAB, 100 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% (w/v) PVP, 1% (w/v) dithiothreitol) per 100-200 mg of mycelium, the mixture was vortexed and then incubated at 65°C for 1 h. After addition of an equal volume of chloroform/isoamyl alcohol solution (ratio 24:1), the mixture was mixed at 150 rpm and incubated in ice for 1-2 h. The solution was then centrifuged at 9,000 rpm for 15 min, and an equal volume of isopropanol with 1/10 in volume of sodium acetate 3 M pH 5.2 were added to the supernatant. After incubation at -20°C for 20 min, the sample was centrifuged at 9,000 rpm for 10 min to precipitate DNA, and the pellet obtained was washed with cooled ethanol 70%, dried and redissolved in 400 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA). Contaminating RNA was removed using RNase A (Fermentas, Milano, Italy).

For Southern blot analysis approximately 3 µg of genomic DNA was digested with *Xba* I (Promega, Milano, Italy), separated on a 1.0% (w/v) agarose/TAE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Milano, Italy). Digoxygenin (DIG)-labeled (Roche, Mannheim, Germany) DNA probes specific for the knocked-out genes and the hygromycin gene were alternatively used for overnight hybridization at 65°C. The

probes were generated with gene specific primers (Table 1) using genomic DNA as template. The PCR reaction, performed using DIG-11-dUTP (Roche, Mannheim, Germany) in a 50 μl volume, was initiated by denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 58°C for 30 sec and 72°C for 1 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, USA).

Table 1 – Primers used for the preliminary screening of mutants and for amplification of the DIG-labeled DNA probes.

Hyg5PRB	CTCGATGAGCTGATGCTTTG	Hygromycin gene
Hyg3PRB	CTTGTTCGGTCGGCATCTAC	
PGIIISF	TGACTTTACAAGGCCTGACC	*F. verticillioides pgIII* gene
PGIIISR	TGCTGATGTTGGTAAGAGCA	
fg11011-5int	ATCTCCGGCGCTGTCGTCAA	*F. graminearum pg1* gene
fg11011-3int	GCAGCTAGCGCAAAGAATGTA	
fg03194-5int	GCCCAAGTTCTTCGCTGCTCACA	*F. graminearum pg2* gene
fg03194-3int	GAAGGCCGCTGGTAGGGTCTCC	

2.2.3. Construction of gene replacement vector and fungal transformation-mediated gene disruption

To generate the constructs for disruption of the *F. graminearum pg1* and *pg2* genes and *F. verticillioides pgIII* gene, flanking homology regions of each gene were amplified by PCR using genomic DNA of each fungus as template. The flanking homology regions are necessary to obtain targeted homologous recombination.

In details, specific oligonucleotides were designed to amplify upstream (primers 1 and 2) and downstream (primers 3 and 4) flanking regions of each gene (Table 2 and Fig. 1A). Lengths of upstream and downstream flanking sequences were about 1,000 bp for *F. graminearum pg* genes and 750 bp for *F. verticillioides pg* gene. The amplifications of the flanking regions were performed in a 50 μl volume using genomic DNA as template. The PCR conditions were as follows: 94°C 3 min, followed by 35 cycles of denaturation at

94°C for 1 min, annealing (Table 3) for 30 sec and extension at 72°C for 1 min. The amplicons obtained were cut from agarose gel, purified and used in a second PCR to fuse the homologous flanking regions with the hygromycin resistance gene *hph* (hygromycin B phosphotransferase gene) used as selection marker (Punt et al., 1987). The fusion PCR reaction (Fig. 1B) was performed in a 50 μl volume using 100 ng of the purified flanking regions containing tails homologous to the 5' and 3' region of the *hph* gene and 300 ng of *hph* gene cut with *Sma* I (Fermentas, Milano, Italy) from pGEM-T vector (Promega, Milano, Italy). 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 where the primers 5 and 6 were used to obtain the full construct, and primers pairs 5-8 and 7-6 for the two split-marker constructs, respectively (Table 2 and Figure 1C). The nested PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing (Table 3) for 30 sec and extension at 72°C for 4 min. The amplicons obtained were cut from agarose gel, purified and then cloned into pGEM-T easy vector (Promega, Milano, Italy) following the manufacturer's instruction, to obtain enough amount for transformation. Full or split-marker constructs, cleaved from the vector by using specific restriction enzymes, were used to transform protoplasts of *F. graminearum* and *F. verticillioides* wild-type strain (Table 4). Protoplast formation was carried out according to Royer et al. (1995); fungal transformation was performed according to Jenczmionka et al. (2003). Transformants were selected on CM Regular medium-agar [0.2% (w/v) Yeast casein-Mix (0.1% (w/v) Yeast extract, 0.1% (w/v) Enzymic hydrolysed casein), 34% (w/v) Saccharose, 1.6% (w/v) Difco^{TM} Agar granulated (Becton, Dickinson and Company, Sparks, MD, USA)] containing 100 and 220 μg/ml of hygromycin B (Duchefa, Haarlem, The Netherlands) for *F. graminearum* and *F. verticillioides* transformants, respectively. Hygromycin resistant mutants were single-conidiated and preliminarily screened by PCR amplification using the following primers: fg11011-5int and fg11011-3int for *Fgpg1* gene, fg03194-5int and fg03194-3int for *Fgpg2* gene, and PGIIISF and PGIIISR for *FvpgIII* gene (Table 1). Transformants with *hph* gene replacing the *pg* coding region were then tested by Southern blot hybridization for single insertion of the disruption construct due to homologous recombination.

Table 2 – Primers used for deletion constructs by fusion PCR method.

|	*Fgpg1* gene	
1	fg03194-5up	TCGTCGTCGCAAACCCTGATACT
2	fg03194-3up	AGATGCCGACCGAACAAGAGCTGTCCCCCATGA ACGCCGAGCCTGAAAATG
3	fg03194-5down	TCAATGCTACATCACCCACCTCGCTCCCCCAACT CTTGTCTCCACTCCCTCTGA
4	fg03194-3down	GTCTGGCGAACATACAATAAAG
5	fg03194-5upnested	GCAACTTGTGGCTGGAAAAC
6	fg03194-3downnested	GGACCCCTACCCGAAGAT
	Fgpg2 gene	
1	fg11011-5up	CCGCGTCGATGAGAACAGAAATAG
2	fg11011-3up	AGATGCCGACCGAACAAGAGCTGTCCCCCATAA AGGCCCGTGTCAGTGTCCAG
3	fg11011-5down	TCAATGCTACATCACCCACCTCGCTCCCCCCTGG CAAAAGACAAAGGAAAATG
4	fg11011-3down	CAAAAGCCGGAAGAATGTCG
5	fg11011-5nested	ACCCCACCCCAATTCTGTCGTA
6	fg11011-3nested	AATGTCCATCCTCGCTCGTTCC
	FvpgIII gene	
1	FvUPF1	ATATTGGGGGTGGCTTATCC
2	FvUPR1	AGATGCCGACCGAACAAGAGCTGTCCCCCGAAA CGAAGAGATGCCGAAG
3	FvDOWNF	TCAATGCTACATCACCCACCTCGCTCCCCCTGTG GGGACTGTCAATTCAA
4	FvDOWNR	TGGTTGTTGGGAATGTGAGA
5	Fv-NESTF2	GAGAAGAATTATGGTGCG
6	FvNESTR	GACATGGGGCTCGATAAAAA
	Hph gene	
7	HygFOR	GTTGGCGACCTCGTATTGG
8	HygREV	CTTACCACCTGCTCATCACCT
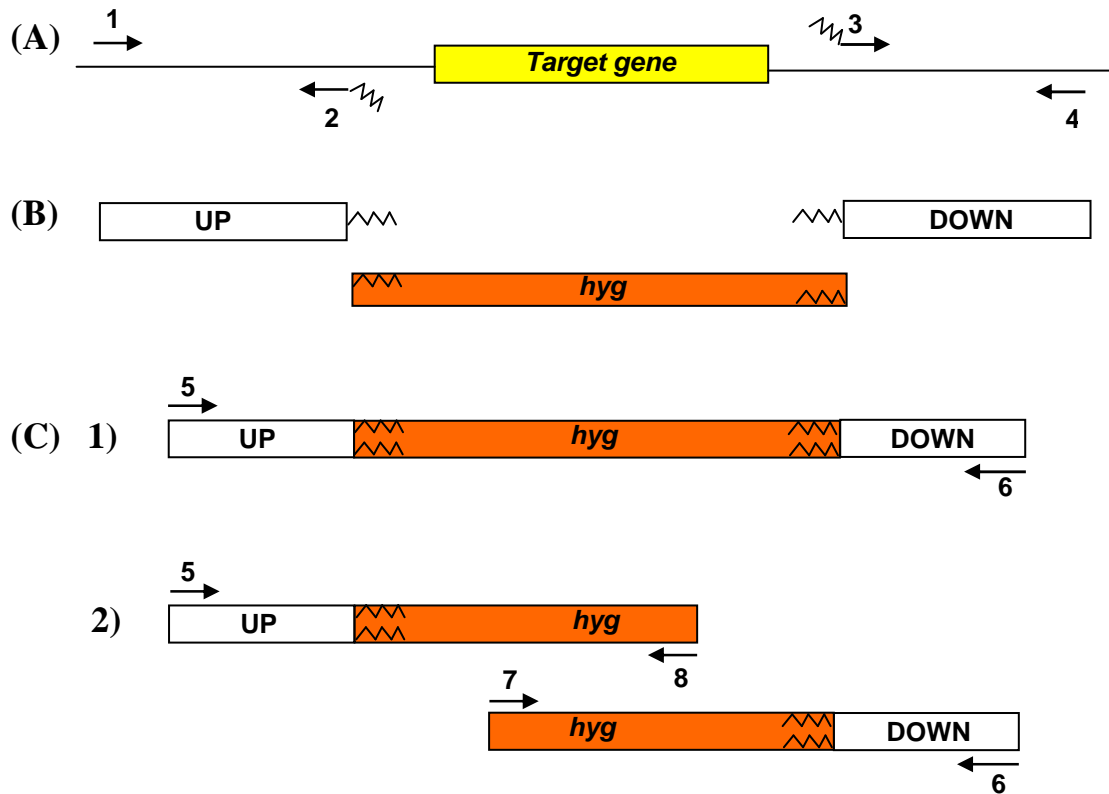
Figure 1 – Schematic illustration of the PCR-based construction of gene replacement vectors: **(A)** Flanking homology regions of *F. graminearum pg1* and *pg2* genes and *F. verticillioides pgIII* gene were amplified by PCR using specific primers for each gene: primers 1 and 2 were used for the amplification of the upstream region (UP), and primers 3 and 4 for the downstream region (DOWN). **(B)** Fusion PCR: UP and DOWN amplicons were fused with the hygromycin resistance gene *hph* by PCR using as primers the tails (∧∧∧) of primers 2 and 3, complementary to the 5' and 3' *hph* regions, respectively. **(C)** Nested PCR: fusion PCR product was amplified by PCR using primers 5 and 6 to obtain full construct (**1**), and primers pairs 5-8 and 7-6 to obtain the two split-marker constructs (**2**). Target genes were disrupted by homologous recombination: two crossing-over events were necessary with the full construct, and three crossing-overs with the split-marker constructs.

Table 3 – Annealing temperatures for the amplification of upstream and downstream flanking regions and for nested PCRs.

	Ta (°C)
Fgpg1 UP region	58
Fgpg1 DOWN region	53
Fgpg2 UP region	53
Fgpg2 DOWN region	52
FvpgIII UP region	60
FvpgIII DOWN region	60
Fgpg1 nested	56
Fgpg2 split-marker up	53
Fgpg2 split-marker down	60
FvpgIII nested	55

Table 4 – Type of construct, length and amount used in the transformation.

F. graminearum pg1	2.2 kb / 2.2 kb	Split-marker constructs	32 μg
F. graminearum pg2	3.4 kb	Full construct	60 μg
F. verticillioides pgIII	3.1 kb	Full construct	70 μg

2.2.4. Analysis of the PG pattern produced by mutants

F. graminearum and *F. verticillioides* wild-type and mutant strains were grown in 50 ml CM medium for 3 days at 24°C at 150 rpm and then mycelia were transferred in 50 ml Szécsi medium [$NH_4H_2PO_4$ 0.09% (w/v), $(NH_4)_2HPO_4$ 0.2% (w/v), $MgSO_4 \cdot 7H_2O$ 0.01% (w/v), KCl 0.05% (w/v), citrus pectin (ICN) 1% (w/v)] for 4 days at 24°C at 150 rpm to induce PG production (Szécsi, 1990). The culture filtrates, collected from the flasks containing Szécsi medium, were filtered through Sartorius MGA and cellulose acetate filters with pore size 0.8 μm, 0.45 μm and 0.2 μm (Sartorius, Milano, Italy), concentrated and dialyzed three times with 150 ml of Milli-Q quality water using the membrane

Vivaflow 200 (10,000 MWC PES) (Sartorius, Milano, Italy) to a final volume of 50 ml. The samples were analyzed by analytical IEF using a 0.8 mm thick polyacrylamide (PAA) gel containing two carrier ampholytes, one covering the pH range 6.0-8.0 (1.6% (v/v); Sigma-Aldrich, Milano, Italy) and the other covering the range 8.0-10.5 (1.6% (v/v); Amersham Biosciences, Uppsala, Sweden). PG isoforms were detected by the agarose overlay technique described by Ried and Collmer (1985).

2.2.5. Infection of wheat plants

Wheat seeds (cv. Bobwhite) were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 20 min, and then rinsed thoroughly in sterile water. Plants were grown in climatic chamber with a 16 h photoperiod and 19/16°C day/night temperature. Two opposite spikelets per spike were inoculated at anthesis (Zadoks stage 65-67; Zadoks et al., 1974) with *F. graminearum* wild-type or mutant strains using 10 μl of the conidial suspension ($2x10^5$ conidia/ml). 7 plants were inoculated with *F. graminearum* wild-type strain, 6 with Δfgpg1 mutant and five with Δfgpg2 mutant, respectively. After inoculation, the spikes were covered with a plastic bag to maintain a moist environment for 72 h. Plants were then moved into a growth chamber with 85% relative humidity under a 16/8 h day/night photoperiod at a day-time temperature of 24°C and a night-time temperature of 19°C. Symptom development on inoculated spikes was monitored up to 3 weeks post-inoculation.

2.2.6. Pathogenic tests on maize

Corn husks, taken from field plants (inbred line PR36B08) grown approximately 2 months (Silking stage), were inoculated with 10^6 conidia of either *F. verticillioides* wild-type or mutant strain. The symptoms were monitored up to 10 days after inoculation (dai).

7-days-old maize seedlings (inbred line PR36B08) were wounded and inoculated at the stem base with 10 μl of conidial suspension ($8x10^4$ conidia) of *F. verticillioides* wild-type and mutant strains respectively. As negative control, seedlings were wounded and inoculated with pure water. All seedlings were incubated at 24°C in high humidity chamber. The inoculated tissue of seedlings, sampled 5 dai, and corn husks were analysed

by analytical IEF, as reported in Sella et al. (2005), using the two carrier ampholytes above reported.

2.3. Results

2.3.1. *F. graminearum* and *F. verticillioides* transformation and characterization of the secreted PG activity in the mutant strains

Twenty-one *F. graminearum* and eighteen *F. verticillioides* transformants were analyzed by PCR, using primers specific for each *pg* gene (Table 1), in order to determine if the target genes were effectively knocked-out: PCR did not produce any amplification product when the *pg* gene was disrupted. For *F. graminearum* mutants, the complete *pg1* gene was disrupted in 3 of 11 transformants and the *pg2* gene was disrupted in 5 of 10 transformants (data not shown). For *F. verticillioides* mutants, the complete *pgIII* gene was disrupted in 1 of 18 transformants (data not shown). After single conidiation of PCR positive transformants, high-stringency Southern blot analysis of genomic DNA was performed and showed homologous integration of the disruption construct in all of them; no ectopic integration was observed in these transformants (Fig. 2, 3 and 4).

To verify if the disruption of the *pg* gene was effective, the secreted PG activity of each mutant strain was characterized. *F. graminearum* and *F. verticillioides* wild-type and knock-out mutants were grown in liquid culture to induce PG production and then the culture filtrates were analysed by analytical IEF. The *F. graminearum* knock-out mutants Δfgpg1 strain 813.3 and Δfgpg2 strain 825.3 produced respectively only PG2 and PG1, compared to the wild-type strain (Fig. 5). The *F. verticillioides* knock-out mutant ΔfvpgIII strain 14.1 did not secrete the PGIII isoform which was instead produced by the wild-type strain (Fig. 6).
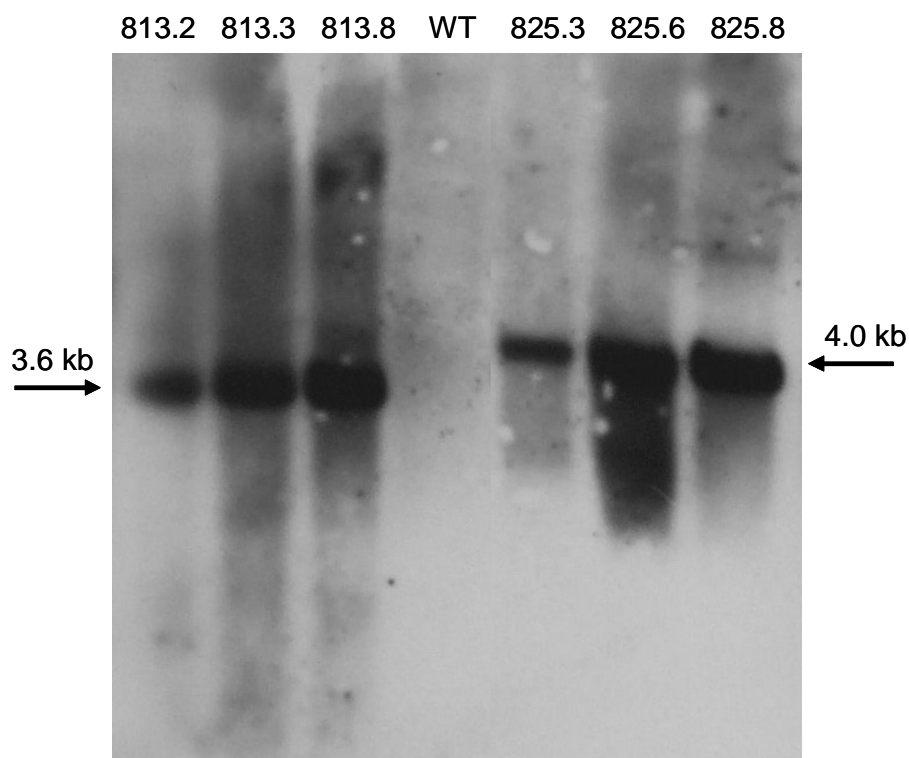
Figure 2 – Southern blot analysis of genomic DNA from *F. graminearum* wild-type and mutant strains. DNA was digested with *Xba* I. The blot was probed with the 516 bp internal fragment of *hph* gene. The Δfgpg1 mutant strains (813.2, 813.3, 813.8) show hybridization signal at 3.6 kb; the Δfgpg2 mutant strains (825.3, 825.6, 825.8) show hybridization signal at 4.0 kb; the wild-type strain gave no hybridization signal.
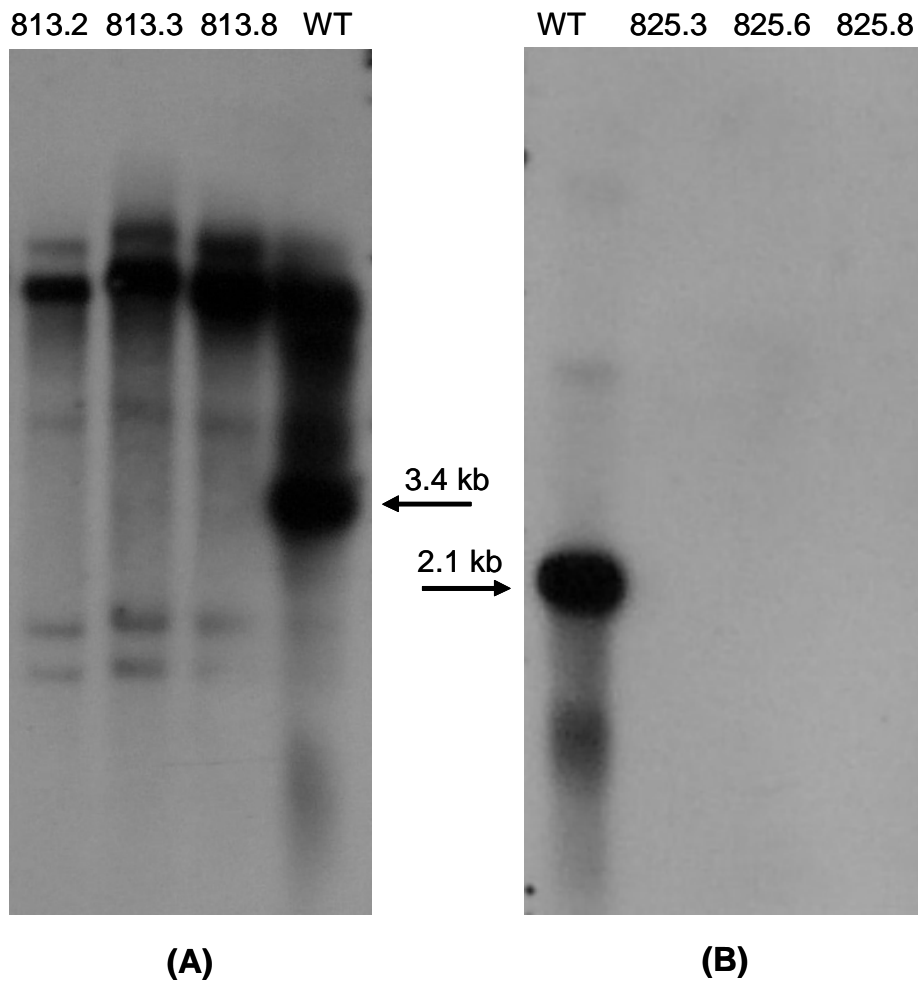
Figure 3 – Southern blot analysis of genomic DNA from *F. graminearum* wild-type and mutant strains. DNA was digested with *Xba* I. **(A)** Blot probed with the 423 bp internal fragment of *pg1* gene. Only the wild-type strain showed an hybridization signal of 3.4 kb, compared to the Δfgpg1 mutant strains (813.2, 813.3, 813.8). **(B)** Blot probed with the 541 bp internal fragment of *pg2* gene. The Δfgpg2 mutant strains (825.3, 825.6, 825.8), differently from the wild-type strain, did not show the hybridization signal of 2.1 kb.
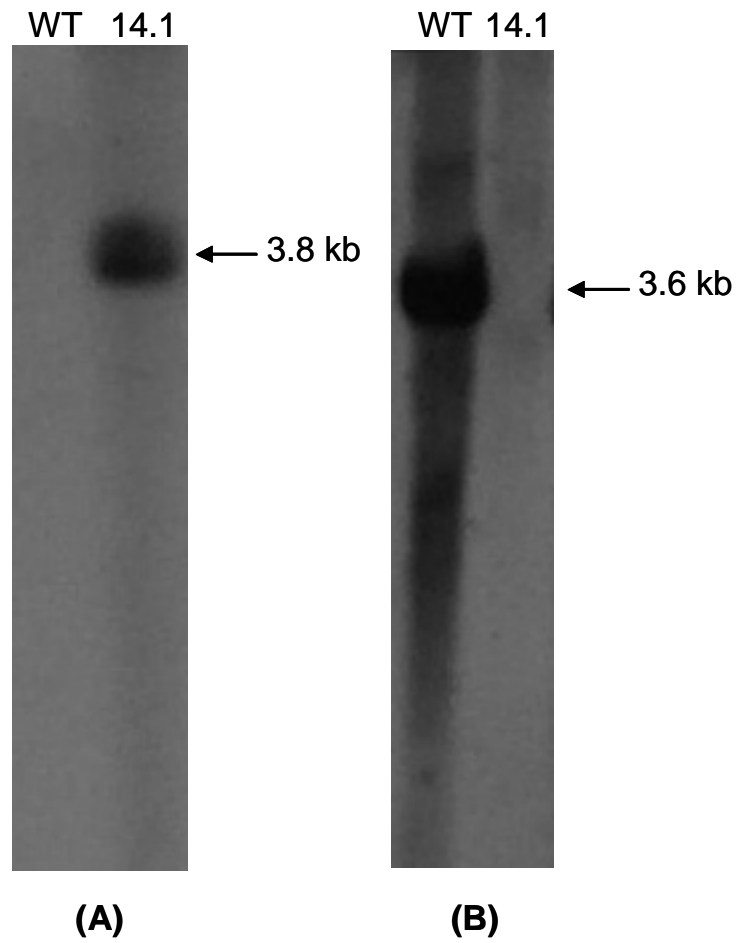
Figure 4 – Southern blot analysis of genomic DNA from *F. verticillioides* wild-type and mutant strains. DNA was digested with *Xba* I. **(A)** Blot probed with the 516 bp internal fragment of *hph* gene. Only the ΔfvpgIII mutant strain 14.1 showed an hybridization signal of 3.8 kb. **(B)** Blot probed with the 568 bp internal fragment of *pgIII* gene. The ΔfvpgIII mutant strain 14.1 did not show the hybridization signal of 3.6 kb specific of the wild-type strain.
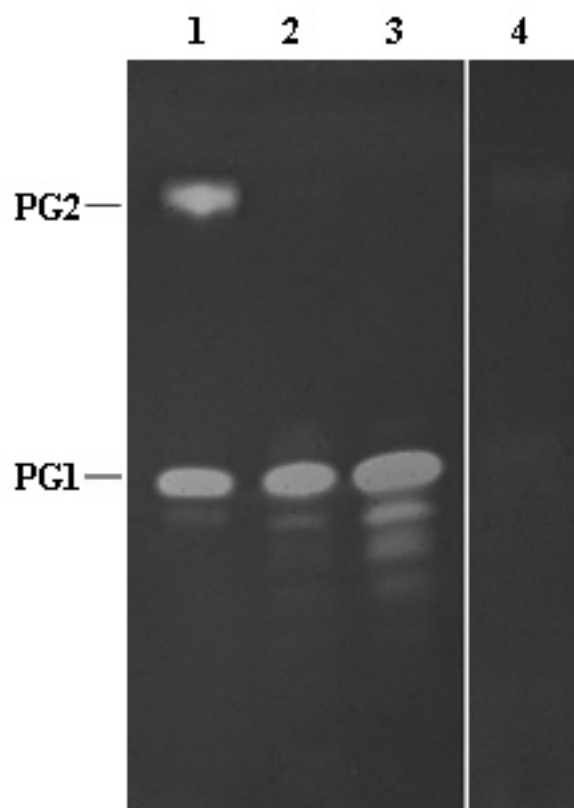
Figure 5 – Thin layer IEF in the pH range 6.0-10.5 of crude PG activity produced by *F. graminearum* wild-type and mutant strains in liquid culture. The Δfgpg1 strain 813.3 (lane 4) and the Δfgpg2 strain 825.3 (lane 3) produced only PG2 and PG1, respectively, when compared to the wild-type strain (lane 2). PG2 isoform is barely visible both in Δfgpg2 and wild-type strains. The purified PG1 and PG2 were loaded in lane 1 for reference. 0.83 nkat of PG activity were loaded in each lane.
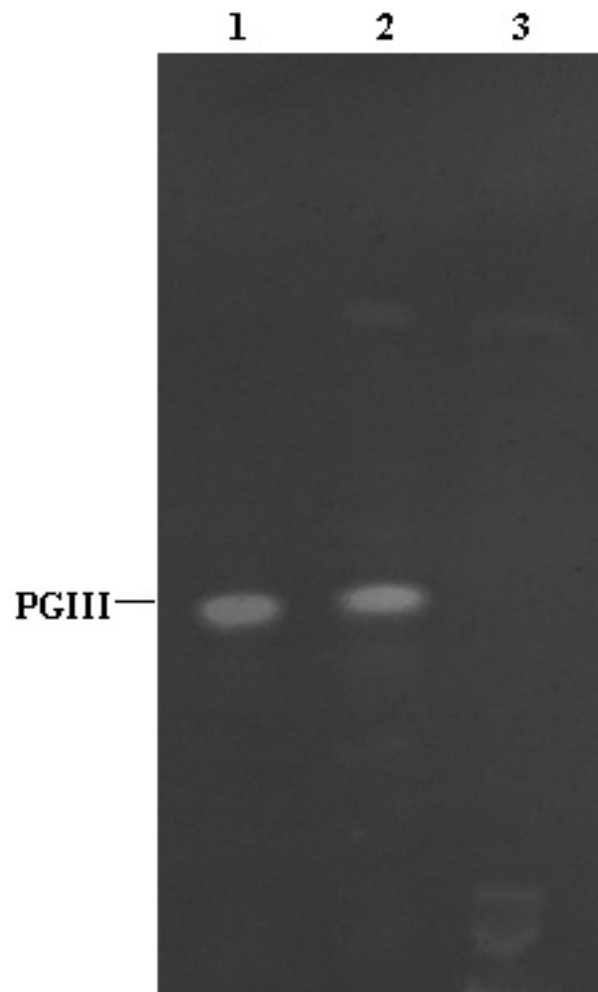
Figure 6 – Thin layer IEF in the pH range 6.0-10.5 of crude PG activity produced by *F. verticillioides* wild-type and mutant strains in liquid culture. The ΔfvpgIII strain 14.1 (lane 3) did not secrete the PGIII isoform, which was produced by the wild-type strain (lane 2). The purified PGIII was loaded in lane 1 as control. 0.83 nkat of PG activity were loaded in each lane.

2.3.2. Pathogenicity of *F. graminearum* Δfgpg1 and Δfgpg2 strains on wheat

To determine whether *F. graminearum* PG1 and PG2 are involved in pathogenicity or virulence, wheat spikes were infected with conidia from Δfgpg1 strain 813.3, Δfgpg2 strain 825.3 and wild-type strain. The knock-out mutants maintained the capability to infect plants, and no apparent differences in the virulence among wild-type and mutant strains were observed, although Δfgpg2 colonization of wheat spikes seemed slowly reduced (Fig. 7). However, due to the low number of infected wheat plants (only 7 with the wild-type strain, 6 and 5 with the Δfgpg1 and Δfgpg2 strains, respectively), further infection tests are needed to evaluate if there are slight differences in the virulence of mutants.

2.3.3. Pathogenicity of *F. verticillioides* ΔfvpgIII strain on maize

In order to ascertain the possible involvement of PGIII in the pathogenicity of *F. verticillioides*, maize seedlings and corn husks were inoculated with conidia from ΔfvpgIII strain 14.1 and wild-type strain. The knock-out mutant maintained the capability to infect the plant tissue in both inoculation systems, but it showed a clearly visible reduction of virulence compared to the wild-type strain. In particular, infections of maize seedlings were monitored for 5 days after inoculation and showed that symptoms development caused by ΔfvpgIII mutant proceeded slower than wild-type strain (Fig. 8A). A subsequent IEF analysis performed by loading fragments of inoculated seedlings directly on the PAA gel surface confirmed that this mutant, differently from the wild-type strain, did not produce the PGIII isoform during infection (Fig. 8B). Also infection experiments performed on corn husks showed an evident delay in the progression of symptoms when using the ΔfvpgIII mutant, and a clear reduction of necrotic symptoms compared to the wild-type strain (Fig. 9). Also in this case an IEF analysis was performed by loading fragments of husks inoculated with the wild-type and mutant strains: as expected, the PGIII isoform was not detected in the tissue infected with the mutant and without necrotic symptoms, while it was detected in the husk fragments infected with the wild-type strain and showing evident necrotic symptoms (Fig. 10A-B).

Figure 7 – Wheat spikes inoculated with *F. graminearum* wild-type and mutant strains 15 days after inoculation. Two opposite spikelets per spike were inoculated with 10 µl of conidial suspension ($2\text{x}10^5$ conidia/ml).
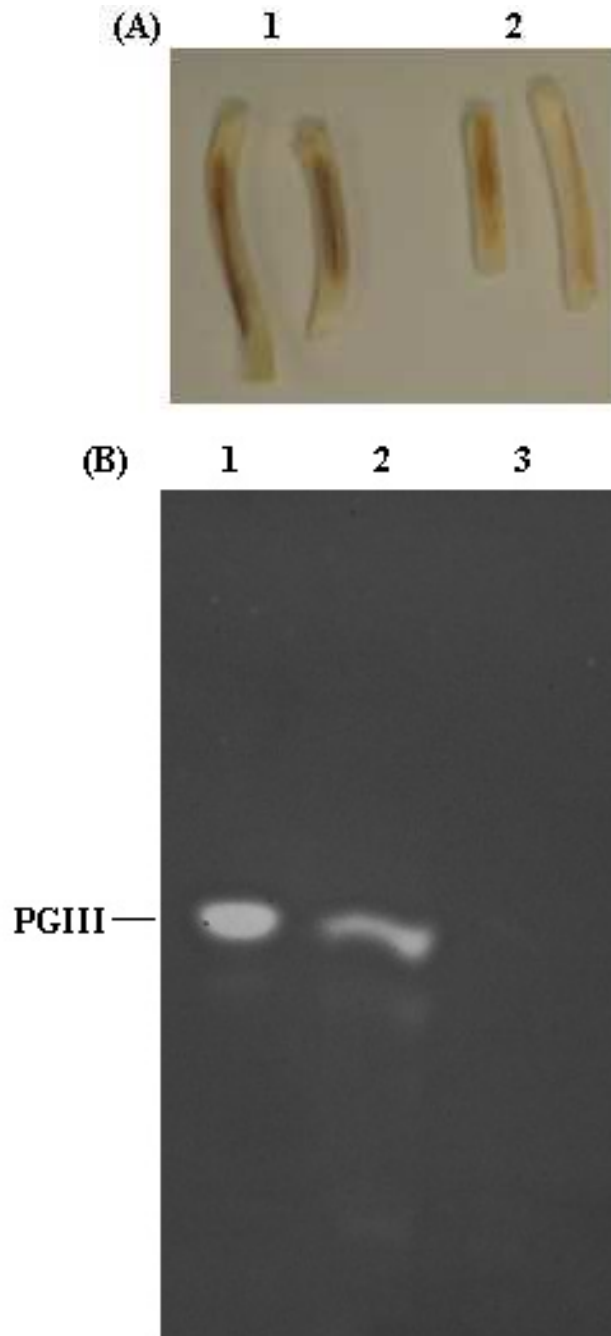
Figure 8 –**(A)** Maize seedlings 5 days after inoculation (dai) with *F. verticillioides* wild-type (1) and knock-out ΔfvpgIII strain 14.1 (2). 10 μl of conidial suspension (8×10^4 conidia) were used to infect wounded seedlings. **(B)** Thin layer IEF in the pH range 6.0-10.5 loaded with maize seedling fragments harvested 5 dai with *F. verticillioides* wild-type (lane 2) and ΔfvpgIII strain 14.1 (lane 3). The knock-out mutant, differently from the wild-type strain, did not produce PGIII during infection. 0.42 nkat of purified PGIII were loaded in lane 1 as control.
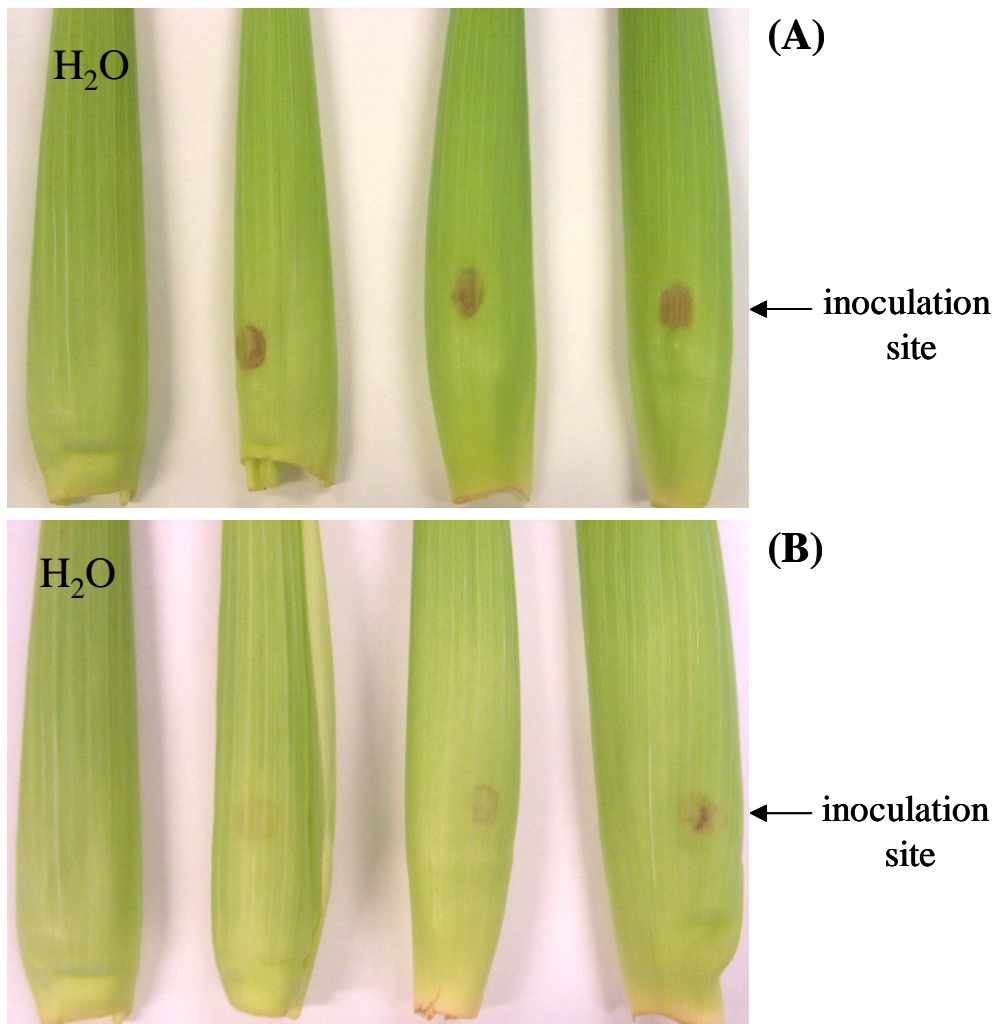
Figure 9 – Corn husks inoculated with 10^6 conidia of *F. verticillioides* wild-type (A) and mutant strains (B) 3 days after inoculation. Corn husks inoculated with ΔfvpgIII strain 14.1 showed an evident slowdown in the progression of symptoms and a clear reduction of necrotic symptoms compared to the wild-type strain.
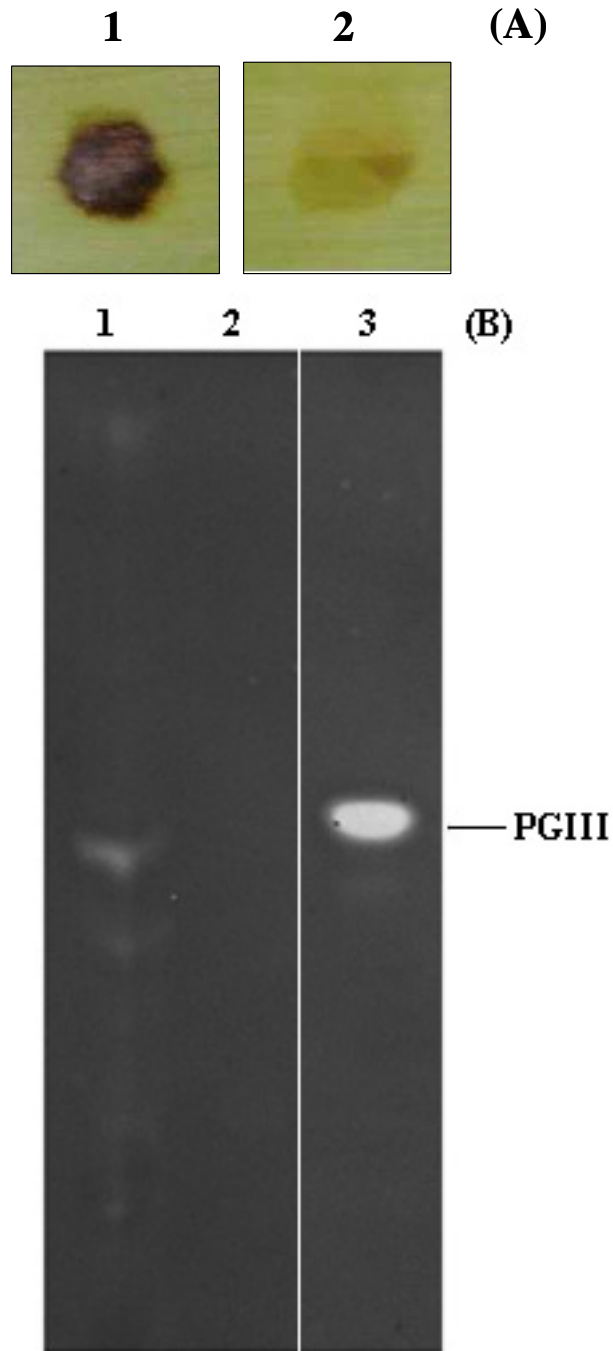
Figure 10 – **(A)** Corn husks inoculated with *F. verticillioides* wild-type (1) and mutant strains (2) showing different levels of symptoms. **(B)** Thin layer IEF in the pH range 6.0-10.5 loaded with corn husk fragments harvested 5 days after inoculation with *F. verticillioides* wild-type strain (lane 1: corn husk showing necrotic symptoms) and ΔfvpgIII strain 14.1 (lane 2: corn husk without necrotic symptoms). 0.42 nkat of purified PGIII were loaded in lane 3 as control.

2.4. Discussion

Gene disruption is a fundamental genetic approach to ascertain the role of specific genes and it has been used in the last years in order to determine the contribution of a number of fungal genes to pathogenicity or virulence of important fungal pathogens (Idnurm et al., 2001). In particular, concerning pectinolytic enzymes of phytopathogenic fungi, the majority of the studies focused on polygalacturonases (PGs). In fact, their clear involvement in fungal pathogenicity is still debated: several targeted mutants showed no reduction in pathogenicity (Gao et al., 1996; Scott-Craig et al., 1998), while others demonstrated that some pectinolytic enzymes were essential for fungal pathogenicity or virulence (Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Oeser et al., 2002; ten Have et al., 2002). However, mutants that did not show any reduction in virulence still contained a residual PG activity in planta because other *pg* genes were expressed (Gao et al., 1996; Scott-Craig et al., 1998).

It is widely recognized that PG may be an important virulence factor of fungi attacking dicot plants, because these hosts contain a high amount of pectin (Carpita and Gibeaut, 1993) and give a negligible contribution to virulence of pathogens of grass plants. However, it was recently demonstrated that PG is a pathogenicity factor of the biotrophic rye pathogen *Claviceps purpurea*. This result, however, could depend from the specific characteristic of its natural substrate: the pectin rich ovary tissue (Oeser et al., 2002).

The ovary tissue is also a target of *F. graminearum* mostly at the initial stage of infection (Miller et al., 2004; Goswami and Kistler, 2004) and therefore its pectinase activity may be involved in the infection process. As shown in the previous chapter, the expression analysis of the two *F. graminearum pg* genes during wheat infection is consistent with a possible contribution of PG to fungal virulence mostly in the early stage of host tissue infection. To address the role of the PG activity in wheat infection, as a first step I obtained mutants disrupted in the PG1 or PG2 enzyme function. The *F. graminearum* knock-out mutants maintained the capability to infect wheat plants, and no apparent differences in the virulence were observed compared to the wild-type. However, due to the limited number of infected plants, a slight reduction in virulence can not be ruled out, and therefore the infection experiments should be repeated with a larger number of plants. Otherwise, it has to be considered that the loss of activity due to the knock-out of one PG could be

compensated during plant infection by the activity of the remaining PG, although in liquid culture the disruption of a *pg* gene did not modify the production of the other PG. Therefore, double knock-out mutants should be obtained to fully clarify the role of the two *F. graminearum* PGs during the infection process. Besides, it is worth noting that the loss of PG activity in the mutants could be compensated also by the activity of other pectic enzymes, like pectin lyase, expressed during wheat infection (Paper et al., 2007; Güldener et al., 2006; First chapter of this thesis).

As far as *F. verticillioides* is concerned, the penetration strategies used by this fungus during the infection process is less characterized. Silks and then interior or exterior immature kernels seem to be the major routes of infection of maize ears. In addition, direct access to the kernels can be gained trough punctures initiated by insects (Munkvold et al., 1997; Sutton et al., 1980).

In liquid culture also *F. verticillioides* secretes high levels of PG encoded by a *pg* gene named *pgIII*, which has been previously characterized together with its encoded protein (Sella et al., 2004; Raiola et al., in press) and has now been successfully disrupted. The veto to use genetically modified organism in field trials and the unavailability of a suitable growth chamber did not allow to perform pathogenicity assay on maize plants. Therefore, infection experiments were conducted only in laboratory on maize seedlings and ear husks. Infection of husk leaves is a technique used to differentiate susceptibility to *F. verticillioides* of maize ears (Clements et al., 2003) and thus may be appropriate to distinguish *F. verticillioides* strains with different virulence. *F. verticillioides pg* knock-out mutant maintained the capability to infect maize seedlings and corn husks, but it showed a clearly visible reduction of necrotic symptoms compared to the wild-type strain. Interestingly, the appearing of necrotic symptoms in maize tissue infected with the wild-type strain seemed related with the presence of PGIII. Therefore this PG could be responsible for the induction of the necrotic symptom. Recently, Zuppini et al. (2005) provided evidence for the occurrence of programmed cell death (PCD) in soybean cells induced by a PG produced early during plant infection by the fungal pathogen *Sclerotinia sclerotiorum*. Whether the *F. verticillioides* PGIII is able to induce PCD in maize tissue has to be confirmed by specific experiments. The *F. verticillioides pg* mutant could be also

used to infect other monocotyledonous plants, like asparagus, leek and onion, which have a pectin rich cell wall similar to that of dicotyledonous plants.

Conclusions

The aim of this thesis was to clarify the role of the PG produced by the fungal pathogens *F. graminearum* and *F. verticillioides* during infection of host plants.

The biochemical features of the two *F. graminearum* PGs purified and here characterized along with the measured alkalinization of the host tissue determined by the fungus are consistent with a role of these PGs in tissue degradation and in the progression of infection. Besides, the expression analysis of *pg* genes during wheat infection suggests a possible role of PGs in the initial phase of host tissue colonization. Though taken together these data seem to support a role for *F. graminearum* PGs in pathogenesis, preliminary wheat infection experiments performed with *pg* knock-out mutants of the fungus did not confirm this hypothesis. In fact, the *F. graminearum* Δfgpg1 and Δfgpg2 mutants maintained the capability to infect wheat plants, and no apparent differences in the virulence among wild-type and mutant strains were observed. Therefore, the function of *F. graminearum* PGs during the infection process remains still unclear. It is possible that the loss of activity due to the knock-out of one PG could be compensated during plant infection by the activity of the remaining PG or by pectin lyase activity, whose genes are also expressed during spike infection. Double knock-out mutant is thus needed to fully clarify the role of the two *F. graminearum* PGs.

Janni et al. (personal communication) showed that wheat plants expressing a bean polygalacturonase inhibiting protein (PGIP) show a reduction of spike symptoms induced by *F. graminearum*, and bean PGIP is able to inhibit *F. graminearum* PGs *in vitro* (data not shown). Therefore, the resistance of these transgenic plants to *F. graminearum* infection could be due to the inhibitory effect of bean PGIP against the fungal PGs, thus indicating that these PGs might play a role during infection. However, Joubert et al. (2007) showed that PGIP can protect plant tissue from PG activity independently from its recognition capability. The infection of PGIP transgenic plants with the double *pg* knock-out mutant could clarify whether the cause of increased resistance of PGIP transgenic plants is due to PG inhibition and/or to other unknown protection effects of PGIP.

During liquid culture *F. verticillioides* is known to express high amount of a previously characterized PG isoform (Sella et al., 2004; Raiola et al., in press). This isoform was detected here during infection of maize husks. By targeted homologous recombination the

pg encoding gene has been disrupted. The knock-out mutant maintained the capability to infect maize seedlings and corn husks, but along with the disappearance of the PG from infected tissue it determined a delayed and reduced necrotic symptom compared to the wild-type strain. Thus the *F. verticillioides* PG is likely the factor responsible of this necrotic symptom.

It will be interesting to assay the virulence of this *F. verticillioides* mutant strain against other host plants like the Asparagales. In fact, *Asparagus* and *Allium* spp. possess a pectin rich cell wall more similar to that of Dicot plants, and it is feasible that on these hosts the fungal PG could affect more strongly the cell wall architecture. Differently to the *F. graminearum* PGs, a PGIP-based strategy to increase resistance of host plants to *F. verticillioides* could be ineffective since all the PGIPs so far characterized, included bean PGIP, appear unable to effectively inhibit the PG of *F. verticillioides* (Sella et al., 2004; Raiola et al., in press).

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