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## ***A FUSARIUM GRAMINEARUM* PG IS REQUIRED FOR VIRULENCE DURING WHEAT INFECTION**

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02.February.2010

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## Riassunto

*F. graminearum* è l'agente causale della fusariosi della spiga, una malattia descritta per la prima volta in Inghilterra nel 1884 ed ora una delle più studiate in quanto colpisce le tre principali risorse di cibo mondiale frumento, mais e riso. Questo fungo può causare la distruzione del raccolto in poche settimane determinando ingenti perdite economiche. In aggiunta, il prodotto ricavato dalle piante infette presenta elevati livelli di micotossine, in particolare tricoteceni quali il deossinivalenolo (DON). Questa tossina è un potente inibitore delle sintesi proteica negli eucarioti e risulta dannoso per la salute umana e animale.

*F. graminearum* penetra nell'ospite durante la fase di antesi attraverso l'ovario e colonizza la superficie interna di lemma e palea. Una volta penetrato esso è in grado di diffondere nella spiga in modo sistemico. Durante la penetrazione e la colonizzazione dei tessuti della spiga, il fungo produce e rilascia numerosi enzimi degradativi della parete cellulare; tra questi, le endo-poligalatturonasi (PGs) sono tra i primi enzimi secreti ma il loro ruolo durante l'infezione non è stato ancora chiarito.

Nel database genomico di *F. graminearum* sono stati identificati due putativi geni codificanti *pg*. Le isoforme codificate da questi geni (chiamate PG1 and PG2) sono state caratterizzate *in vitro* e *in vivo* (Tomassini et al., 2009). L'obiettivo del presente lavoro di tesi è chiarire l'importanza di queste PG prodotte durante l'infezione della pianta ospite attraverso l'ottenimento di mutanti knock-out ottenuti attraverso ricombinazione omologa target del gene target.

Due mutanti  $\Delta$ PG1 e due mutanti  $\Delta$ PG2 (producenti rispettivamente solo PG2 e PG1) sono stati ottenuti e caratterizzati *in vitro* e durante l'infezione della pianta ospite. In coltura liquida il mutante  $\Delta$ PG1 è risultato produrre scarsa attività PG rispetto al fungo WT e al mutante  $\Delta$ PG2. Tuttavia i mutanti analizzati non hanno mostrato differenze in peso secco dopo crescita in terreno liquido contenente pectina come unica fonte di carbonio.

I mutanti sono stati in seguito saggiati in esperimenti di infezione di piante di frumento. Si è osservato che tutti i mutanti testati mantenevano la loro capacità di infettare la spiga. Tuttavia, il mutante  $\Delta$ PG1 presentava una ridotta virulenza (circa 75%

in meno di spighe infettate) rispetto al WT mentre il mutante  $\Delta$ PG2 non mostrava alcuna riduzione di virulenza.

E' stata inoltre quantificata la micotossina DON prodotta dai mutanti durante l'infezione della pianta ospite: il mutante  $\Delta$ PG1 produceva da 8 a 15 volte meno DON rispetto a WT e mutante  $\Delta$ PG2, probabilmente a causa della ridotta colonizzazione della spighetta da parte di questo mutante.

Il ceppo WT e un mutante  $\Delta$ PG1 sono stati inoltre trasformati in modo da esprimere costitutivamente la green fluorescent protein (GFP). I mutanti così ottenuti sono stati utilizzati per studi istologici delle prime fasi di infezione. Rispetto al WT il mutante  $\Delta$ PG1 era in grado di colonizzare l'ovario, un tessuto ricco in pectina, molto più lentamente; anche la colonizzazione da parte del mutante del tessuto conduttore nel rachide risultava più lenta. Questo rallentamento nella crescita del fungo nella spiga potrebbe essere dovuto alla ridotta attività PG da parte del mutante  $\Delta$ PG1 e potrebbe permettere alla pianta di attivare più efficacemente le risposte di difesa. Nel complesso i risultati ottenuti nel presente lavoro sembrano indicare che la PG1 di *F. graminearum* svolge un importante ruolo durante la patogenesi e può quindi essere considerato un fattore di virulenza.

## SUMMARY

*Fusarium graminearum* is a relevant pathogen of monocot species and is the main causal agent of Fusarium Head Blight (FHB), a devastating disease which commonly affects cereals. Besides, this fungus produces in the infected grains significant levels of mycotoxins, such as the trichotecenes deoxinivalenol (DON). *F. graminearum* penetrates in the host tissue during anthesis and once inside the tissue is able to spread systemically through the vascular vessels. During penetration and colonization of wheat spike tissues, the fungus secretes several cell wall degrading enzymes (CWDE). In particular, endopolygalacturonases (PGs) have been shown to be early secreted in wheat plants, but, their role during the infection process has not been ascertained yet.

Two *pg* encoding genes have been previously identified in the *F. graminearum* genome database and the isoforms encoded by these two genes (named PG1 and PG2) have been characterized both in vitro and in vivo.

Aim of the present work was to clarify the importance of these *F. graminearum* PGs during infection of host wheat plants through the knock-out of their corresponding encoding genes, obtained by targeted homologous recombination.

Two  $\Delta$ PG1 mutants and two  $\Delta$ PG2 mutants strains (producing only PG2 and PG1, respectively) were obtained and characterised both in vitro and during plant infection. The PG activity produced in liquid culture by the  $\Delta$ PG1 mutants was negligible compared to that produced by WT and  $\Delta$ PG2 mutants. However, no difference in dry weight was obtained when growing WT and mutant strains in the presence of pectin as the sole carbon source.

Infection experiments of wheat host plants were also performed. We observed that all the knock-out mutants tested maintained the capacity to infect the plant, but the  $\Delta$ PG1 strains showed a significant reduction of virulence (about 75% less infected spikelets) compared to WT strain, while no reduction of virulence was observed with the  $\Delta$ PG2 mutants.

We also quantified the amount of DON mycotoxin produced by the fungus during wheat infection: the  $\Delta$ PG1 strains produced from 8 to 15 times less DON mycotoxin compared to WT and  $\Delta$ PG2 strains, probably due to their reduced colonization of the plant tissue.

*F.graminearum* WT and a  $\Delta$ PG1 mutant strain were also transformed to constitutively express the green fluorescent protein (GFP). The obtained mutants were used to perform a

comparative study of the early events of wheat colonization. Compared to WT, the  $\Delta$ PG1 mutant colonized more slowly the ovaries, tissues rich of pectin,, and also the colonization of the vascular vessels of the rachis by this mutant was strongly delayed compared to WT. In particular, the retarded colonization of the ovary by the  $\Delta$ PG1 mutant might be due to the reduced production of PG activity. The fungal growth in the infected wheat tissue could result therefore slowed down allowing the plant to initiate its defence reactions. Taken together, these results seems to indicate that PG1 play an important role during pathogenesis and therefore can be considered a virulence factor.

## 1. INTRODUCTION

For most necrotrophic fungi, a relevant role in pathogenesis is played by enzymes degrading the plant cell wall (Cooper, 1988), a physical barrier to pathogen penetration and colonization.

Among cell wall degrading enzymes (CDWE), *endo*-polygalacturonases (PGs) are expressed in the early stages of host infection and strongly contribute to the virulence or the pathogenicity of several phytopathogenic fungi by degrading the pectin component of the cell wall and middle lamella (Clay *et al.*, 1997; ten Have *et al.*, 1998; Isshiki *et al.*, 2001). Therefore, pectic enzymes have an important role especially in the disease of dicotyledonous plants that have a cell wall rich in pectin. However pectinases have been shown to be implicated also in the pathogenesis of some cereal pathogens, although monocot plants have developed a cell wall consisting of small amount of pectin (Carpita and Gibeaut, 1993). For example pectic enzymes are necessary for *Fusarium culmorum* to break down the major cell wall components during infection and spreading in the host tissues (Kang and Buchenauer, 2000); besides Douhaier *et al.* (2007) showed that, during wheat infection, *Mycosphaerella graminicola* secretes high and early amount of polygalacturonase activity in the infected leaf allowing the breakdown of pectic material contained in the cell wall, and demonstrated a correlation between PG activity and the lesion frequency in wheat leaves. Furthermore, Wanyoike *et al.* (2002) showed by gold labelling that wheat infection by *Fusarium graminearum* resulted in reduced and irregular labelling of the middle lamella and the primary cell wall of cells between the ovary and lemma, thus indicating a pectin degradation.

*F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] is a relevant pathogen of monocot species and is the main causal agent of the *Fusarium* head blight (FHB) disease. FHB is a devastating disease which commonly affects cereals such as wheat, barley and other small grains causing high yield loss (McMullen *et al.*, 1997). Besides, this fungus produces in the infected grains several CDWE (Kikot *et al.*, 2009) and significant levels of mycotoxins, such as the trichotecenes deoxinivalenol (DON) and nivalenol (NIV), dangerous for human and animal health (McMullen *et al.*, 1997, Parry *et al.*, 1995). In particular, DON is one of the few *F. graminearum* virulence factors identified so far, since it has been shown to be crucial for the spread of the

fungus within the wheat spike (Bai *et al.*, 2001). Another factor shown to be important for *F. graminearum* virulence is a secreted triacylglycerol lipase (Voigt *et al.*, 2005), a CWDE able to catalyze the hydrolysis of triacylglycerols into glycerol and free fatty acids.

The characterization of *F. graminearum* genes involved in virulence or pathogenicity is an essential step for better understating the mechanisms of fungal pathogenesis.

Analyzing the *F. graminearum* genome database (<http://mips.gsf.de/genre/proj/fusarium/>) we have previously identified two genes encoding *endo*-PGs. Expression analysis during *F. graminearum*-*T. aestivum* interaction has shown that transcription of both *pg* genes occurs within the first 12 h after spike inoculation and peaks at 24 h (Tomassini *et al.*, 2009). The *endo*-PGs isoforms encoded by these two genes (named PG1 and PG2) have been also purified and characterized (Tomassini *et al.*, 2009) and show different functional properties. In particular, these PGs are secreted both *in vitro* and *in vivo*, during wheat infection, with the activity of PG1 largely exceeding that of PG2 (Tomassini *et al.*, 2009). The PG expression during the early stages of wheat infection seems consistent with the characteristics of the tissue initially infected after spikelet inoculation. In fact, *F. graminearum* is known to penetrate wheat spikelets through the ovary and the inner surface of lemma and palea (Goswami and Kistler, 2004). In particular, the fungus appears to affect the ovary within 12 h (Miller *et al.*, 2004), and homogalacturonans and methyl-esterified homogalacturonans have been shown to be abundant constituents of ovary cell wall in grasses as rye (Tenberger *et al.*, 1996). Thus the degradation of the spikelet soft tissue may be achieved with the contribution of the PG activity produced by *F. graminearum* during the infection process.

Aim of the present work was to evaluate the importance of *F. graminearum* PGs during the infection process of host plants like wheat through the knock-out of the two corresponding *pg* genes by targeted homologous recombination.

Gene disruption is a fundamental genetic approach that have been hugely used in the recent years to ascertain the role of specific genes. The main strategy to disrupt genes in fungi (May, 1992) is based on a vector cassette where the marker gene for 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, 2003). In this case, disruption of the target gene occurs by a triple crossover event.

In this PhD thesis I obtained the disruption of the *F. graminearum pg* genes encoding the PG1 and PG2 isoforms purified and characterized in the previous work (Tomassini et al., 2009). To verify the effective disruption of the *pg* genes, the PG pattern produced by the  $\Delta$ PG mutants obtained was analysed in liquid culture and during wheat infection. These  $\Delta$ PG mutants were then characterized *in vitro* by measuring the dry weight and the PG activity produced when grown in liquid culture with pectin as the carbon source, and *in vivo* by infection experiments of host plants like wheat and maize and also of *Arabidopsis thaliana*. *Arabidopsis* has been previously used as a model system since some papers have revealed similarities in the requirements for *F. graminearum* disease in *Arabidopsis* and wheat: for example, this fungus is able to infect both plants through the flower and anthers are required for infection (Urban et al., 2002). However, an important difference have been also highlighted between *Arabidopsis* and wheat pathosystems: the DON mycotoxin produced by *F. graminearum*, although not required to colonize the *Arabidopsis* floral tissue (Cuzick et al., 2008), is considered a virulence factor in wheat infection because it is necessary for the spread of the fungus within the wheat spike (Bai et al., 2001). Therefore the DON mycotoxin produced during wheat infection by WT and  $\Delta$ PG mutants was also measured.

Since infection results showed that a  $\Delta$ PG mutant was reduced in virulence, this one and the *F. graminearum* WT were transformed to express the green fluorescent protein (GFP), a marker naturally fluorescent derived from jellyfish (*Aequorea victoria*) (Lorang et al 2001) that functions in a wide variety of transgenic systems. A number of phytopathogens have been successfully transformed to express GFP (Du et al.1999, Lagopodi et al.2002, Maor et al.1998, Spellig et al.1996 and Van den Wymelenberg et al.1997) allowing the process of fungal infection to be monitored in living systems. The obtained *F. graminearum* GFP-expressing strains were used to perform a comparative

study of the early events of wheat infection, allowing to follow with time the infection of wheat spikes directly in situ on different sections of tissues, thus minimizing artefacts.

## 2. MATERIALS AND METHODS

### 2.1. Fungal strains and culture conditions

*Fusarium graminearum* strain 3827.4 was cultured at 24°C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA).

To obtain mycelium for DNA extraction, WT and mutant strains 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 without aminoacids and ammonium sulfate] for 3 days at 150 rpm and 24 °C.

For PG activity induction, 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  $\text{NH}_4\text{H}_2\text{PO}_4$  0.09% (w/v),  $(\text{NH}_4)_2\text{HPO}_4$  0.2% (w/v),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.01% (w/v), KCl 0.05% (w/v), citrus pectin (ICN) 1% (w/v) (Szécsi, 1990). The cultures were grown for 4-7 days at 24°C on an orbital shaker (about 100 rpm).

For plant inoculation, conidia of *F. graminearum* WT and mutant strains were obtained by culturing 5 mycelium discs (5 mm diameter) in 50 ml of CMC liquid medium [(0.1%  $\text{NH}_4\text{NO}_3$ ; 0.1%  $\text{KH}_2\text{PO}_4$ ; 0.05%  $\text{MgSO}_4$ ; 0.1% yeast extract; 1.5% carboxymethylcellulose] (Cappellini and Peterson, 1965). After 5-7 days, liquid cultures were filtrated and conidia were collected and diluted to obtain approx.  $2 \times 10^5$  conidia  $\text{ml}^{-1}$ . Alternatively, *F. graminearum* WT and mutant strains were cultured on SNA agar plates [(0.1%  $\text{KH}_2\text{PO}_4$ ; 0.1%  $\text{KNO}_3$ ; 0.1%  $\text{MgSO}_4$ ; 0.05% KCl; 0.02% glucose; 0.02% saccharose; 2% bactoagar; 200 ppm biotin; 200 ppm thiamine] (Urban et al., 2002) to induce conidiation. Conidia were recovered from agar plates with sterile water using a sterile glass rod.

### 2.2. Plants growth conditions

Wheat seeds (cv. Bobwhite and cv. Nandu) were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 10 min, rinsed thoroughly in sterile water and incubated for 3 days in the dark on wet filter paper for germination. Seedlings were then vernalized at 4 °C for 7-10 days before transplant in soil. Plants were grown in climatic

chamber, 1 month approximately with a 14 h photoperiod and 19/17°C day/night temperature, 1 month approximately with a 14 h photoperiod and 22/20°C day/night temperature.

*Arabidopsis thaliana* seeds were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 5 min and washed thoroughly two times in sterile water. A diluted sterilized seeds suspension was sown in soil, three drops of seeds suspension for pot. Plants were grown in climatic chamber with a 12 h photoperiod and 22/20°C day/night temperature.

Maize plants (cv. DEKALB DK6530) were grown in field and ears were collected at flowering stage for inoculation experiments.

### **2.3. Nucleic acid extraction**

Genomic DNA from *F. graminearum* wild-type and mutant strains was extracted from the mycelium obtained in liquid culture. After addition of 1 ml of a 2X CTAB stock solution [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 (DTT)] 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 3M 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 500 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA). Contaminating RNA was removed by treating DNA with 0.04 µg/ml of RNase A (Fermentas, Milano, Italy) for 2 hours at 37 °C.

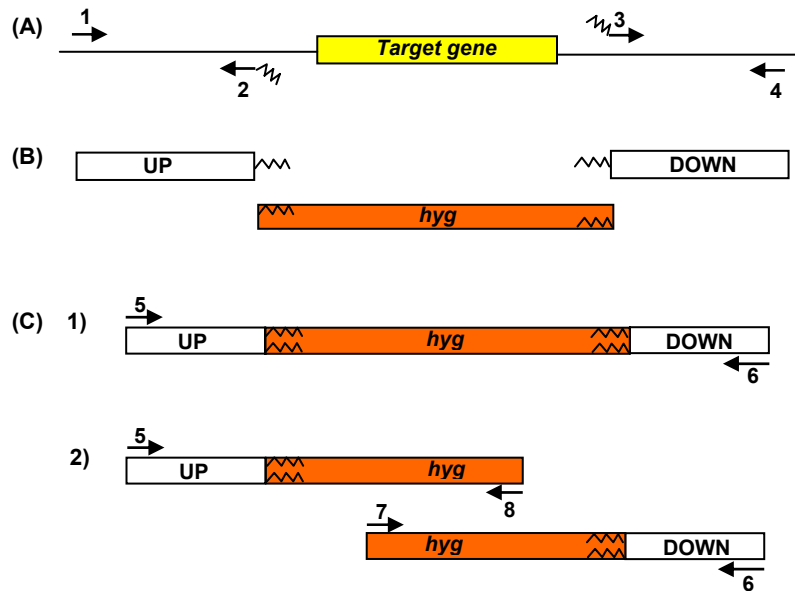
#### **2.4. Construction of gene replacement vectors and fungal transformation-mediated gene disruption**

To generate the constructs for disruption of the *F. graminearum* *pg1* and *pg2* genes, flanking homology regions of each gene were amplified by PCR using *F. graminearum* genomic DNA 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 1 and figure 1A). Lengths of upstream and downstream flanking sequences were about 1000 bp. The amplification of the flanking regions was performed in a 50 µl volume. The PCR conditions were as follows: 94°C 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, a suitable annealing temperature (table 2) 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) used as selection marker (Punt et al., 1987). The fusion PCR reaction (figure 1B) was performed in a 50 µl volume using 200 ng of the purified flanking regions containing tails homologous to the 5' and 3' region of the *hph* gene and 400 ng of *hph* gene cut with *Sma* I (Fermentas, Milano, Italy) from a pGEM-T vector (Promega, Milano, Italy) kindly provided by Prof. Schäfer of the University of Hamburg. 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, or primer pairs 5-8 and 7-6 for obtaining the two split-marker constructs, respectively (table 1 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, a suitable annealing temperature (table 2) 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 of construct for transformation the E.coli transformer with pGEM-T vector with amplicons was growth in LB-broth high salt culture (Fluka, BioChemika) and after over night incubation at 37°C, the

plasmid DNA was extracted according to MIDI Nucleobond<sup>®</sup> Ax (Macherey-Nagel GmbH & Co.) kit manual. Full or split-marker constructs were then cleaved from the pGEM-T vector by using suitable restriction enzymes (table 3) and were used to transform protoplasts of *F. graminearum* wild-type strain as reported below. Length and type of constructs used are also reported in table 3. Protoplast formation and fungal transformation were performed according to Jenczmionka and Schäfer (2004) and Proctor et al. (1995) with some modification. 100 ml of YEPD medium (0.3% yeast extract, 1% bacto peptone, 2% glucose) were inoculated with  $1 \times 10^6$  conidia of *F. graminearum* and incubated overnight at 28°C and 150 rpm. The mycelium was harvested by filtration through a Wilson-sieve (100 µm) and washed with sterile water. Mycelium was then collected and dried on a sterile filter paper. Thereafter, 0.5/1 g of dried mycelium was resuspended in 20 ml of enzyme solution [2.5% driselase (Sigma-Aldrich, Milano, Italy) and 0.5% lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, Milano, Italy) dissolved in 1.2M KCl] and incubated at 30°C for 3 hours to obtain the digestion of the fungal cell wall. The obtained protoplasts were then filtered two times (100µm and 50µm), centrifuged and resuspended at the final concentration of  $1 \times 10^8$  protoplasts/ml. 10 µg of digested DNA construct were then added to 200µl of the protoplast solution ( $2 \times 10^7$  protoplasts), mixed well and incubated for 20 minutes. After incubation, 1ml of PEG-Co solution [40% PEG4000, 60% STC-Co (20% saccharose, 10mM Tris-HCl pH8, 50mM CaCl<sub>2</sub>)] was added and the mixture was mixed and incubated for further 20 minutes. After that, 5 ml of TB3 solution (20% saccharose, 0.3% yeast extract, 0.3% casein acid hydrolysed) were added and the mixture was shaken over night at 28°C for cell wall regeneration. The following day protoplasts with regenerated cell wall were spinned down and resuspended in 1ml of TB3 solution. The protoplasts mixture was then divided into 5 falcons and 10 ml of 1.4% Granulated agar (Difco<sup>™</sup>, Becton, Dickinson and Company, Sparks, MD, USA) dissolved in TB3 solution and supplemented with 150 µg/ml hygromycin B (Duchefa Biochemie) were added to each falcon. After mixing, the content of each tube was plated in a 96mm petri dish. After over night incubation at 28°C, 10 ml of 1.4% granulated agar dissolved in TB3 solution and supplemented with double amount of Hygromycin (300 µg/ml hygromycin B) were poured into each plate. After 3-10 days of incubation at 28°C the

colonies growing in the plates were collected and transferred to 30 mm plates containing CM Regular medium-agar [0.1% (w/v) yeast extract, 0.1% (w/v) enzymic hydrolysed casein, 34% (w/v) saccharose, 1.6% (w/v) granulated agar supplemented with 100 µg/ml of hygromycin B (Duchefa, Haarlem, The Netherlands). Hygromycin resistant mutants were then single-conidiated and preliminarily screened by PCR using the following primers: fg11011-5int and fg11011-3int for *Fgpg1* gene, fg03194-5int and fg03194-3int for *Fgpg2* gene (table 4). Transformants without the *pg* coding region were then tested by Southern blot hybridization for single insertion of the disruption construct.



**Figure 1** – Schematic illustration of the PCR-based construction of gene replacement vectors: **(A)** Flanking homology regions of *F. graminearum pg1* and *pg2* genes 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)**, or 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.

## 2.5. Southern blot analysis

For Southern blot analysis, approximately 5/7 µg of genomic DNA were digested with suitable restriction enzyme, separated on a 1.0% (w/v) agarose/TAE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Milano, Italy). Digoxigenin (DIG)-labeled (Roche, Mannheim, Germany) specific probes were generated with gene specific primers using genomic or plasmid DNA as template (table 5) and were alternatively used for overnight hybridization at 65 °C.

The PCR reaction, performed in a 50 µl volume and using DIG-11-dUTP (Roche, Mannheim, Germany), consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, a suitable annealing temperature (table 5) for 1 min and 72°C for 2 min.

Southern hybridization and detection of the DIG-labeled probes were performed according to manufacturer's instruction. Membranes were exposed to X-ray film (X-Omat AR, Kodak, Rochester, NY, USA) for approximately 3 hours.

## 2.6. Analysis of the PG pattern produced by mutants

*F. graminearum* WT and mutant strains were grown for 4 days in 50 ml Szécsi medium containing 1% citrus pectin (ICN) (w/v) (Szécsi, 1990). The culture filtrates were 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, Germany). The culture filtrates were heated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 20% of the saturation value and after 3 h they were centrifuged for 30 min at 4°C at 10,000 g. The supernatants were salted out with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 75% saturation overnight at 4°C. The mixtures were then centrifuged for 40 min at 4°C at 25,000 g and the pellets were 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, obtained by mixing equal volumes of ampholytes covering the pH ranges 6.0-8.0 (Sigma-Aldrich, Milano, Italy) and 8.0-10.5 (Amersham Biosciences, Uppsala, Sweden). PG isoforms were detected on a pectate agarose overlay gel buffered at pH 5.0, according to Ried and Collmer (1985).

The PG pattern produced by  $\Delta$ PG1 and  $\Delta$ PG2 mutants was analysed also *in vivo* by grinding 12 infected ovaries with floret glumes (from cv. Bobwhite) collected 4 days after spikelet inoculation, and loading the poured tissue on a PAA gel prepared as reported above.

## **2.7. Characterization of mutant strains and dry weight experiments**

For dry weight experiments and PG activity assays, conidia of WT and mutant strains were placed in 100-ml Erlenmeyer flasks containing 20 ml of Szécsi medium (Szécsi, 1990) supplemented with 1% (w/v) pectin to obtain a final concentration of  $1 \times 10^4$  conidia  $\text{ml}^{-1}$ . 3 flasks per strain were prepared in each experiment. The cultures were then grown for 7 days at 24°C on an orbital shaker at 150 rpm. 500  $\mu\text{l}$  aliquots were harvested daily from each flask to assess the PG activity produced by the fungal strains. At the end of the experiment, the content of each flask was transferred into 50-ml pre-weighed tubes and centrifuged at 8500  $\times g$  for 20 min. The tubes containing the mycelium were washed two times with water, oven dried at 80°C for 3 days and then weighed. The dry weight experiment was repeated 3 times. Data were statistically analyzed applying the two-tailed Student's t-test.

## **2.8. PG activity assays**

PG activity was determined 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 0.5 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 6.0, and 1.5 ml of the culture filtrate collected as above reported and suitably diluted in the same buffer. Micro-Ostwald capillary viscosimeters (i.d. = 0.70 mm), connected to AVS 310 system (Schott Geräte, Mainz, Germany), were used. One viscosimetric unit (VU) was defined as the amount of enzyme causing a 50% decrease of the initial relative viscosity of the reaction mixture in 1,000 min. Reducing end-groups assay was performed by the method described by Milner and Avigad (1967) by incubating for 30 minutes 5 or 10  $\mu\text{l}$  of the culture filtrate collected as above reported in a 200  $\mu\text{l}$  reaction mixture containing PGA 0.25% as

substrate. One reducing unit (RU) was defined as the amount of enzyme required to release 1  $\mu\text{mol}/\text{min}$  of reducing groups using D-galacturonic acid as standard.

## **2.9. Infection of wheat plants**

Wheat spikes (cv. Bobwhite) were inoculated at anthesis (Zadoks stage 65-67; Zadoks et al., 1974) with *F. graminearum* wild-type or mutant strains. Spikes were inoculated by dropping between the bracts of two florets of two opposite spikelets 10  $\mu\text{l}$  of a fresh conidial suspension, containing approximately 2,000 conidia. At least 3 independent experiments were performed by inoculating at least 10 plants with each strain. After inoculation, the spikes were covered for 3 days post-infection with a plastic bag to maintain a moist environment. 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 22°C and a night-time temperature of 20°C. Symptom development on inoculated spikes was monitored up to 3 weeks post-inoculation. Data were statistically analyzed applying the two-tailed Student's t-test.

## **2.10. Infection of *Arabidopsis thaliana* flowers**

8-week-old *Arabidopsis* plants were inoculated with *F. graminearum* WT, Split10.4 ( $\Delta\text{PG1}$ ) and 2.8.1 ( $\Delta\text{PG2}$ ) strains by placing 10  $\mu\text{l}$  of a conidial suspension ( $1.5 \times 10^5$  conidia/ml) supplemented with 0.5% Tween 20 (Sigma-Aldrich, Milano, Italy) on the principal open flower and rosette.

The infected plants were covered with a plastic bag for 4 dpi to maintain high humidity and were incubated at 20°C, monitoring the symptom development up to 10 days post-inoculation, when the disease symptoms on *Arabidopsis* is maximal. Three independent infection experiments were performed with least 10 infected plants per strain.

According to the observed symptoms, plants showing superficial mycelium covering the principal flower and rosette and with the main stem dried and constricted were defined as “dead inflorescences”. Plants with symptoms restricted to the inoculated flowers and with the apical meristem still viable were defined as “surviving inflorescences”. Data were statistically analyzed applying the chi-square test.

### **2.11. Infection of maize ears**

Maize ears collected from field were infected with *F. graminearum* WT, Split10.4 ( $\Delta$ PG1) and 2.8.1 ( $\Delta$ PG2) strains. Ten maize ears per strain were inoculated in 3 points with 200  $\mu$ l of the conidial suspension ( $1 \times 10^6$  conidia/ml). After inoculation, ears were covered with a plastic bag to maintain a moist environment and were incubated at room-temperature, monitoring the symptom development for 10 days post-inoculation. One independent experiment was performed.

### **2.12. DON mycotoxin quantification**

Concentration of DON produced by *F. graminearum* WT and mutant strains in the infected wheat spikelets (cv. Bobwhite) and in liquid medium was estimated by using the RIDASCREEN DON enzymatic immunoassay (R-Biopharm AG, Darmstadt, Germany). The basis of the test is the antigen-antibody reaction. The microtiter wells contained in the test kit are coated with capture antibodies directed against anti-deoxynilvalenol antibodies. Free deoxynilvalenol and deoxinilvalenol enzyme conjugate compete for the doexynilvalenol antibody binding sites. At the same time, the DON antibodies are also bound to the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. After addition of the substrate/chromogen to the wells, bound enzyme conjugate convert the chromogen into a blue product. The addition of the stop solution leads to a colour change from blue to yellow. The absorbance inversely proportional to the deoxynivalenol concentration in the sample, was measured photometrically at 450 nm.

To check the mycotoxin concentration produced in liquid culture, WT and mutant strains were grown in Sczècsi medium with 1% pectin as the sole carbon source. After 4 days of growth, 500  $\mu$ l of the filtrated cultures were concentrated by vacuum to 50  $\mu$ l of final volume, and then tested for DON content.

To check the mycotoxin produced by WT and mutants strains in infected wheat plants, infected spikes were collected at 21 dpi. Only the two inoculated spikelets per spike were analyzed for DON content at least. Three repetition were performed for each strain, each repetition consisting of 6 spikelets from 3 different spikes. Spikelets were homogenized with mortar and pestle diluting the tissue 1:20 or 1:10 (w/v) with water.

The homogenized tissue was then centrifuged at 8000 rpm for 5 min; the supernatants were collected in a new falcon and centrifuged again at 8000 rpm for 5 minute. The supernatants was finally diluted 1:100 and different volumes were tested. Data were statistically analyzed applying the two-tailed Student's t-test.

### **2.13. Fungal transformation with GFP construct**

*F. graminearum* WT and Split10.4 mutant ( $\Delta$ PG1) strains were transformed to express the gene encoding for the green fluorescent protein (GFP) from jellyfish. GFP is a naturally fluorescent, non toxic protein and a vital marker allowing the progress of fungal infection to be monitored in living system. The protein fluoresces in response to illumination with UV, so the fungus is visible without prior manipulation of the tissue.

The GFP mutants were obtained by non-homologous integration in the genome of the pII99 plasmid (kindly provided by Prof. Schäfer of the University of Hamburg) linearized with Hind III; the pII99 plasmid contained the GFP encoding gene under the control of the isocitrate-lyase promoter of *Neurospora crassa* and the geneticin resistance gene. Transformation was conducted as above reported. The protoplast mixture was plated in a 96mm petri dish containing 10 ml of 1.4% granulated agar dissolved in TB3 solution and supplemented with 100  $\mu$ g/ml geneticin (Geneticin G-418 sulphate, GIBCO®). After over night incubation at 28°C, 10 ml of 1.4% granulated agar dissolved in TB3 solution supplemented with double amount of geneticin (200  $\mu$ g/ml geneticin) were poured into the plate. After 3-10 days of incubation at 28°C the growing colonies were collected and transferred to plates containing CM Regular medium-agar supplemented with 100  $\mu$ g/ml of geneticin. GFP mutants were preliminarily screened by PCR amplification (GFPfor/GFPprev, table 4) to check the presence of the complete GFP gene. PCR positive transformants were then tested by Southern blot hybridization (table 5) to verify the number of insertions. The GFP mutants with maximum 3 insertions of *gfp* gene were checked by fluorescence microscopy. The mutants with homogeneous fluorescence in mycelium and conidia were selected for infection experiments.

#### **2.14. Histological examination of infected wheat spikelets**

Wheat infection experiments for histological examination were conducted on two wheat cultivars (Nandu and Bobwhite) as above described. Histology with cv. Nandu was carried out during a stage at the Biocenter Klein Flottbeck of the University of Hamburg. The first infection experiment was performed to examine the progression of mutants expressing GFP within the wheat tissue. Both WT and  $\Delta$ PG1 GFP expressing strains maintained a similar behaviour in plant compared to the correspondent strains without GFP.

Spikelets and rachis of wheat spikes infected with WT and  $\Delta$ PG1 GFP expressing strains were collected between 5 and 10 days after inoculation (dai), hand-sectioned and monitored by fluorescence microscopy following the constitutively expressed GFP signal.

The microscopical examinations were conducted with the following fluorescence microscopes and filter sets: A Zeiss Axio Imager.Z1 equipped with following filter sets: GFP: exc.: 450-490 nm, beam splitter: 495 nm, em.: 500-550 nm; DAPI: exc.: 335-383 nm, beam splitter: 395 nm, em.: 420-470 nm and the fluorescence camera AxioCam MRm. Confocal laser scanning microscopy was performed by a Zeiss LSM 710. The GFP was excited with a 488-nm laser-line, and fluorescence was detected at 505–530 nm. Autofluorescence of cell walls was excited with a 488 nm laser-line and detected at 580–620nm.

**Table 1.** Primers used to produce the deletion constructs by fusion PCR method.

<i>Fgpg1 gene</i>		
1	Fg11011-5'up	CCGCGTCGATGAGAACAGAAATAG
2	Fg11011-3'up	AGATGCCGACCGAACAAGAGCTGTCCCCATAAAGGCCCGTGTTCAGTGTCCAG
3	Fg11011-5'down	TCAATGCTACATCACCCACCTCGCTCCCCCTGGCAAAGACAAAGGAAAATG
4	Fg11011-3'down	CAAAAGCCGGAAGAATGTCTG
FULL CONSTRUCT		
5	Fg11011-5'nested	AGCCACCACATGCGATTCT
6	Fg11011-3'nested	CGAGCGTTGGGATTATGC
SPLIT MARKER CONSTRUCT		
5	Fg11011-5'up nested	ACCCACCCCAATTCTGTCTGTA
6	Fg11011-3'down nested	AATGTCCATCCTCGCTCGTTCC
7	HygFOR	GTTGGCGACCTCGTATTGG
8	HygREV	CTTACCACCTGCTCATCACCT
<i>Fgpg2 gene</i>		
1	Fg03194-5'up	TCGTCGTCGCAAACCCTGATACT
2	Fg03194-3'up	AGATGCCGACCGAACAAGAGCTGTCCCCATGAACGCCGAGCCTGAAAATG
3	Fg03194-5'down	TCAATGCTACATCACCCACCTCGCTCCCCAACTCTTGTCTCCACTCCCTCTGA
4	Fg03194-3'down	GTCTGGCGAACATAACAATAAAG
5	Fg03194-5'upnested	GCAACTTGTGGCTGGAAAAC
6	Fg03194-3'downnested	GGACCCCTACCCGAAGAT

**Table 2.** Annealing temperatures (Ta) for the amplification of upstream and downstream flanking regions and for nested PCRs.

<u>PG1 construct</u>	Ta
Fgpg1 up region	53 °C
Fgpg1 down region	52 °C
Fgpg1 nested	53 °C
Fgpg1 split-marker up	53 °C
Fgpg1 split-marker down	60 °C.
<u>PG2 construct</u>	
Fgpg2 UP region	58 °C
Fgpg2 DOWN region	53 °C
Fgpg2 nested	56 °C

**Table 3.** Restriction enzymes used to cleave the construct from pGEM-T vector with type and length of the construct obtained.

Construct for	Construct length	Type of construct	Restriction enzymes
<i>pg1</i>	2.2 kb / 2.2 kb	Split-marker constructs	<i>Apa I / Sal I</i>
<i>pg1</i>	3.5 kb	Full construct	<i>Apa I / Sal I</i>
<i>pg2</i>	3.5 kb	Full construct	<i>Apa I / Not I</i>

**Table 4.** Primers for preliminary screening of mutants and annealing temperature (Ta) used.

Primer name	Primer sequence	Gene amplified	Ta
fg11011-5'int	ATCTCCGGCGCTGTCGTCAA	<i>pg1</i>	55°C
fg11011-3'int	GCAGCTAGCGCAAAGAATGTA		
fg03194-5'int	GCCCAAGTTCTTCGCTGCTCACA	<i>pg2</i>	55°C
fg03194-3'int	GAAGGCCGCTGGTAGGGTCTCC		
GFPfor	CCACTAAATACCCACGACAT	GFP	52°C
GFPprev	CGCGCTATATTTTGTCTTCT		

**Table 5.** Primers used for amplification of the DIG-labeled DNA probes and corresponding annealing temperature (Ta).

Primer name	Primer sequence	Template	Ta
Hyg5PRB	CTCGATGAGCTGATGCTTTG	Plasmid DNA	55°C
Hyg3PRB	CTTGTTTCGGTCGGCATCTAC		
GenPRBf	AGGATCTCCTGTCATCTCA	Plasmid DNA	54°C
GenPRBr	CCAAGCTCTTCAGCAATA		
GFP PRBf	CTGACCCTGAAGTTCATTT	Plasmid DNA	54°C
GFP PRBr	ACTGGGTGCTCAGGTAGT		
Fg11011-Fprobe	CCTTTACTGATGCTGCTTCT	Genomic DNA	55°C
Fg11011-Rprobe	CATGTGGACGTTGTAGACAC		
Fg03194-5'int	GCCCAAGTTCTTCGCTGCTCACA	Genomic DNA	55°C
Fg03194-3'int	GAAGGCCGCTGGTAGGGTCTCC		

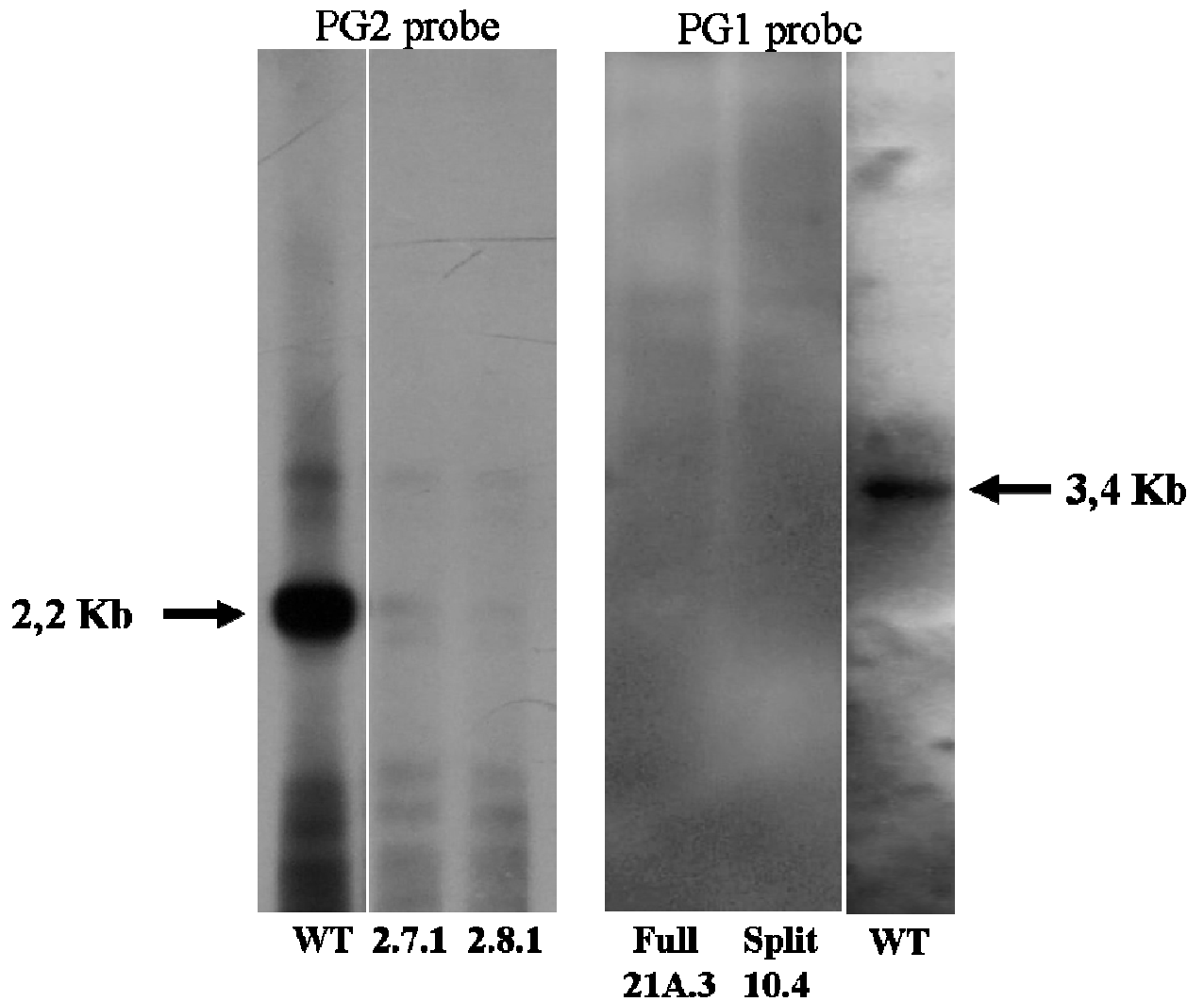


### 3. RESULTS

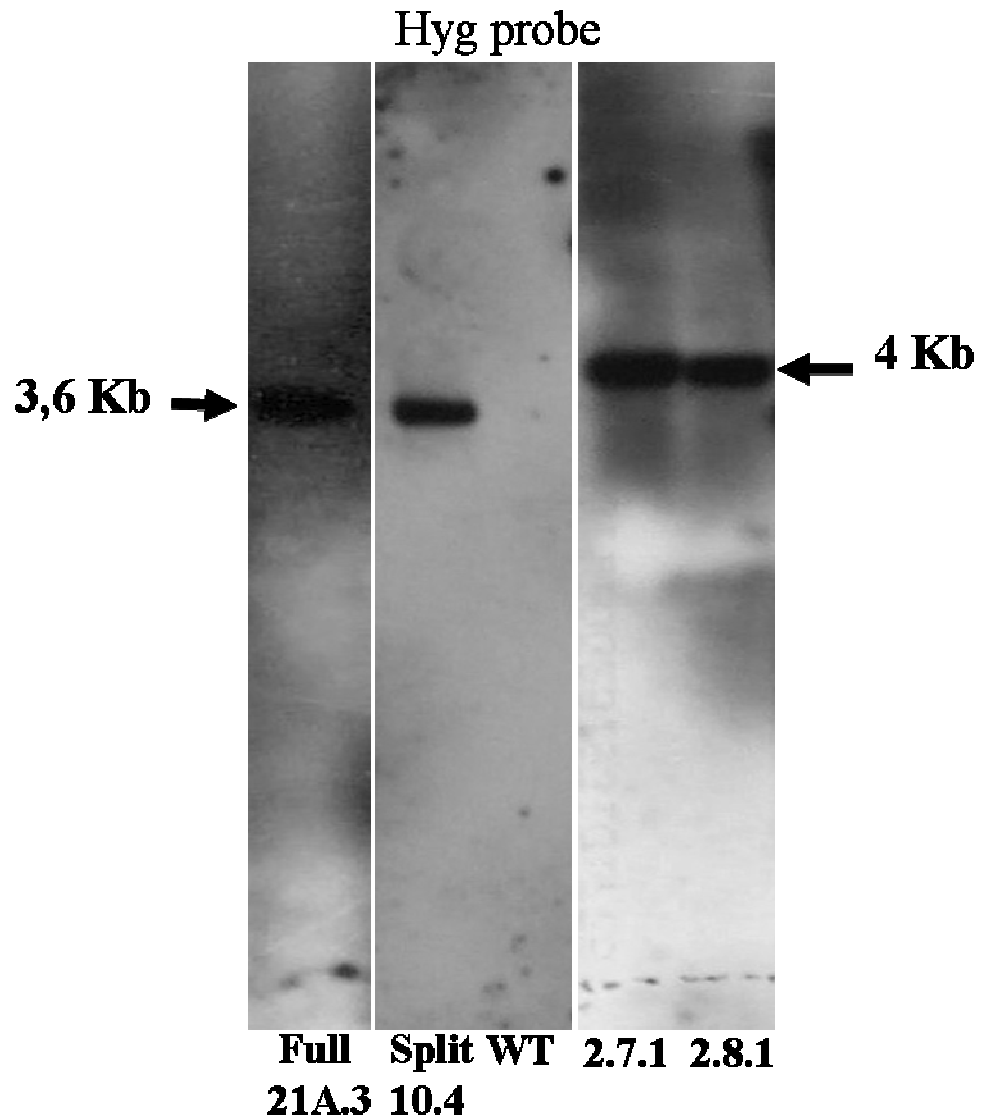
#### 3.1. *F. graminearum* WT transformation to obtain single PG knock-out mutants

The *F. graminearum* WT strain was transformed with specific constructs in order to obtain the targeted integration of the hygromycin resistance gene to replace the *pg1* and *pg2* genes, respectively. In total, sixty *F. graminearum* transformants were analyzed by PCR to determine if the *pg* genes were effectively knocked-out. In particular, the complete *pg1* gene was not amplified by PCR in 2 of 10 transformants obtained with the split-marker construct and in 10 of 22 transformants obtained with the full construct. *Pg2* gene was not amplified in 14 of 28 transformants (data not shown). After single conidiation of some selected PCR positive transformants (5 putative  $\Delta$ PG1 and 7 putative  $\Delta$ PG2), high-stringency Southern blot analysis of genomic DNA was performed and showed a single homologous integration of the disruption construct in 3  $\Delta$ PG1 and 4  $\Delta$ PG2 mutant strains analysed (figures 2 and 3); ectopic integration of the hygromycin resistance gene was observed in the other mutants (data not shown).

To check that these mutations did not impair mycelium growth, mutant strains were transferred to PDA and SNA plates, and to CM and CMC liquid media. No differences in growth compared to WT strain were observed, neither conidial production differed from CMC liquid culture and SNA plates (data not shown).



**Figure 2** – Southern blot analysis of genomic DNA from *F. graminearum* wild-type and mutant strains. Genomic DNA was digested with *Xba I* (Promega) and a PG1 or PG2 probe was alternatively used. Only the wild-type strain showed an hybridization signal of 3.4 kb corresponding to the *pgl* gene, compared to the  $\Delta$ PG1 mutant strains (Full21A.3 and Split10.4). The  $\Delta$ PG2 mutant strains (2.7.1 and 2.8.1), differently from the wild-type strain, did not show the hybridization signal of 2.2 kb.



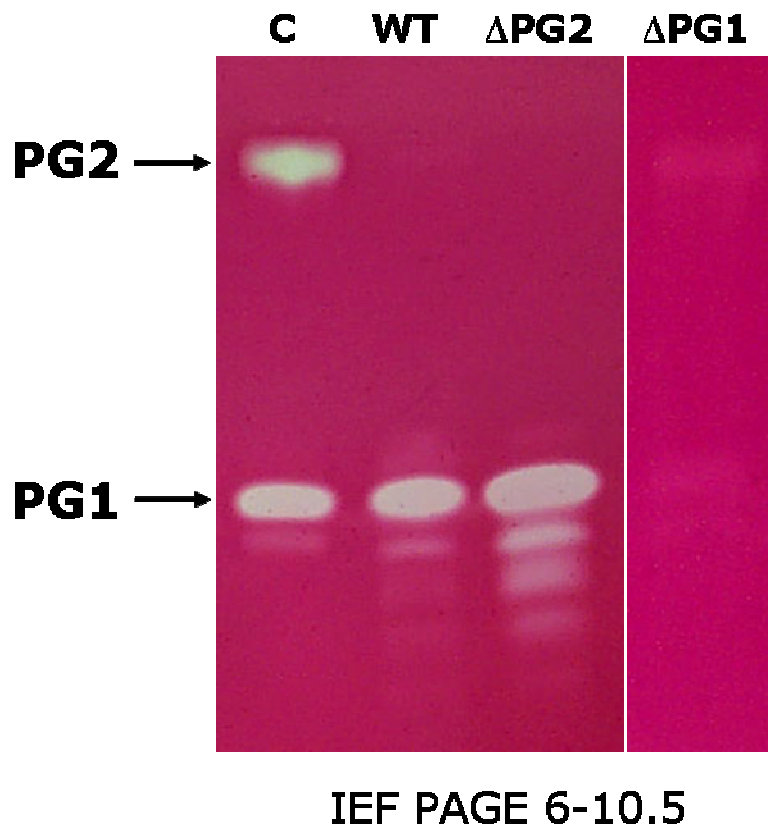
**Figure 3** – Southern blot analysis of genomic DNA from *F. graminearum* wild-type and mutant strains. Genomic DNA was digested with *Xba I* (Promega) and a fragment of the *Hyg* resistance gene was used as probe. The  $\Delta$ PG1 mutant strains (Full21A.3 and Split10.4) showed a single hybridization signal at 3.6 kb; the  $\Delta$ PG2 mutant strains (2.7.1 and 2.8.1) showed a single hybridization signal at 4.0 kb; the wild-type strain gave no hybridization signal.

### 3.2. In vitro characterization of $\Delta$ PG1 and $\Delta$ PG2 mutant strains

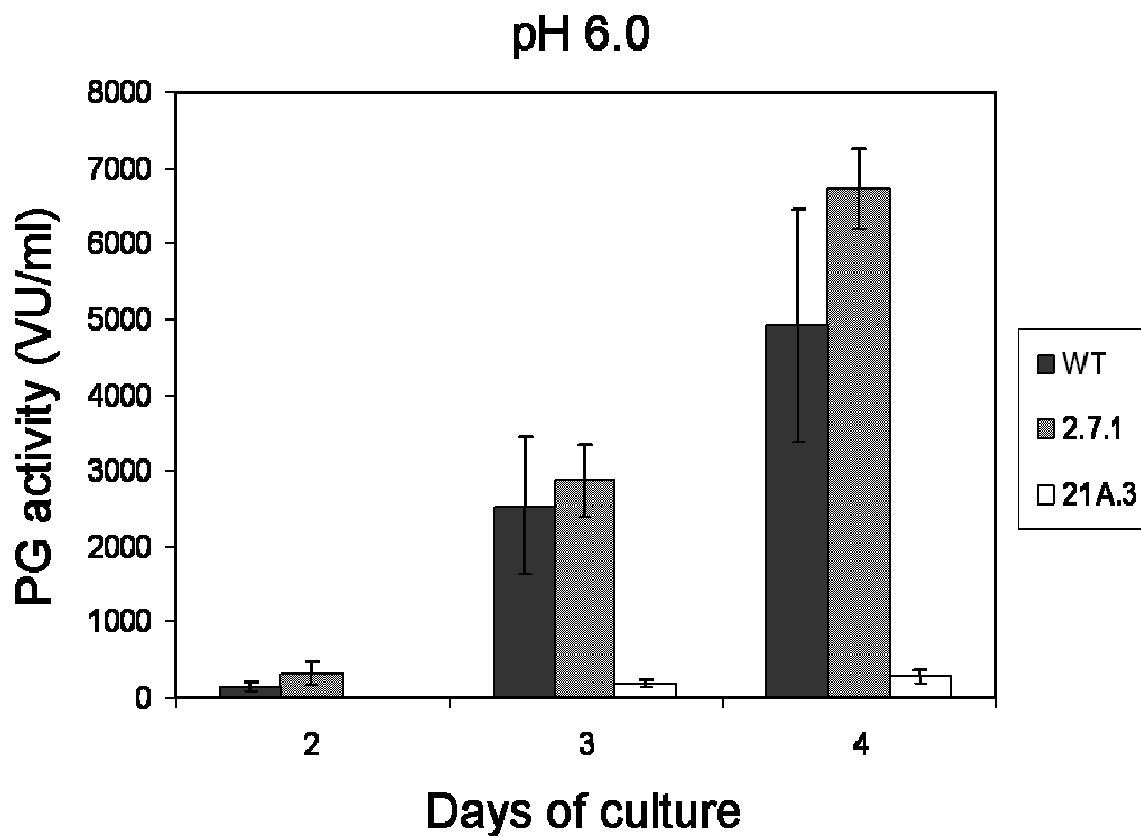
To verify if the disruption of the *pg* gene was effective, we characterized the secreted PG activity of  $\Delta$ PG1 mutant strain (Split10.4 and Full21A.3) and two  $\Delta$ PG2 mutant strains (2.7.1 and 2.8.1). *F. graminearum* wild-type and knock-out mutants were grown in liquid culture containing pectin as the sole carbon source to induce PG production. Then the culture filtrates were analysed by analytical IEF and by PG assays. IEF analysis confirmed that the *F. graminearum*  $\Delta$ PG1 and  $\Delta$ PG2 knock-out mutants produced respectively only PG2 and PG1 compared to the wild-type strain (figure 4). Besides IEF analysis showed that all the isoforms about pI 8.15 were encoded by the *pg1* gene and were not produced by the  $\Delta$ PG1 mutant (figure 4).

Viscosimetric and reducing-ends group assays were then performed to measure the PG activity produced by the two mutants at 2, 3 and 4 days of culture. In particular, the viscosimetric assay showed that the PG activity produced by the  $\Delta$ PG1 mutant was negligible compared to that produced by WT and  $\Delta$ PG2 mutant at all the time points tested (figure 5). The reducing-ends group assay showed that after 3 days of culture the PG activity of  $\Delta$ PG1 mutant was similar to that of WT and  $\Delta$ PG2 strain. However, after 4 days, the PG activity of WT and  $\Delta$ PG2 was maximum and 4/6 times higher than that produced by  $\Delta$ PG1, whose activity was lower than that measured at 3 days (figure 6).

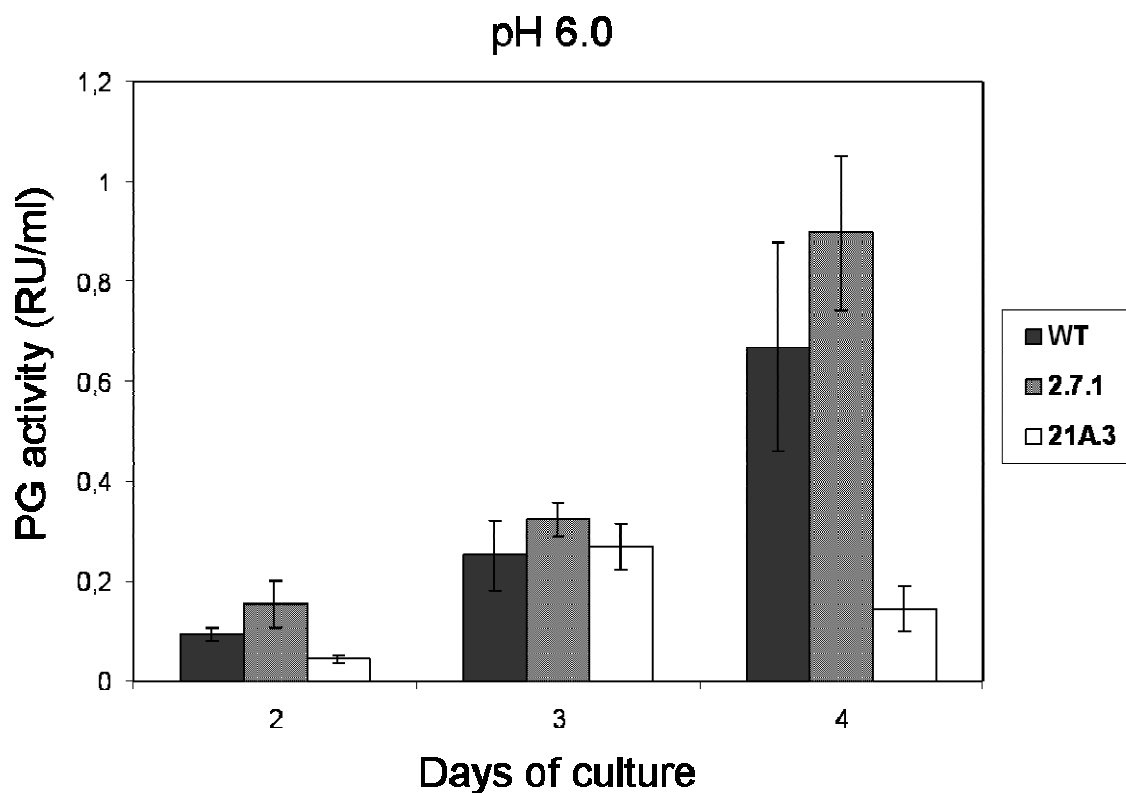
We also evaluated the dry weight obtained by growing WT and mutant strains in liquid culture with pectin as the sole carbon source. After 7 days of culture, we did not find any significant difference between all the strains tested (figure 7).



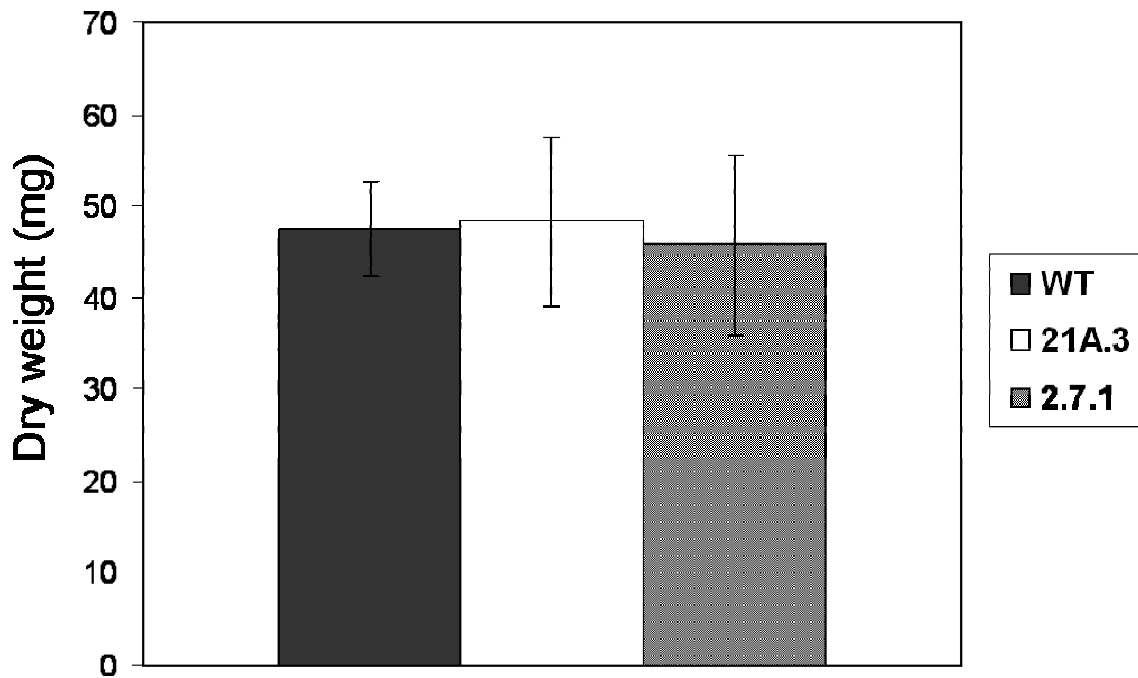
**Figure 4** – Thin layer IEF in the pH range 6.0-10.5 of PG activity produced by *F. graminearum* wild-type and mutant strains in liquid culture containing pectin as the sole carbon source. 4 days-old liquid cultures were filtrated, concentrated and dialyzed. The  $\Delta$ PG2 mutant (strain 2.7.1 in the figure) and the  $\Delta$ PG1 mutant (strain Full21A.3 in the figure) produced only PG1 and PG2, respectively, when compared to the wild-type strain. PG2 isoform is barely visible in  $\Delta$ PG1 and wild-type strains. The Split10.4 and the 2.8.1 strains showed the same behaviour as Full21A.3 and 2.7.1 respectively (data not shown).



**Figure 5** – PG activity produced by *F. graminearum* WT and mutant strains in Szécsi liquid culture with 1% pectin as the sole carbon source and measured by viscosimetric assay at pH 6. The viscosimetric activity was expressed in viscosimetric units per millilitre (VU/ml). Each data point represents the mean of three determination carried out on three different flasks. Bars indicate the standard error of the mean (SEM).



**Figure 6** – PG activity produced by *F. graminearum* WT and mutant strains produced in Szécsi liquid culture with pectin as the sole carbon source and measured by reducing-end groups assays at pH 6. Reducing end-groups were expressed in reducing units per millilitre (RU/ml). Each data point represents the mean of three determinations carried out on three different flasks. Bars indicate the standard error of the mean (SEM).



**Figure 7** – Dry weight experiment.  $1 \times 10^4$  conidia/ml of WT and mutant strains were grown in 20 ml of Szècsi liquid medium supplemented with 1% pectin; the fungi were grown for 7 days on orbital shaker at 150 rpm at 24°C. At the end of the experiment the cultures were filtrated, washed and oven dried at 80°C for 3 days and then weighed. Dry weights are expressed in milligrams (mg). The Split10.4 and the 2.8.1 strains showed the same behaviour as Full21A.3 and 2.7.1 respectively (data not shown). Probability (p) that there is no significant difference between WT and mutant strains, as determined by the two-tailed T-Student's test:

Full21A.3	p > 0.94
2.7.1	p > 0.88

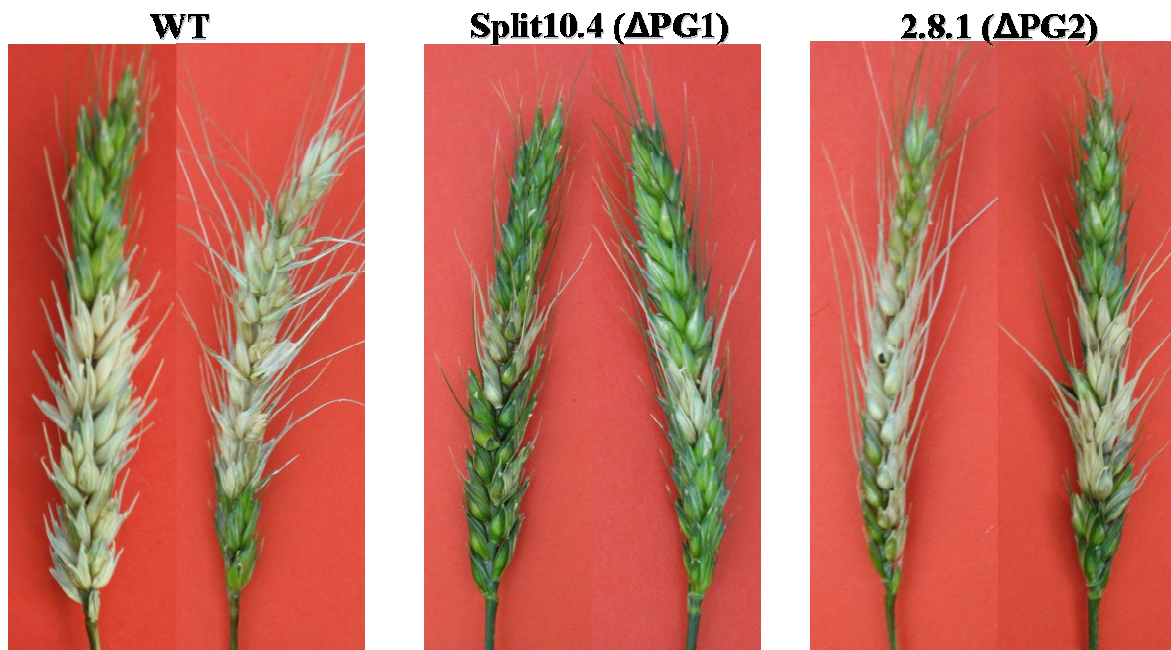
Bars indicate the standard error of the mean (SEM).

### 3.3. In vivo characterization of $\Delta$ PG1 and $\Delta$ PG2 strains

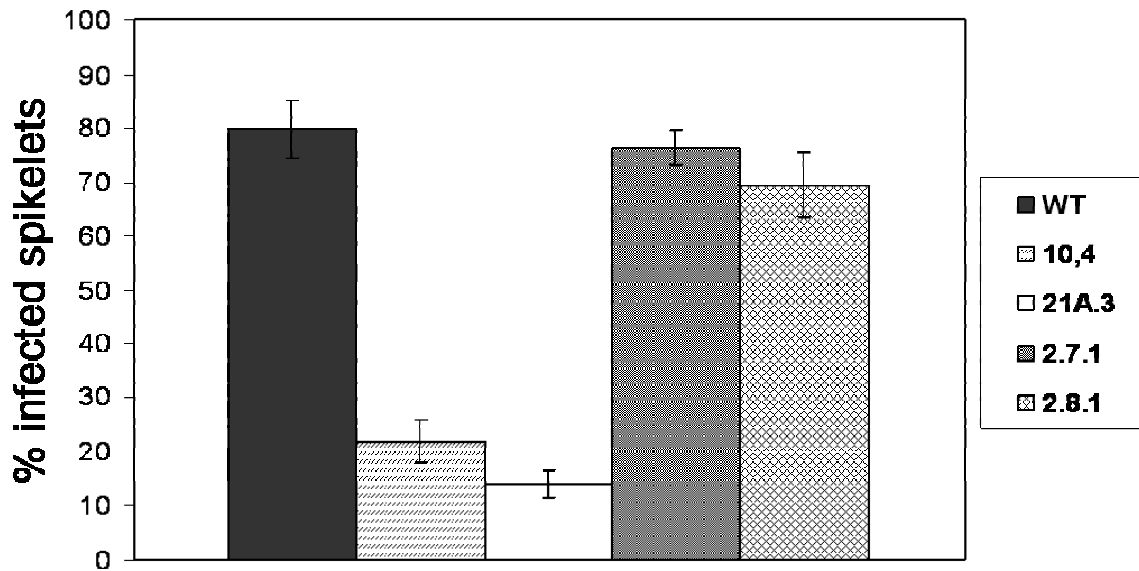
#### 3.3.1. Wheat infection experiments

To determine whether *F. graminearum* PG1 and PG2 are involved in virulence, infection experiments of wheat spikes (cv. Bobwhite) were performed. Plants were point inoculated at anthesis with a spore suspension of wild-type and mutant strains. In particular, we used both  $\Delta$ PG1 strains (Split10.4 and Full21A.3) and both  $\Delta$ PG2 strains (2.7.1, 2.8.1). Several infection experiments were performed and results were assessed 20 days post infection. All the knock-out mutants tested maintained the capability to infect wheat plants; however, the  $\Delta$ PG1 mutant strains showed a significant reduction of virulence compared to the wild-type strain (about 75-80% less infected spikelets), while no reduction of virulence was observed with the  $\Delta$ PG2 mutant strains (figures 8 and 9). In particular, infections with the  $\Delta$ PG1 mutants were often restricted to the initially inoculated spikelet and only occasionally this mutant spread to the adjacent spikelets (figure 10).

These results seems to indicate that PG1 likely play an important role during pathogenesis, instead PG2 could be dispensable.



**Figure 8** – Wheat spikes infected with *F. graminearum* at 14 days post inoculation. The spikelets infected with WT were almost blight; the same symptoms were observed in spikelets infected with 2.8.1 ( $\Delta$ PG2) mutant. The spikelets infected with split10.4 ( $\Delta$ PG1) strain showed a clearly evident reduction of virulence compared to the wild-type strain.



**Figure 9** – Infected wheat spikelets symptoms at 20 dpi. Disease symptoms were assessed by counting the number of visually diseased spikelets and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets. At least 3 independent infection experiments were performed and at least 10 plants were inoculated in each independent experiment. Total number of infected plants: 68 inoculated with WT; 45 with Split10.4; 47 with Full21A.3; 37 with 2.7.1 and 35 with 2.8.1.

Probability (p) that there is no significant difference between WT and mutant strains, as determined by the two-tailed T-Student's test:

Split10.4	p < 0.00003
Full21A.3	p < 0.00008
2.7.1	p > 0.59
2.8.1	p > 0.22

Bars indicate the standard error of the mean (SEM).



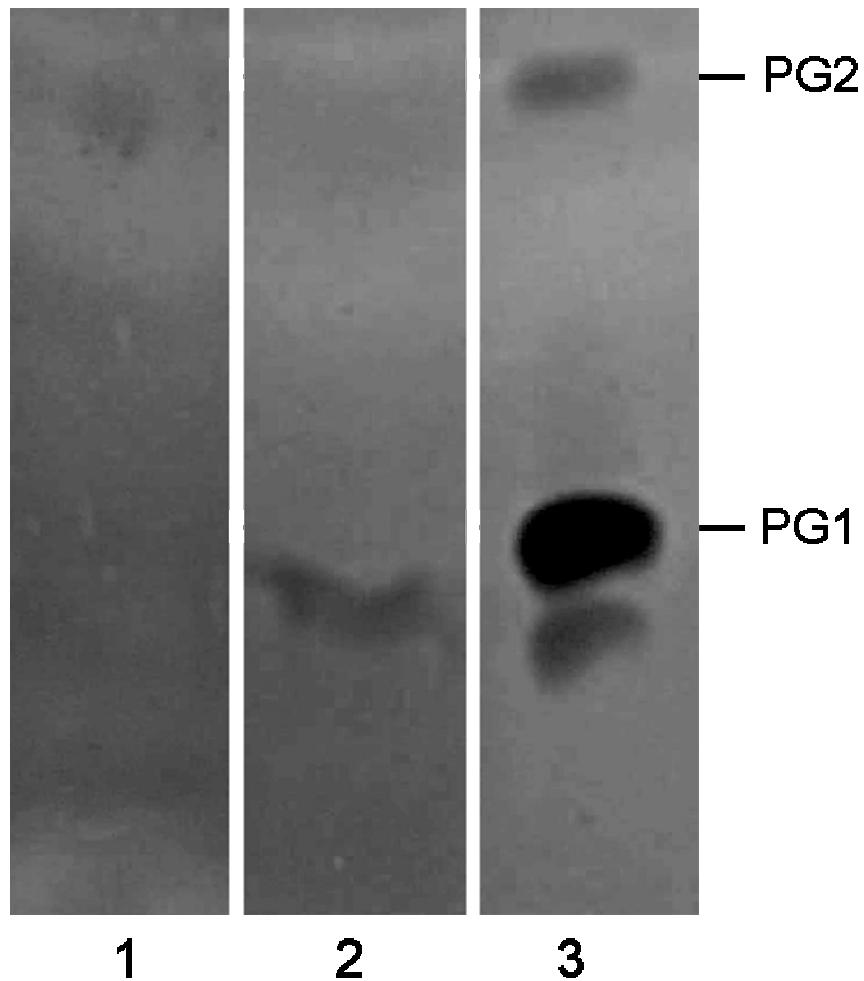
**Figure 10** – Wheat spikes inoculated with *F. graminearum* wild-type and  $\Delta$ PG1 mutant strains 20 days after inoculation. The spikelets infected with  $\Delta$ PG1 mutants were often restricted to the initially inoculated spikelet and only occasionally this mutant spread to the adjacent spikelets.

### 3.3.2. PG isoforms in infected wheat ovaries

The presence of the PG isoforms produced by the  $\Delta$ PG1 and  $\Delta$ PG2 mutants in the infected wheat ovaries was checked in a gel activity assay at pH 5.0 by loading ground ovaries with floret glumes collected 4 days after wheat spikelet inoculation. In the ovary tissue infected with the 2.7.1 strain ( $\Delta$ PG2 mutant), only the PG1 isoform was clearly evident, while PG2 was not detectable (figure 11, lane 2). Instead, the PG1 isoform was undetectable in the ovary tissue infected with the Split10.4 strain ( $\Delta$ PG1 mutant), which only produced a faintly visible band (as an undefined spot) corresponding to the PG2 isoform (figure 11, lane 1). No PG band was visible by loading healthy ground ovaries (data not shown).

### 3.3.3. *Arabidopsis* flower infection experiment

A preliminary infection experiment was initially performed with *Arabidopsis* 8-week-old plants: about 10 plants were inoculated with *F. graminearum* WT, Split10.4 ( $\Delta$ PG1) and 2.8.1 ( $\Delta$ PG2) strains. Results 10 dpi showed that strain 2.8.1 showed a virulence comparable to that displayed by WT (data not shown), as previously observed in wheat infection experiments; the virulence of the  $\Delta$ PG1 mutant was instead reduced. To better clarify this reduction of virulence, two further infection experiments were therefore performed with WT and Split10.4 strains. 10 dpi the  $\Delta$ PG1 mutant showed a significant reduction of virulence of about 40% compared to the WT strain (table 6).



**Figure 11** – Thin-layer IEF in the pH range 6.0–10.5 of *F. graminearum*-infected ovaries collected 4 days after inoculation. Lane 1 was loaded with 12 ovaries infected with the strain Split10.4 ( $\Delta$ PG1 mutant). Lane 2 was loaded with 12 ovaries infected with the strain 2.7.1 ( $\Delta$ PG2 mutant). An aliquot of a chromatographic fraction containing both PG1 and PG2 was loaded on lane 3. PG isoforms were detected by pectate agarose overlay buffered at pH 5.0 (Ried & Collmer, 1985). The PG2 isoform *in vivo* was barely detectable, as an undefined spot (lane 1). The image is shown in inverted colours.

**Table 6. Infection experiment of Arabidopsis plants with *F. graminearum* WT and  $\Delta$ PG1 mutant.**

Strain <sup>a</sup>	Dead inflorescences <sup>b</sup>	Surviving inflorescences <sup>c</sup>	N <sup>d</sup>	P <sup>e</sup>
WT	20	13	33	
Split10.4	13	23	36	<0.05

<sup>a</sup> WT: *F. graminearum* wild-type strain; Split10.4:  $\Delta$ PG1 mutant strain.

<sup>b</sup> Number of inoculated plants showing symptoms in the floral stem (main stem dried and constricted). Data were obtained from three independent experiments.

<sup>c</sup> Number of inoculated plants with the apical meristem still viable. Data were obtained from three independent experiments.

<sup>d</sup> Number of infected plants (at least 10 plants in each independent experiment)

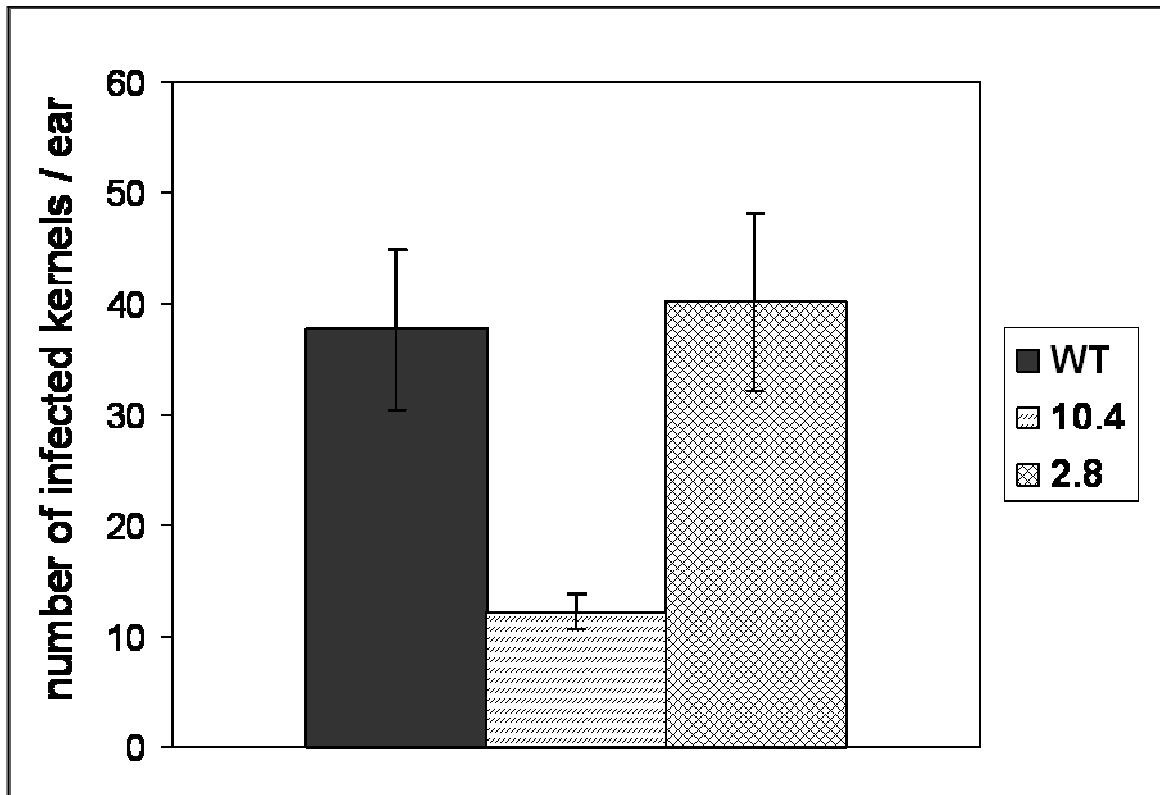
<sup>e</sup> Probability that there is no significant difference between WT and  $\Delta$ PG1 mutant, as determined by the Chi-square test with 1 degree of freedom.

#### 3.3.4. Maize ears infection experiment

An infection experiment of maize ears was also performed. Ears were collected from field at the flowering stage and were inoculated in 3 points with a spore suspension of wild-type and mutant strains. In particular, we used one  $\Delta$ PG1 strain (Split10.4) and one  $\Delta$ PG2 strain (2.8.1). Results after 10 days of inoculation showed also in this case a significant reduction of virulence of the  $\Delta$ PG1 mutant strain compared to WT. In particular, the number of infected kernels was reduced by about 60%. Instead, the  $\Delta$ PG2 mutant strain appeared as infective as WT (figures 12 and 13).

#### 3.4. Assays to quantify the mycotoxin produced by WT and mutant strains during wheat infection

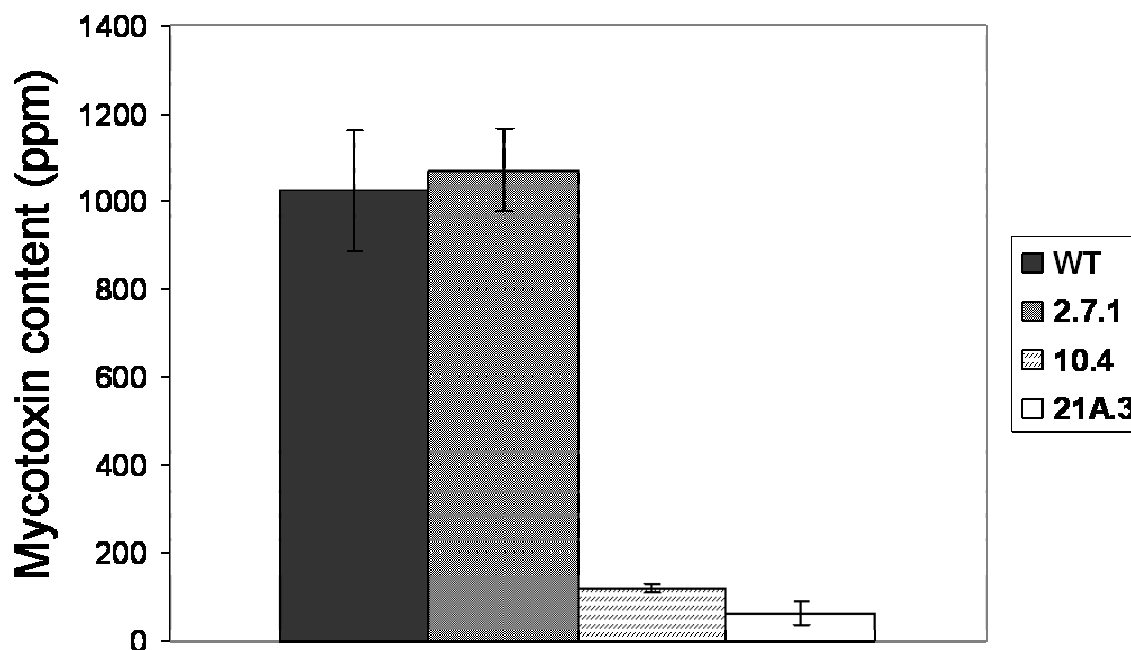
Wheat spikelets (cv.Bobwhite) infected with WT and mutant strains were used to quantify the amount of DON mycotoxin produced by the fungus during infection. DON was extracted from the two infected spikelets per spike and was measured with the RIDASCREEN DON enzymatic immunoassay. Results indicated that the  $\Delta$ PG1 strains (Split10.4 and Full21A.3) produced from 8 to 15 times less DON mycotoxin compared to WT strain while the  $\Delta$ PG2 strains (2.7.1 and 2.8.1) produced on amount of DON comparable to that of WT (figure 14). We also tested the amount of DON produced by these strains in liquid culture containing pectin as the sole carbon source. Since *F. graminearum* is known to produce very low levels of DON in liquid culture, in order to measure the amount of DON we had to concentrate 10X the liquid culture. Our data indicate that *F. graminearum* WT and mutant strains produce a similar amount of mycotoxin in liquid culture (about 2 ppb) (data not shown).



**Figure 12** – Symptoms on infected maize ears observed after 10 dpi and expressed as number of infected kernels per ear. Results showed a significant reduction of virulence of the  $\Delta$ PG1(Split10.4) mutant strain compared to WT: the number of infected kernels was reduced by about 60%. Instead, the  $\Delta$ PG2 (2.8.1) mutant strain appeared as infective as WT. Bars indicate the standard deviation (SD).



**Figure 13** – Maize ears inoculated with *F. graminearum* wild-type and mutant strains 10 days after inoculation. The virulence of the  $\Delta$ PG1(Split10.4) mutant strain is clearly reduced compared to WT while the symptoms caused by the  $\Delta$ PG2 mutant are similar to those determined by WT.



**Figure 14** – Mycotoxin content in wheat infected spikelets. The DON content was analysed by Ridascreen DON kit (R-Biopharm). At least 3 repetitions were performed for each thesis, every thesis consisting of 6 spikelets of 3 different spikes. Mycotoxin amount is expressed in parts per million (ppm = mg/Kg). The strain 2.8.1 produced an amount of mycotoxin comparable to that of 2.7.1 strain (data not shown). Probability (p) that there is no significant difference between WT and mutant strains, as determined by the two-tailed T-Student's test:

Split10.4  $p < 0.0002$   
 Full21A.3  $p < 0.003$   
 2.7.1  $p > 0.78$

Bars indicate the standard error of the mean (SEM).

### 3.5. GFP expression in WT and $\Delta$ PG1 strain for histological analysis

The *F. graminearum* WT and a  $\Delta$ PG1 mutant strain (Split10.4) were transformed to constitutively express the GFP in order to localize the fungus in the plant tissue. In total, 26 *F. graminearum* transformants obtained from WT and 22 transformants obtained from Split10.4 were analyzed by PCR: the *gfp* gene was amplified in 16 transformants obtained from WT and 17 from the Split10.4 strain (data not shown). After single conidiation of some selected PCR positive transformants, high-stringency Southern blot analysis of genomic DNA was performed and showed integration of the GFP encoding gene in all the mutant strains analysed (figure 15). 5 mutant strains with maximum 3 integration of the GFP construct, respectively three WT GFP expressing strains (1.1, 5.3 and M3.4) and two Split10.4 GFP expressing strains (1.1.2 and 1.1.3), were selected and analysed by fluorescence microscopy to confirm the expression of the green fluorescent protein. The GFP signal was detected homogeneously in mycelium and conidia of all the 5 mutant strains tested (figure 16). All the GFP mutants were then tested for growth in PDA, SNA and CM medium and also in wheat infection experiments, exhibiting a behaviour similar to that of the starting strains (data not shown). The obtained mutants were then used to perform a comparative study of the early events of wheat colonization and to examine the progression of the fungus within the wheat tissue. Two wheat cultivars (Bobwhite and Nandu) were point inoculated at anthesis with a spore suspension of three selected GFP mutants: 5.3, M3.4 (WT GFP expressing strains) and 1.1.2 ( $\Delta$ PG1 GFP expressing strain). Heads were harvested for microscopy at several time points during the first 10 days after inoculation and spikelets sections (figure 17) were examined for GFP expression by fluorescence microscopy.

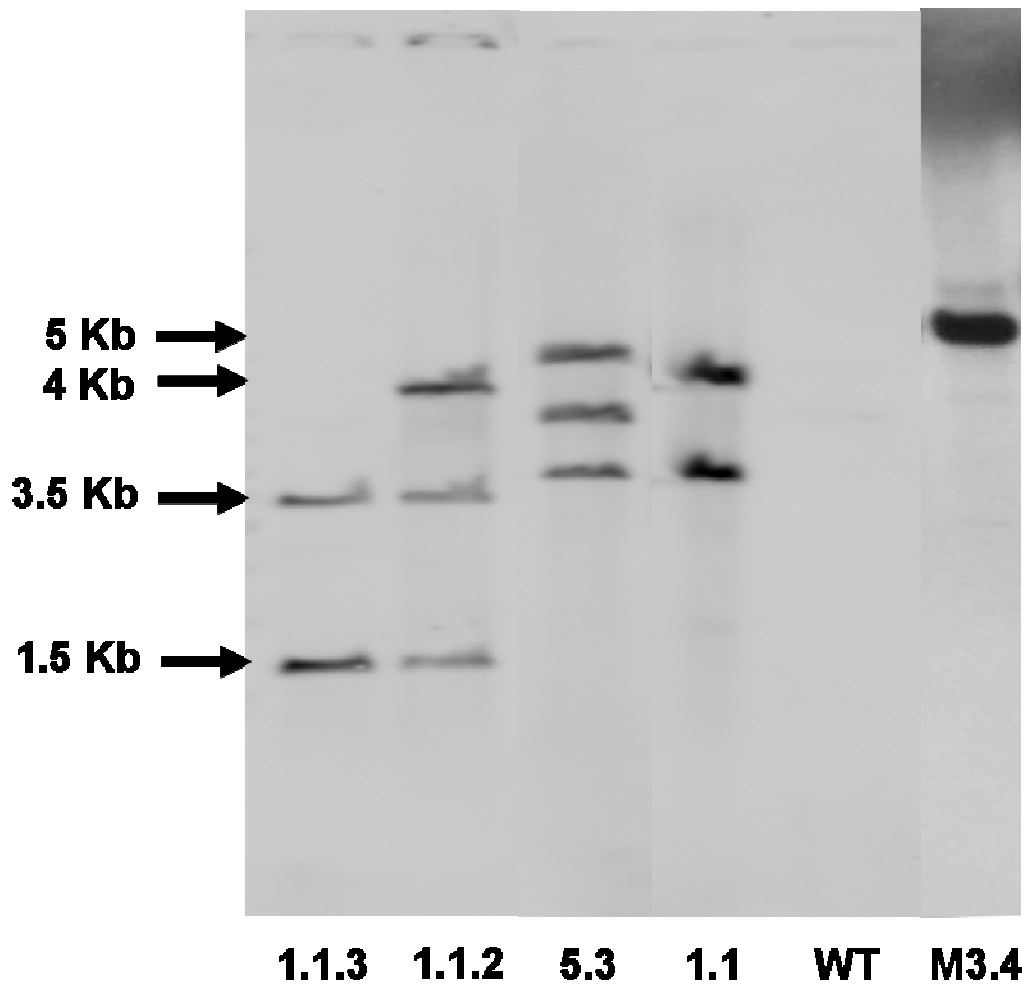
The first observations were performed on cultivar Nandu. At the first time point analysed (5 days post-inoculation), hyphae were clearly visible at the inoculation point inside the floret. At this time the mycelium was colonizing the ovary and the first difference between WT and  $\Delta$ PG1 strain was visible: in particular WT had colonized all the ovaries of the infected spikelet while the mutant growth was slowed (figure 18). At the point of inoculation with WT strain the ovary was also completely dry and blight, while that of the spikelet infected with the  $\Delta$ PG1 mutant did not show any symptom (figure 18).

After 7 days, the soft tissues of the ovaries infected with WT appeared thoroughly colonized and the fungus was present in the basal zone of the spikelet. Moreover, 3 of the 4 ovaries of the spikelets were completely dry and blight. Differently, the  $\Delta$ PG1 mutant had not entirely colonized the ovary yet and had caused the completed blight only of the first inoculated ovary (figure 19).

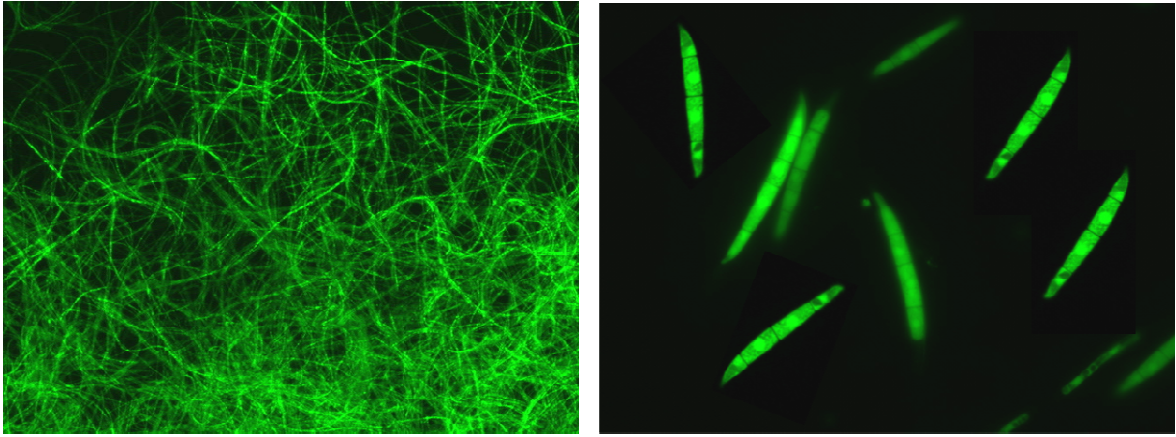
A cross section through the rachis and the rachis node was also performed at 7 dpi: under the WT inoculated spikelet a strong necrotic symptom was clearly evident in the vascular tissues of the rachis node while in the sections obtained from spikelets inoculated with the  $\Delta$ PG1 mutant strain, the necrotic symptom was much less widespread (figure 20 A-C). Besides, also the sections through the rachis showed a clear symptom of necrosis in the vascular vessels of WT infected spikelets while no symptoms were visible in the vascular vessels of  $\Delta$ PG1 infected spikelet (figure 20 B-D). This necrotic symptoms was likely due to the effect of DON mycotoxin produced by the fungus during plant infection.

10 days post infection the WT mycelium had moved into the rachis; in particular, hyphae were found growing in the vascular vessels of the rachis, where the spread of the  $\Delta$ PG1 mutant strain was much less extensive being detected only occasionally (figure 21).

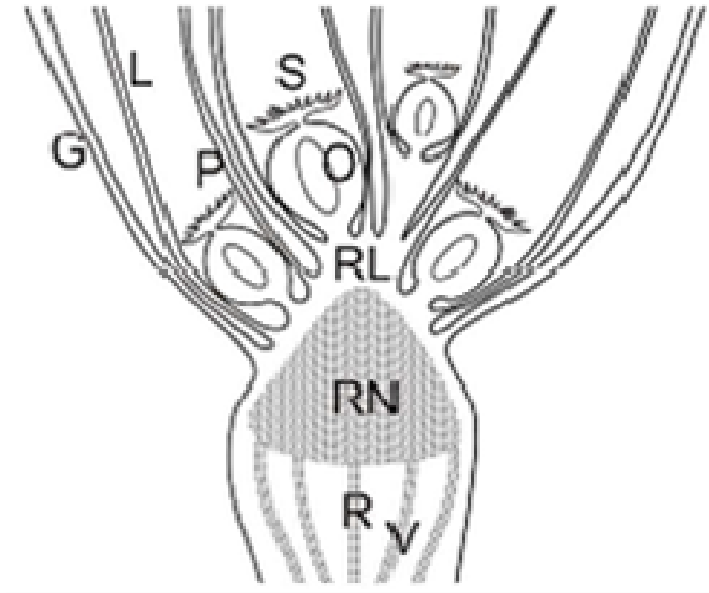
The histological analysis was also performed on cv. Bobwhite. A cross section through the rachis of infected spikelets was carried out at 7 dpi and showed that more necrotic symptoms were evident in the vascular vessels of WT inoculated spikelets (figure 22 A-B) compared to the sections inoculated with the  $\Delta$ PG1 mutant (figure 22 C). Moreover, at 10 dpi, the WT mycelium was clearly detected in the vascular vessels of the rachis while the mycelium of the  $\Delta$ PG1 mutant was not visible yet (figure 23).



**Figure 15** – Southern blot analysis of genomic DNA from *F. graminearum* wild-type and mutant strains transformed to express the GFP encoding gene. 1.1.3 and 1.1.2 strains were obtained from  $\Delta$ PG1 (Split10.4) mutant; 5.3, 1.1 and M3.4 strains were obtained from WT. DNA was digested with *Sac I* (Promega), which cuts only once in the GFP vector, before the GFP gene sequence. A fragment of the GFP gene was used as probe. The molecular size markers (1 kb DNA Ladder, Fermentas) are shown on the left.

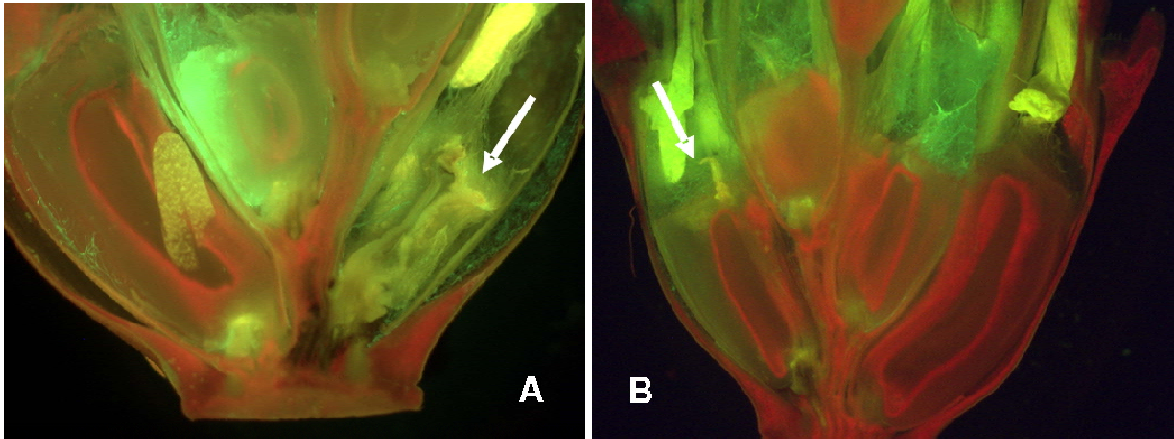


**Figure 16** – Mycelium and conidia of a GFP mutant strain analyzed by fluorescence microscopy. The GFP was excited with a 488-nm laser-line, and fluorescence was detected at 505–530 nm. The GFP signal was detected homogeneously in mycelium and conidia of all the selected *gfp* expressing mutants.



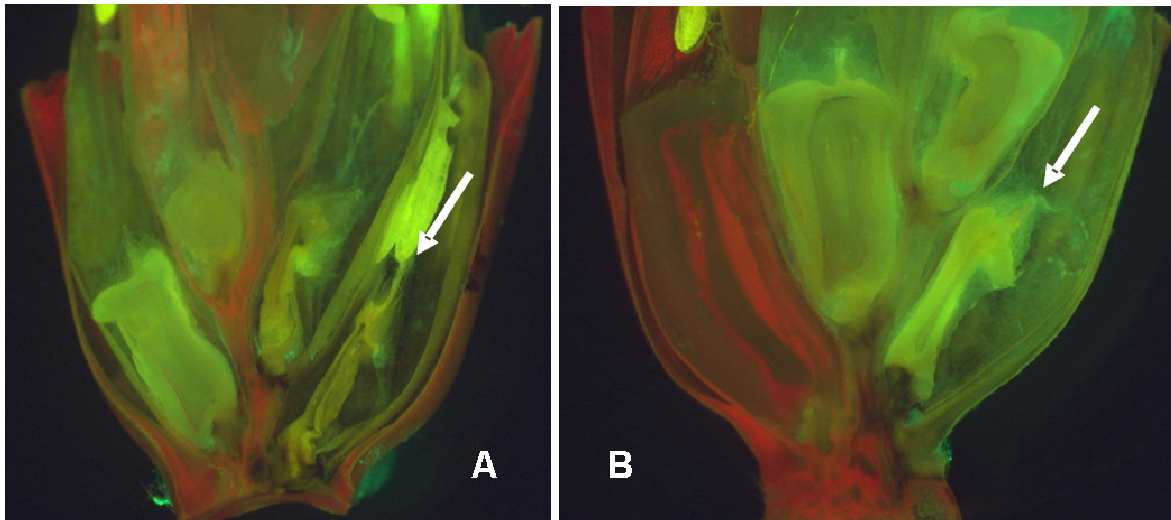
**Figure 17** - Schematic drawing of a wheat spikelet (from Ilgen et al., 2009) showing glume (G), lemma (L), palea (P), stigma (S), ovary (O), rachilla (RL), rachis node (RN), rachis (R) and vascular vessels (V).

5 dpi

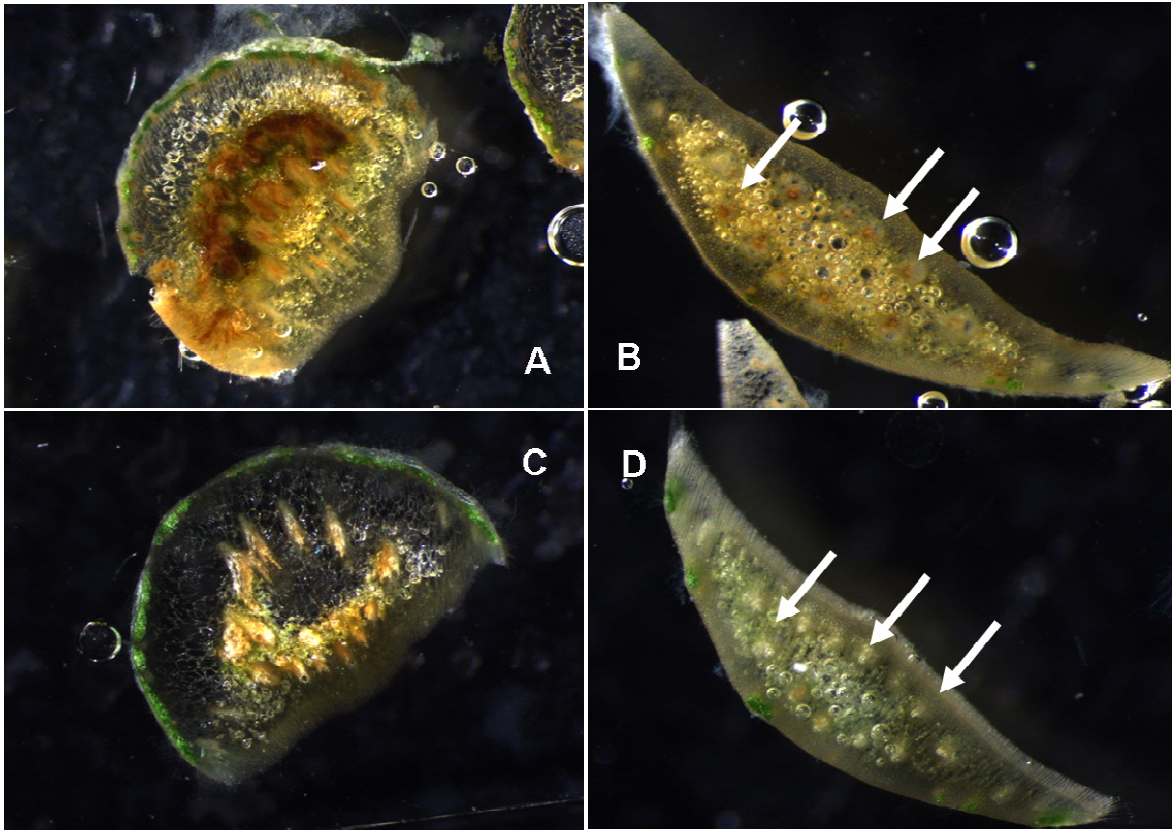


**Figure 18** – Fluorescence pictures of wheat spikelets (cv. Nandu) inoculated with WT (A) and  $\Delta$ PG1 mutant strain (B) after 5 dpi. Inoculation point is indicated by an arrow. The trans-section of the inoculated spikelets was examined under GFP exciting conditions. The WT strain was able to colonize the ovaries faster than the  $\Delta$ PG1 mutant; the ovary inoculated with WT strain was completely dry and blight, while the ovary of the spikelet infected with the mutant did not show any symptom.

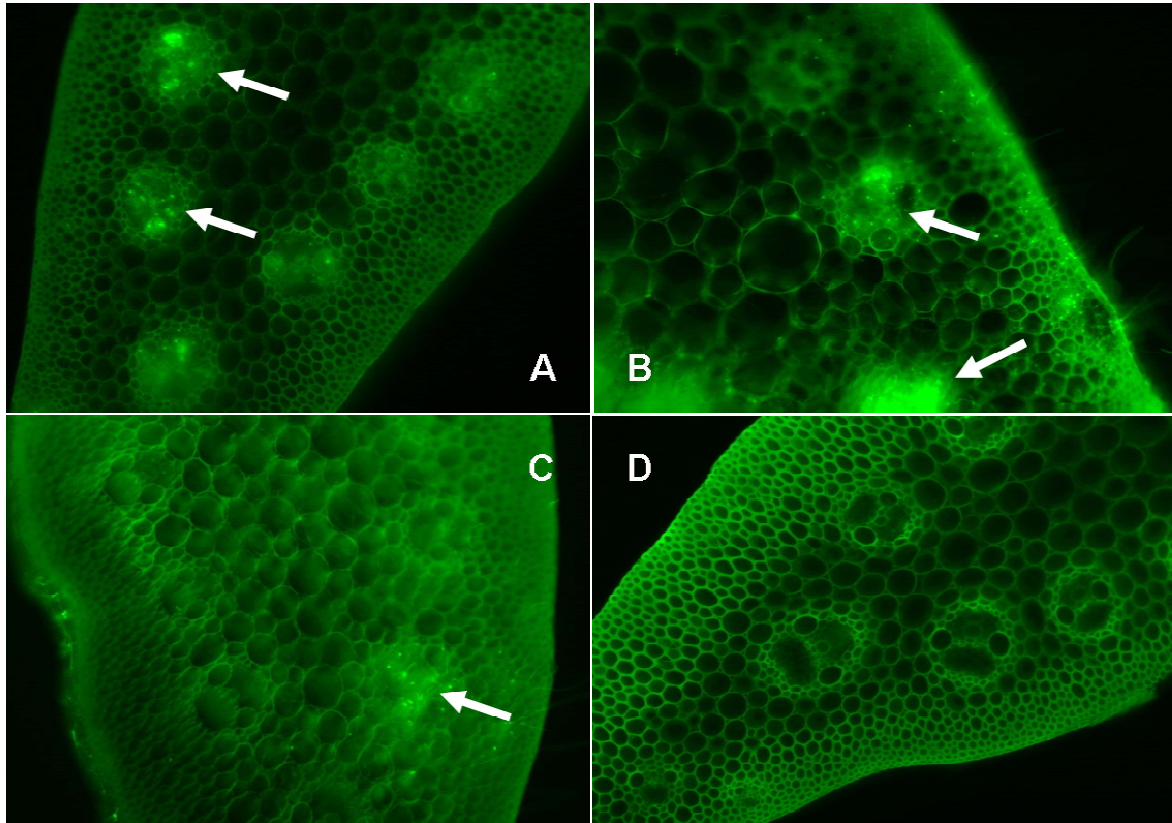
7 dpi



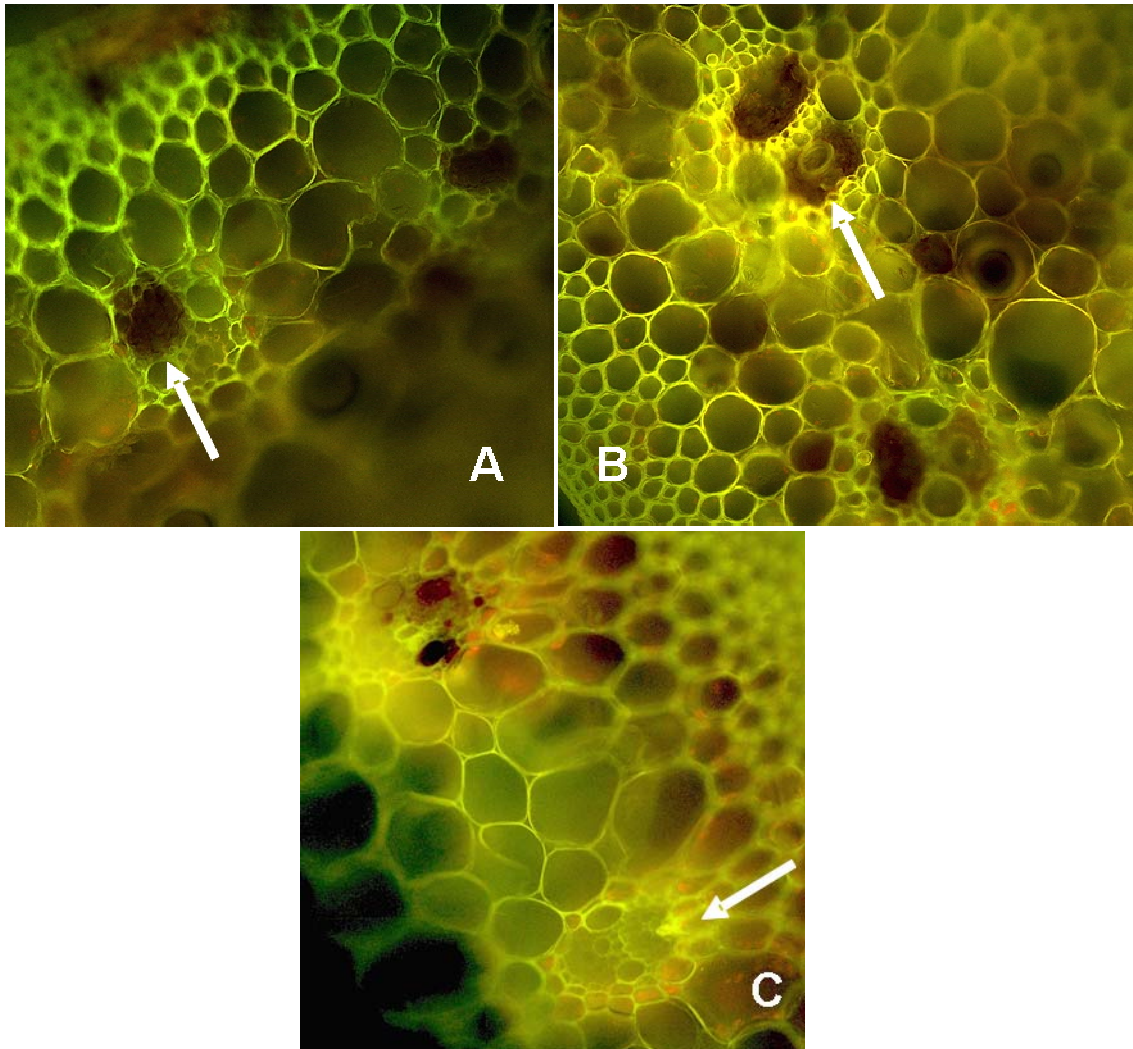
**Figure 19** – Fluorescence pictures of wheat spikelets (cv. Nandu) inoculated with WT (A) and  $\Delta$ PG1mutant strain (B) 7 dpi. The trans-section of the inoculated spikelets was examined under GFP exciting conditions. The soft tissues of the ovaries of WT inoculated spikelets appeared thoroughly colonized while the  $\Delta$ PG1 mutant had not entirely colonized the ovary yet.



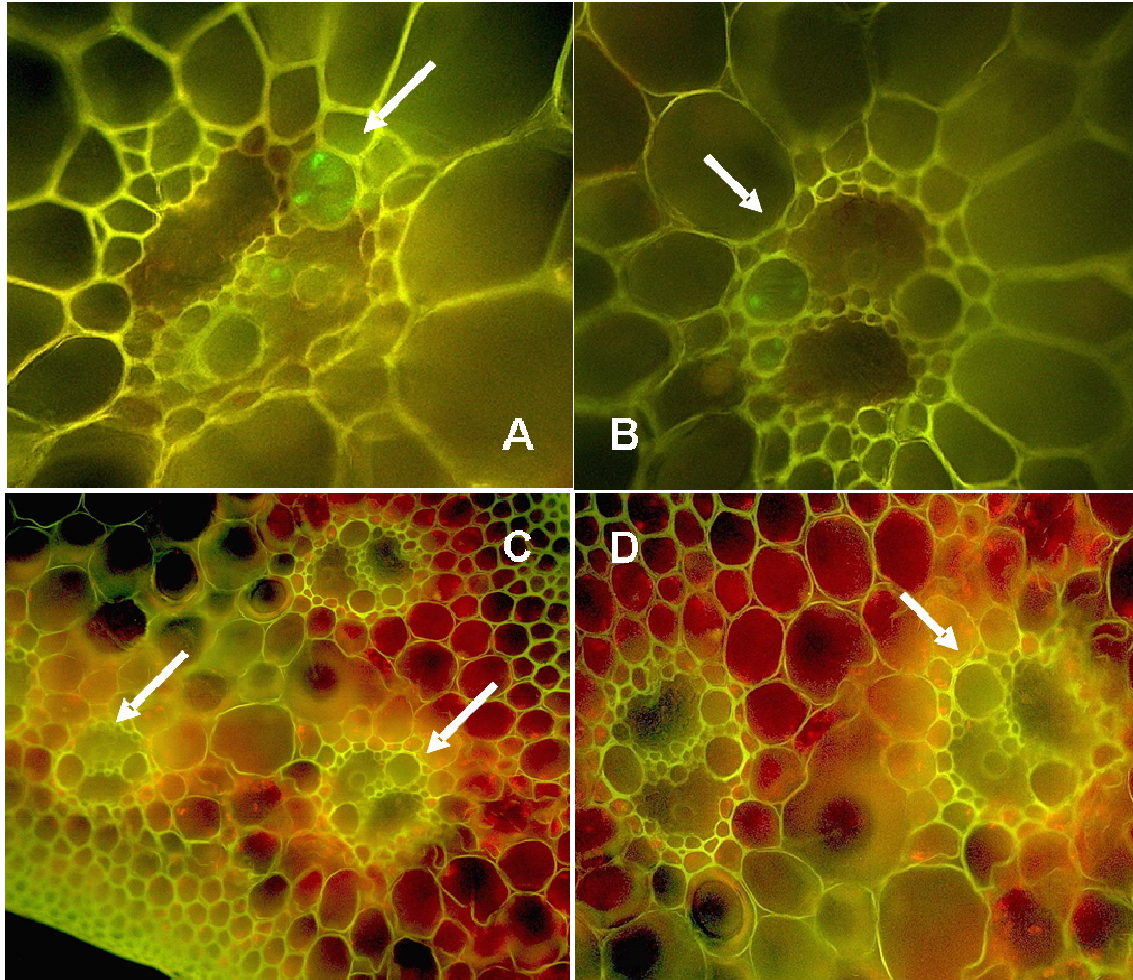
**Figure 20** – Top view of a cross section through the rachis node (A-C) and the rachis (B-D) of inoculated spikelets of wheat (cv. Nandu) 7 dpi. Sections of WT inoculated spikelets (A-B) showed stronger necrotic symptoms compared to the sections of spikelets inoculated with the  $\Delta$ PG1 mutant (C-D). Arrows indicate the vascular vessels.



**Figure 21** – Cross section of a rachis part adjacent to the inoculated spikelets of wheat (cv. Nandu) at 10 dai. A-B: rachis under the spikelet inoculated with WT, C-D: rachis under the spikelet inoculated with  $\Delta$ PG1 mutant. The cross section was examined under GFP exciting conditions. The rachis and in particular the vascular tissue appear completely colonized by the WT strain. Instead, the  $\Delta$ PG1 mutant is only occasionally detected in the vascular vessels of the rachis (arrows indicate the GFP signal in the vascular vessels).



**Figure 22** – Top view of a cross section through the rachis of the inoculated spikelets of wheat (cv. Bobwhite) 7 dpi. Sections of WT inoculated spikelets (A-B) showed more necrotic symptoms in the vascular vessels compared to the sections of spikelets inoculated with the  $\Delta$ PG1 mutant (C). Arrows indicate the vascular vessels.



**Figure 23** – Cross section of a rachis part adjacent to the inoculated spikelets of wheat (cv. Bobwhite) at 10 dai. A-B: rachis under the spikelet inoculated with WT, C-D: rachis under the spikelet inoculated with  $\Delta$ PG1 mutant. The cross section was examined under GFP exciting conditions. The rachis appears colonized by the WT strain, whose mycelium is growing particularly in the vascular tissue. Instead, the  $\Delta$ PG1 mutant is not detected in the vascular vessels of the rachis (arrows indicate the vascular vessels).

#### 4. DISCUSSION

Gene disruption is a fundamental genetic approach to ascertain the role of specific genes and it has been recently used in the case of important fungal pathogens in order to determine which fungal genes could be considered as pathogenicity or virulence factors. 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; Di Pietro and Roncero, 1998; Scott-Craig *et al.*, 1998), while others demonstrated that some pectinolytic enzymes were important for fungal attack (Clay *et al.*, 1997; Shieh *et al.*, 1997; ten Have *et al.*, 1998; Isshiki *et al.*, 2001; Oeser *et al.*, 2002; ten Have *et al.*, 2002).

Although PGs seem to play an important role especially in the diseases of dicotyledonous plants with a pectin rich cell wall, some pectinases produced by pathogens infecting cereal monocot plants, which have small amount of pectin in their cell wall (Carpita and Gibeaut, 1993), have been previously shown to be involved in pathogenicity (Oeser *et al.*, 2002; Kang and Buchenauer, 2000).

*Fusarium graminearum* is a relevant pathogen of cereal species like wheat, but the importance of the PGs produced by this fungus during the infection process has not been clarified yet. The PG activity secreted by *F. graminearum* is likely involved in pathogenesis since a degradation of pectin has been observed in the middle lamella and primary cell wall of wheat tissue infected by this fungus (Wanyoike *et al.*, 2002). Also the expression analysis of *F. graminearum* *pg* genes during wheat infection supports a possible role of PGs in the early stage of host tissue penetration, since both genes are early expressed with a maximum transcript level at 24 hours post infection (Tomassini *et al.*, 2009). *F. graminearum* is known to penetrate wheat spikelets through the ovary (Goswami and Kistler, 2004), a tissue particularly rich in pectin (Oeser *et al.*, 2002.); in particular, Miller *et al.* (2004), by using a *F. graminearum* strain transformed with a green fluorescent protein, hypothesized that pollen and anthers are the major targets in the initial stages of infection, with the fungus quickly progressing towards the soft tissues of the ovary.

To fully clarify the role of *F. graminearum* PGs in the infection process, in the present PhD thesis the two *F. graminearum* *pg* genes, encoding the PG1 and PG2 enzymes characterized by Tomassini et al. (2009), were disrupted by targeted homologous recombination and the mutants obtained were characterized *in vitro* and *in vivo*. In particular, two  $\Delta$ PG1 and two  $\Delta$ PG2 mutant strains were selected for further experiments after analysis by PCR and Southern blot to confirm the deletion of the *pg* gene and the single homologous integration of the disruption construct.

The  $\Delta$ PG1 and  $\Delta$ PG2 knock-out mutants were analysed by analytical IEF and, as expected, produced respectively only PG2 and PG1 compared to the WT strain. PG2 resulted difficult to detect in WT and  $\Delta$ PG1 mutants because its activity produced in liquid culture was very low compared to PG1. Viscosimetric and reducing-ends group assays showed that the overall PG activity produced by the  $\Delta$ PG1 mutant was very low compared to that of WT and  $\Delta$ PG2 mutant. However, as inferred by dry weight experiment, the low activity of the  $\Delta$ PG1 mutant did not influence the growth of the fungus in a medium with pectin as the sole carbon source. This result suggests that the lack of PG1 activity does not affect the ability of the  $\Delta$ PG1 mutant to degrade pectin and obtain enough carbon source for its growth. Probably, other pectinase activities such as exo-polygalacturonases acting on the pectic substrate used in this experiment are secreted by the  $\Delta$ PG1 mutant during liquid culture and repair the lack of PG1.

The knock-out mutants were also characterized *in vivo* by infecting host plants like wheat and maize and the model plant *Arabidopsis*: these infection experiments shed new light upon the possible role of *F. graminearum* PGs in the infection process. All the knock-out mutants maintained the capability to infect these plants. However the  $\Delta$ PG1 mutant showed always a significant reduction of virulence compared to the WT strain; in wheat the reduction of virulence observed was about 75-80%, while in *Arabidopsis* and maize about 40-60%. The IEF analysis of wheat infected ovaries confirmed that the PG1 isoform was undetectable in the tissue infected by the  $\Delta$ PG1 mutant, and only a band corresponding to the PG2 isoform was faintly visible, thus indicating that the overall PG activity produced by the  $\Delta$ PG1 mutant is very low also during wheat infection. Taken together, these results seem to indicate that the absence of PG1 could be responsible of the reduced virulence observed. On the contrary, the infection

experiments highlighted the negligible role of PG2 in the infection process since no significant reduction of virulence was observed with the  $\Delta$ PG2 mutant.

Since *F. graminearum* is known to produce DON mycotoxin in the infected grains, the ability of the  $\Delta$ PG mutants to produce this mycotoxin was tested both in liquid culture containing pectin as carbon source and in infected spikelets. As previously reported by other authors (Mudge et al., 2006; Voigt et al., 2007), the levels of mycotoxin observed in most *in vitro* culture conditions are greatly lower than levels measured during wheat infection. In particular, Gardiner et al. (2009) observed a lack of induction with any carbon source used. In fact, the DON level measured in liquid culture was very low (about 2 ppb) and resulted similar for  $\Delta$ PG and WT strains. Instead, the DON contained in wheat spikelets inoculated with WT and  $\Delta$ PG2 mutant strains was about 1000 ppm, while the  $\Delta$ PG1 mutant produced 8 to 15 times less mycotoxin (about 100 ppm). Since the  $\Delta$ PG1 mutant is reduced in virulence compared to WT, the different amount of DON measured in the infected spikelets could be related to the fungal biomass and might depend from the lower colonization of the spike by the  $\Delta$ PG1 mutant.

The importance of PG1 and its possible role in the infection process have been better elucidated by infection experiments with the GFP expressing mutants. The  $\Delta$ PG1 mutant resulted able to colonize more slowly the ovaries compared to WT, and it was also demonstrated that the colonization of the vascular vessels of the rachis by this mutant was strongly delayed compared to WT. In fact, 10 dpi the WT mycelium was clearly evident in the vascular tissues of both wheat cultivar analysed (Nandu and Bobwhite), while no hyphae were visible in the vascular vessels of  $\Delta$ PG1 infected spikelet.

The retarded fungal colonization of the ovary, particularly rich in pectin, is likely due to the reduced production of PG activity by the  $\Delta$ PG1 mutant. The growth of this mutant in the infected wheat tissue is therefore slowed down probably allowing the plant to initiate its defence reactions, which require gene activation and expression. For example, cell wall thickening or deposition of amorphous material within the vascular bundles of wheat has been shown to slow the growth of *F. graminearum* (Ribichich et al. 2000).

Taken together these results seem to indicate that *F. graminearum* PG1 may play an important role during pathogenesis and could therefore be considered a virulence factor. Since Janni et al. (2008) have recently demonstrated that wheat plants expressing a bean polygalacturonase inhibiting protein (PGIP) show a reduction of foliar symptoms caused by the pathogen *Bipolaris sorokiniana*, whose PG is inhibited by bean PGIP, the demonstration of the importance of *F. graminearum* PG1 during plant infection might be useful to investigate the effectiveness of a PGIP-based strategy to increase resistance of host cereal plants to infection by this fungus.

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