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FUNGAL CELL WALL DEGRADING ENZYMES AND PLANT INHIBITORS: ROLE DURING PLANT INFECTION AND STRATEGIES TO INCREASE PLANT RESISTANCE

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RIASSUNTO

Durante il processo infettivo i patogeni producono un gran numero di enzimi degradativi della parete cellulare, tra cui endo-xilanasi e poligalatturonasi, così da superare la barriera rappresentata dalla parete cellulare della pianta ospite e ottenere sostanze nutritive. Le xilanasi idrolizzano lo xilano, un polisaccaride particolarmente abbondante nella piante monocotiledoni, e alcune sono state dimostrate essere in grado di causare necrosi e risposta ipersensibile nel tessuto ospite. Le poligalatturonasi sono secrete da microorganismi patogeni durante le prime fasi del processo infettivo e sono implicate nella degradazione della pectina. Le poligalatturonasi e le xilanasi fungine svolgono un ruolo importante durante la patogenesi, ma non si conosce in modo approfondito come contribuiscano alla virulenza del fungo patogeno *Fusarium graminearum*, l'agente causale della fusariosi della spiga. In questo lavoro sono stati deleti, tramite ricombinazione omologa sito-specifica, il fattore trascrizionale *Xyr1* (che regola putativamente l'espressione di diversi geni codificanti per enzimi xilanolitici) e la principale poligalatturonasi del fungo, codificata dal gene *pg1*, producendo i mutanti singoli Δxyr and Δpg. I mutanti derivati dalla delezione del gene *pg1* hanno mostrato una bassissima attività poligalatturonasica e una virulenza ridotta su soia ma non su frumento; il mutante Δxyr ha presentato una drastica diminuzione dell'attività xilanasica, ma una virulenza comparabile al wild type sia su soia che su frumento. Per stabilire quindi un possibile effetto sinergico tra le attività xilanasica e poligalatturonasica di *F. graminearum*, è stato prodotto il doppio mutante ΔΔxyr/pg trasformando i protoplasti del mutante singolo Δpg. I mutanti ΔΔxyr/pg hanno presentato una ridotta capacità di crescere quando sono stati allevati in coltura liquida con xilano come unica fonte di carbonio e una forte diminuzione delle attività xilanasica e poligalatturonasica rispetto al ceppo wild type. La virulenza dei mutanti ΔΔxyr/pg su soia e spighe di frumento è rimasta notevolmente ridotta rispetto a quella dei mutanti singoli. L'effetto sinergico delle attività xilanasica e poligalatturonasica è stato quindi confermato incubando le pareti cellulari di frumento in presenza di PG1 e di due tra le più espresse xilanasi di *F. graminearum* purificate.

Siccome precedentemente è stato dimostrato che la xilanasi FGSG_03624 di *F. graminearum* causa risposta ipersensibile in frumento, è stata indagata l'abilità di questa xilanasi nell'indurre risposte di difesa in *Arabidopsis*. Trattamenti esogeni con questa proteina hanno indotto l'espressione del gene di difesa PDF1.2 e hanno mostrato una riduzione dei sintomi causati dal batterio *Pseudomonas syringae pv. maculicola* ma nessun effetto contro il fungo

Botrytis cinerea. La proteina è stata quindi transientemente espressa in foglie di tabacco e costitutivamente espressa in *Arabidopsis* dopo una mutagenesi nel sito catalitico che ha abolito l'attività xilanasica. Quando infettate con *B. cinerea*, le foglie di tabacco e di *Arabidopsis* hanno mostrato uguale suscettibilità rispetto alle rispettive foglie di controllo. Contrariamente a quanto osservato con i trattamenti esogeni, le piante transgeniche di *Arabidopsis* non erano più resistenti all'infezione di *P. syringae*. Tra i meccanismi di difesa sviluppati per contenere l' infezione di patogeni, le piante possiedono specifici inibitori, solitamente presenti nella parete cellulare, capaci di ridurre o bloccare completamente l'attività degli enzimi degradativi secreti dei funghi patogeni. In *Triticum aestivum* è stata identificata una famiglia di inibitori xilanasici (TAXI) capace di inibire la xilanasi Xyn11A di *B. cinerea*, un fattore di virulenza del fungo. Geni codificanti TAXI sono stati espressi in tabacco e *Arabidopsis* e le piante trasformate sono state infettate con *B. cinerea*. Mentre l'espressione transiente di TAXI nelle foglie di tabacco ha determinato una maggiore resistenza contro il fungo, l'espressione costitutiva in *Arabidopsis* non ha prodotto nessun effetto positivo.

SUMMARY

During the infection process pathogens produce high amounts of cell wall-degrading enzymes (CWDE) such as endo-xylanases and polygalacturonases (PGs), in order to overcome the barrier constituted by the plant cell wall and obtain nutrients. The first class of enzymes hydrolyzes xylan, a polysaccharide particularly abundant in monocot plants, and some of them have been shown to cause necrosis and hypersensitive response (HR) in the host tissue. The PGs are secreted by pathogenic microorganisms at the early stage of the infection process and are involved in the degradation of the pectin polymer. Fungal PGs and xylanases have been shown to play an important role during pathogenesis of some fungal pathogens, but little is known about their contribution to the virulence of the fungal pathogen *Fusarium graminearum*, the causal agent of the Fusarium Head Blight disease. Therefore we focused our attention on the *F. graminearum xyr1* and *pg1* genes (encoding the major regulator of xylanase genes expression and the main PG isoform of the fungus, respectively) by producing single gene disruption mutants (Δxyr and Δpg). Targeted disruption of the *pg1* gene produced a mutant with a PG activity nearly abolished and a reduced virulence on soybean but not on wheat spikes; besides, the Δxyr mutant, although dramatically impaired in xylanase activity, showed a virulence comparable with wild type on both soybean and wheat. In order to establish a possible synergistic effect between the *F. graminearum* xylanase and PG activities, a ΔΔxyr/pg double mutant was produced by transforming protoplasts of a Δpg mutant strain. As expected, when grown on xylan and pectin as carbon sources, the xylanase and PG activities of the double mutant strains were dramatically reduced compared to the wild type strain, while the growth of the double mutant was affected only on xylan containing medium. Infection experiments on soybean seedlings and wheat spikes showed that the virulence of the double mutant strains was significantly reduced compared to the single mutants. The synergistic effect of PG and xylanase activities was confirmed incubating the *F. graminearum* PG1 and two main purified fungal xylanases in presence of wheat cell walls.

Since one of these xylanases (FGSG_03624) has been previously shown to cause HR in wheat tissues, we also evaluated the ability of this xylanase to induce defence responses in *Arabidopsis*. Exogenous treatments with this protein induced the expression of PDF1.2 defense gene, marker of the jasmonate/ethylene pathways. Treatment of xylanase leaves reduced symptoms of the bacterium *Pseudomonas syringae pv. maculicola* but not those of the fungus *Botrytis cinerea*. A site-directed mutagenesis of the FGSG_03624 catalytic site

abolished the xylanase activity. This mutagenized protein was transiently expressed in tobacco leaves and also constitutively expressed in *Arabidopsis* plants. Transformed tobacco and *Arabidopsis* plants were as susceptible as the untransformed plants to *B. cinerea* infections. Differently from what observed with exogenous treatments, transgenic *Arabidopsis* plants were not more resistance to *P. syringae* infection. As a defense mechanism to counteract pathogens infection, plants have evolved specific inhibitors, usually localized in the plant cell wall, able to reduce or completely block the fungal CWDE. A *Triticum aestivum* family of xylanase inhibitor proteins (TAXI) have been shown to inhibit the *B. cinerea* Xyn11A xylanase, a well-known virulence factor of this fungus. TAXI encoding genes were expressed in tobacco leaves and *Arabidopsis* plants and these plants were infected with *B. cinerea*. While tobacco leaves transiently expressing TAXIs showed increased resistance against the fungus compared to control leaves, transgenic *Arabidopsi*s plants resulted as susceptible as the untransformed plants.

INTRODUCTION

The increase of food demand, as a result of the rapid growth in world population, involves the need to analyze several factors that are hindering the productivity of agricultural plants. In particular, biotic stresses caused by fungi, bacteria and viruses have a negative consequence on crops production and understanding plant-pathogen interaction can be helpful to identify new strategies to optimize crop yields. To prevent pathogen infection, plants exploit several constitutive defense barriers against microorganisms like cell wall, epidermal cuticle and bark (Ferreira et al., 2007). In addition, plant cells are able to recognize microbe-associated molecular patterns (MAMPs) thus activating inducible defense mechanisms such as programmed cell death at the site of infection (Coll et al., 2011) and expression of specific inhibitors of hydrolytic enzymes (Lawrence et al., 2000). The plant cell wall is mainly composed of polysaccharides (i.e. cellulose, hemicelluloses and pectins) and different classes of proteins (Carpita et al., 1993; Kikot et al., 2009). During host plant infection, a great variety of organisms, including bacteria and fungi, produce many cell wall degrading enzymes (CWDE) such as cellulases, xylanases, pectinases, cutinases, lipases and proteases in order to colonize the host tissue and also to obtain nutrients (Prins et al., 2000). In particular, endo-β-1,4-xylanases are glycoside hydrolase enzymes able to catalyze the hydrolysis of β-1,4-xylan (Collins et al., 2005; Wong et al., 1988), a structural polysaccharide of plant cell wall and particularly abundant in the primary cell wall of monocot plants (Cooper et al., 1988). At the early stage of infection, pathogenic microorganisms secrete also polygalacturonases (PGs), which are involved in the degradation of pectin. Some fungal PGs and xylanases have been shown to play an important role during pathogenesis by gene disruption approach (Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Oeser et al., 2002; Brito et al., 2006; Noda et al., 2010). For example, the xylanase Xyn11A of *Botrytis cinerea* has been shown to be a virulence factor of the fungus (Brito et al., 2006); however, its contribution to virulence seems ascribable to its necrotizing activity rather than to its enzymatic activity (Noda et al., 2010). The fungal pathogen *Fusarium graminearum*, the causal agent of Fusarium Head Blight (FHB), produces a xylanase (FGSG_03624) which has been shown to induce hypersensitive response (HR) in wheat tissues, but it is not essential to pathogenesis (Sella et al., 2013). Beside, the dispensability of the *F. graminearum* xylanase activity for fungal virulence was recently confirmed by disruption of the *xyr1* gene, encoding for a transcriptional regulator of the xylanase genes (Sella et al., 2016).

Among the defense mechanisms used by plants to counteract microbial pathogens, xylanase inhibitor proteins (XIs) are able to reduce or completely block the fungal endoxylanolytic activity and are usually localized in the plant cell wall. Three inhibitor families with different inhibitory capacities have been identified in wheat: *Triticum aestivum* XI (TAXI) (Debyser et al., 1999), xylanase inhibitor protein (XIP) (McLauchlan et al., 1999) and thaumatin-like XI (TLXI) (Fierens et al., 2007). In particular, wheat TAXIs are able to inhibit the *B. cinerea* Xyn11A xylanase (Brutus et al., 2005).

In this PhD thesis two main research lines were followed. The first was to establish a possible synergistic effect between PG and xylanase activities during plant infection by *F. graminearum*. To this aim several deletion mutants of the *F. graminearum* genes encoding for a fungal PG and the XYR1 regulator were produced by a gene disruption approach. The second objective was to verify the possibility to increase plant resistance by expressing in plant (i) the wheat xylanase inhibitor TAXI or (ii) the *F. graminearum* FGSG_03624 xylanase which is able to activate some defense responses in plant tissue.

Chapter I

Gene disruption approach to investigate the synergistic effect of *Fusarium graminearum* polygalacturonase and xylanase activities during host infection

INTRODUCTION

For most necrotrophic fungi an important role in pathogenesis is played by enzymes degrading the plant cell wall (Cooper, 1988), a physical barrier that pathogens have to overcome to penetrate and colonize the host tissue and obtain nutrients. Among cell wall degrading enzymes (CDWE), endo-polygalacturonases (PGs, EC 3.2.1.15) hydrolyze the homogalacturonan pectic polymers of cell wall and middle lamella by cleaving the internal α-1,4–D-galacturonic acid backbone and are expressed in the early stages of host infection (Reignault et al., 2008). PGs have been shown to strongly contribute to the disease symptoms of several fungi (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 other authors have ruled out an involvement of pectinolytic enzymes in pathogenicity (Gao et al., 1996; Di Pietro and Roncero, 1998; Scott-Craig et al., 1998), pectic enzymes are described as important virulence factors in several diseases of dicotyledonous plants that have a cell wall rich in pectin (Carpita and Gibeaut, 1993). Fungal pectinases have been shown to be also implicated in the infection of cereal plants, although these plants possess a cell wall with a small amount of pectin (Vogel, 2008). In fact, *Fusarium culmorum* breaks down the major cell wall components, including pectin, during spike infection and spreading in the host tissues (Kang and Buchenauer, 2000); Douaiher et al. (2007) showed that, during liquid culture, the wheat pathogen *Mycosphaerella graminicola* secretes high amount of polygalacturonase activity which might allow the breakdown of pectic material contained in the wheat leaf cell wall and also demonstrated a correlation between PG activity and the lesion frequency observed in wheat leaves. Furthermore, Wanyoike et al. (2002) showed by gold labelling a degradation of pectin in the middle lamella and primary cell wall of cells between the ovary and lemma during wheat infection by *Fusarium graminearum*. However, the only demonstration of a PG as a pathogenicity factor is that reported in the rye pathogen *Claviceps purpurea* (Oeser et al., 2002).

Endo-xylanases are another class of CWDEs involved in the hydrolysis of xylan, a major component of hemicelluloses and particularly abundant in the primary cell wall of monocotyledonous plants where it forms a complex structure composed of a D-xylose backbone linked by β-1,4-bridges. In particular, endo-xylanases break down the xylan backbone by catalyzing the hydrolysis of the β-1,4 linkages and can play an important role during plant infection not only by degrading the cell wall xylan but also by inducing necrosis

in the host tissues independently from their enzymatic activity (Brito et al., 2006; Noda et al., 2010).

F. graminearum [teleomorph *Gibberella zeae*] is the main causal agent of Fusarium head blight (FHB), a devastating disease which commonly affects cereals such as wheat, barley and other small grains (Goswami and Kistler, 2004), and is also responsible of root and collar rot of soybean seedlings (Pioli et al., 2004). The infection of wheat spikes occurs at flowering, when sexual or asexual spores of the fungus arrive on spikelets carried by wind and rain. Spores germinate and penetrate in the host tissue by exploiting the natural openings of the ovary and at the bottom of lemma and palea (Pritsch et al., 2001; Bushnell et al., 2003) or by actively penetrating the epidermal cells through hyphae, infection cushions and lobate appressoria (Boenisch and Schäfer, 2011). After floral invasion, intercellular hyphae spread throughout the spikelet down into the rachis node and, subsequently, systemically through the spike (Brown et al., 2010). A histological study of the *F. graminearum* infection process showed that in the first stages of the infection process the fungus seems to establish a biotrophic interaction with the host plant, switching to necrotrophy at later infection stages (Brown et al., 2012). In fact, in the infected grains *F. graminearum* secretes toxic secondary metabolites such as the trichotecenes mycotoxin deoxinivalenol (DON), which is dangerous for human and animal health (Goswami and Kistler, 2004). In particular, DON induces cell death (Desmond et al., 2008) and is a *F. graminearum* secreted virulence factor since it has been shown to be crucial for the spread of the fungus within the wheat spike (Bai et al., 2001).

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

Analyzing the *F. graminearum* genome database (http://mips.gsf.de/genre/proj/fusarium/) two genes encoding for endo-PGs have been previously identified (Tomassini et al., 2009). Expression analysis during *F. graminearum-Triticum aestivum* interaction showed 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 PG isoforms encoded by these two genes (named PG1 and PG2) are secreted both *in vitro* and *in vivo* in the early stages of wheat infection, with the activity of PG1 largely exceeding that of PG2 (Tomassini et al., 2009). In particular, the secretion of high amount of PG1 in the wheat ovary (Tomassini et al., 2009) seems consistent with the characteristics of the tissue initially infected by *F. graminearum*. In fact, 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 (Tenberge et al., 1996; Chateigner-Boutin et al. 2014). Thus, the degradation of the spikelet soft tissue may be achieved with the contribution of the PG1. Other indirect demonstrations of the possible role of *F. graminearum* PG activity during wheat infection are represented by the reduction of disease symptoms caused by the fungus on transgenic wheat plants expressing a polygalacturonase-inhibiting protein effective against PG1 (Ferrari et al., 2012) and by the observation that the *F. graminearum* PME activity contributes to fungal virulence on wheat spike likely favoring PG activity (Sella et al., 2016).

The first aim of the present work was to evaluate the importance of the *F. graminearum* PG1 during wheat infection. Disrupted mutants of the *Fgpg1* gene obtained by targeted homologous recombination by Sella et al. (unpublished) were tested on wheat spikes and soybean seedlings.

The second aim of this work was to verify a possible cooperative effect of *F. graminearum* PG and xylanase in the degradation of plant cell wall and during plant infection. Previously, *F. graminearum* strains disrupted in the *xyr1* gene, a transcriptional regulator of xylanase genes, showed a dramatic reduction of xylanase activity but an unmodified virulence, compared to the wild type, on *Triticum aestivum*, *T. durum* and *Glycine max* (Sella et al., 2016). Therefore, a *F. graminearum* double knock-out mutant of *pg1* and *xyr1* genes was produced and the obtained ΔΔxyr/pg strains were characterized both *in vitro* and *in vivo* by infection experiments of soybean and wheat. Furthermore, *F. graminearum* GFP-expressing strains were produced to perform a comparative study in infection structures formation between the WT and the Δpg and ΔΔxyr/pg mutant strains.

MATERIALS AND METHODS

Fungal and plant growth conditions

F. graminearum wild type (WT strain 3827) and mutants were cultured at 24°C on potato-dextrose agar (PDA, Difco Laboratories, Detroit, USA).

Conidia of *F. graminearum* WT and mutants were produced by culturing 5 PDA discs (5 mm diameter) containing actively growing mycelium in 50 ml of carboxymethyl cellulose (CMC) liquid medium (Cappellini and Peterson, 1965) at 150 rpm and 28°C for 6 days. Liquid cultures were then filtrated with a sterile gauze, washed with sterile water and conidia were collected and counted with the Thoma chamber.

WT and mutants were cultured by inoculating 1×10^4 conidia ml^{-1} in 20 ml of Szécsi medium (Szécsi et al., 1990) supplemented with 0.5% (w/v) beechwood xylan (Sigma-Aldrich, Milano, Italy) or apple pectin (70-75% of esterification; Sigma-Aldrich) at 25°C and 100 rpm. Xylanase and polygalacturonase (PG) activities were measured by assaying cultural filtrates aliquots obtained after 4 and 7 days of growth. Fungal growth was determined after 7 days of growth by weighting the mycelium previously filtered through a Wilson sieve (40 µm), washed twice with deionized water and oven dried at 80°C for 3 days.

Conidiation experiments were performed inoculating synthetic nutrient agar (SNA) plates (Nirenberg, 1981) with a mycelium plug and incubating at 25°C for 10 days.

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

Construction and preparation of the cassettes for gene replacement

F. graminearum genomic DNA was extracted as described by Henrion et al. (1994) and used as template to amplify the flanking homologous regions of the *xyr1* gene necessary for homologous recombination in the Δpg background (strain 3827). The Δpg mutant strains were produced and already available in the laboratory where I carried out my PhD research activity. In the first PCR, the upstream and downstream regions (of about 900 and 1000 bp) were amplified respectively with the primer pairs Fg17662upF/Fg17662genupR and Fg17662gendownF/ Fg17662downR (Table 1) at the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 1 min. The fragments of the expected

sizes were purified with the "NucleoSpin Gel and PCR Clean-up" kit (Macherey-Nagel GmbH&Co KG, Milano, Italy) and used in a second PCR to fuse the homologous flanking regions with the geneticin resistance gene (*gen*) used as selection marker. For the PCR reaction 500 ng of the geneticin gene, cut with *Bgl*II and *Hind*III from pII99 vector (Jansen et al., 2005) and 100 ng of each flanking region containing tails homologous to the 5' and 3' region of the *gen* gene were used. The fusion PCR was performed with Pfu polymerase in a final volume of 50 µl at the following conditions: 94°C for 3 min, 20 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 5 min. To obtain the full construct (of about 4500 bp) the fusion product was used as template in a nested PCR performed with the primers Fg17662gennestF/Fg17662gennestR (Table 1) at these conditions: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 10 min. The nested fragment was purified as above reported and cloned into the pGEM-T vector. Digestion of the recombinant vector obtained from a positively transformed *E. coli* colony was performed using *Apa*I and *Not*I restriction enzymes and confirmed the integration of the construct into the vector.

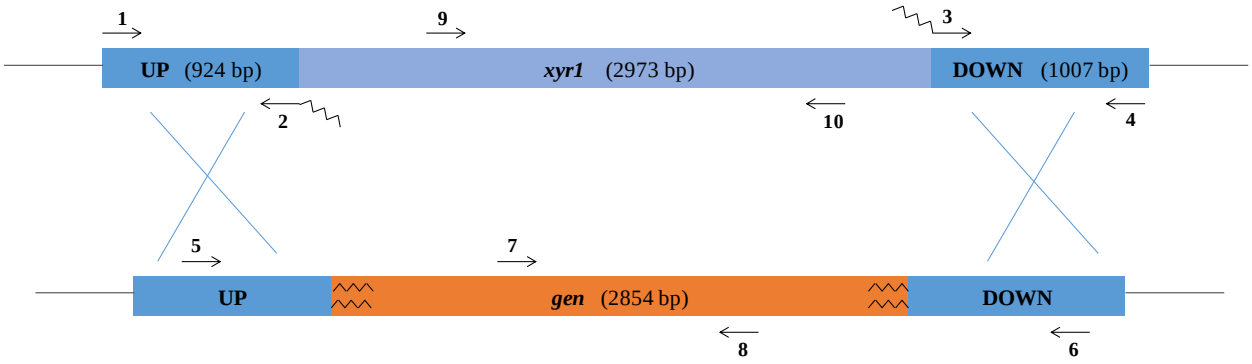


Fig 1. Schematic illustration of the PCR-based construction of the gene replacement vector. Flanking homology regions of the *F. graminearum xyr1* gene were amplified by PCR using specific primers: primers 1 (Fg17662upF) and 2 (Fg17662genupR) were used for the amplification of the upstream region (UP), and primers 3 (Fg17662gendownF) and 4 (Fg17662downR) for the downstream region (DOWN). UP and DOWN amplicons were fused with the geneticin resistance *gen* gene by the "Fusion PCR" technique, using as primers the tails of primers 2 and 3, complementary to the 5' and 3' *gen* regions, respectively. The fusion PCR product was used as template in a subsequent nested PCR reaction, where primers 5 (Fg17662gennestF) and 6 (Fg17662gennestR) were used to obtain the full construct of about 4500 bp. Primer pairs 7-8 (GenPRBf-GenPRBr) and 9-10 (Fg17662intF- Fg17662intR) were used for PCR screening of ΔΔxyr/pg mutant strains and to obtain the *gen* and *xyr1* probes for Southern blot analysis, respectively.

The construction of the *xyr1* gene replacement vector for *F. graminearum* wild type (strain 3827) transformation was performed as reported by Sella et al. (2016).

The GFP-PNR1 plasmid, kindly provided by prof. Schäfer (Hamburg University), was cut with *Hind*III, precipitated with isopropanol and resuspended in water. About 20 µg of the digested construct were used for each fungal transformation.

Fungal transformation

Protoplastation and fungal transformation were performed according to Nguyen et al. (2012). Geneticin-resistant colonies obtained from the Δpg mutant transformation were selected and transferred to 3-mm complete medium (CM) plates (Leslie and Summerell, 2006) supplemented with 200 µg/ml of geneticin. PCR reactions using primer pairs internal to the geneticin and the *xyr1* gene (Table 1) were performed for the preliminary screening of mutant strains, which were then single-conidiated first in water agar (1.6%) plate for 3 hours at 28°C and then on CM plates supplemented with the selection antibiotic at the same concentration. Transformant colonies without the *xyr1* gene were then tested by Southern blot hybridization for single insertion of the disruption construct.

Fungal transformation of the *F. graminearum* wild type (strain 3827) for *xyr1* gene disruption was performed as described by Sella et al. (2016). Hygromycin-resistant colonies were collected, selected in CM supplemented with 200 µg/ml of hygromycin, single-conidiated and preliminarily screened by PCR using the primers reported in Table 1.

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

Southern blot analysis

For Southern blot analysis, approximately 15 µg of genomic DNA extracted as previously reported were digested with specific restriction enzymes, separated on a 0.8% (w/v) agarose/TAE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Milano, Italy). Digoxygenin (DIG)-labeled specific probes were generated by PCR with specific primers (Table 1) using genomic or plasmid DNA as template and were used for overnight hybridization at 68°C. The PCR reaction was performed in a volume of 25 µl using DIG-11-dUTP (Roche, Mannheim, Germany) and consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec 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, USA) for approximately 1 hour.

Xylanase and PG activity assays

Xylanase activity secreted by *F. graminearum* WT and mutants grown in liquid cultures containing xylan as the only carbon source was measured through the dinitrosalicylic acid (DNS) assay (Bailey et al., 1992) using D-xylose (Merck, Milano, Italy) as standard. In particular, reducing sugars released were measured after incubating 50 µl of each fungal culture filtrate in a total reaction mixture of 500 µl containing 0.5% (w/v) beechwood xylan (Sigma-Aldrich) dissolved in 50 mM sodium citrate buffer (pH 5) for 30 min at 37°C. One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose in 1 min under the assay conditions.

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

Enzymatic treatment of wheat cell walls

Triticum aestivum (cv. Concordia) cell walls (1% w/v) were incubated in 50 mM sodium acetate buffer pH 6.0 supplemented with streptomycin (0.2 mg mL^{-1}) and BSA (0.1 mg mL^{-1}). In the 300 µl reaction mixture, 200 U/ml of the *F. graminearum* PG1 isoform [purified as described by Tomassini et al. (2009); one unit of PG activity defined as the amount of enzyme required to release 1 µmol/min of reducing groups using D-galacturonic acid as standard] and 0.1 U/ml for each of the two more expressed *F. graminearum* xylanases [FGSG_03624 and FGSG_10999, respectively purified as reported by Sella et al. (2013) and Tundo et al. (2015)] were tested separately or mixed together. After addition of the enzyme samples, the mixtures were incubated at 30° C for 20 hours and µg of uronides released were measured using the method described by Blumenkrantz and Asboe-Hansen (1973) and D-galacturonic acid as standard.

Plant inoculation

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

WT and mutant strains were used to inoculate *T. aestivum* spikelets at anthesis (Zadoks et al., 1974) pipetting 10 µl of a fresh conidial suspension containing approximately 2×10^5 conidia ml^{-1} between the glumes of two florets of two opposite central spikelets; after inoculation, spikes were covered for 3 days with a plastic bag to keep a moist environment. Symptoms were estimated at 21 days post infection dividing the number of infected spikelets by total number of spikelets per spike.

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

Fluorescence microscopy

Infection structures of WT and mutants were investigated by fluorescence microscopy using Axio Imager Z1 microscope. A UV lamp HAL 100 served as UV light source. GFP was excited with a 405 nm laser and detected at 488 nm. Images were taken with Zeiss AxioCam MRm CCD camera. Image processing were done with Zeiss AxioVision software.

Table 1. List of primers used to produce the deletion constructs and for preliminary screening of mutants. N refers to the number of primer represented in figure 1.

N	PRIMER NAME	PRIMER SEQUENCE (5'-3')
Primers to produce the disruption construct and for preliminary screening of ΔΔxyr/pg mutant		
1	Fgl7662upF	ACTTCCCTCCAGGATCCACT
4	Fgl7662downR	GCAACAACACAAGCGAGAAA
2	UPGEN	AACAACTGGCATGAATTCATCGATGATATCAGATCACATCTTGCCGGTACTCAGG
3	DOWNGEN	GATCCTCTAGAGTCGACCTGCAGGCATGCACAACATACTTGGGGCGTCTT
5	Fgl7662gennstF	CAAGACGTGAAGCATTCGTT
6	Fgl7662gennstR	CTTCCCCTTGCCCTCTTTAT
9	Fgl7662intF	CTGTTGCTGATGGTGTTGCT
10	Fgl7662intR	TTCTTTGCGTGATGCAAGTC
7	GenPRBf	AGGATCTCCTGTCATCTCA
8	GenPRBr	CCAAGCTCTTCAGCAATA
	Primers for preliminary screening of Δpg mutant	
	HygPRBf	AAAGTGCCGATAAACATAAC
	HygPRBr	CTCTCTCAAAGCATCACTCT
	11011int F	ATCTCCGGCGCTGTCGTCAA
	11011intR	GCAGCTAGCGCAAAGAATGTA

RESULTS

Production of *F. graminearum* Δpg disruption mutant and infection experiments

In the laboratory where I carried out my PhD research, several Δpg mutant strains were obtained by gene disruption on wild type 3827 strain background. Gene disruption was confirmed on three of them (Δpg 2.3, Δpg 2.8 and Δpg 2.13) by PCR (Fig. 2) and Southern blot analysis using a fragment of the *pg1* gene and of the *hyg* gene as probes (Fig. 3). To determine whether *F. graminearum pg1* gene is involved in virulence, infection experiments of soybean seedlings and wheat spikes were performed (Fig. 4). On soybean the virulence of the mutant strains was significantly reduced compared to the WT strain by about 30% (Fig. 4A), while on wheat spikes the virulence of WT and mutant strains was comparable (Fig. 4B). Since no significant difference in virulence between the Δpg mutant strains was observed, the strain Δpg 2.13 was selected to produce the double mutant ΔΔxyr/pg.

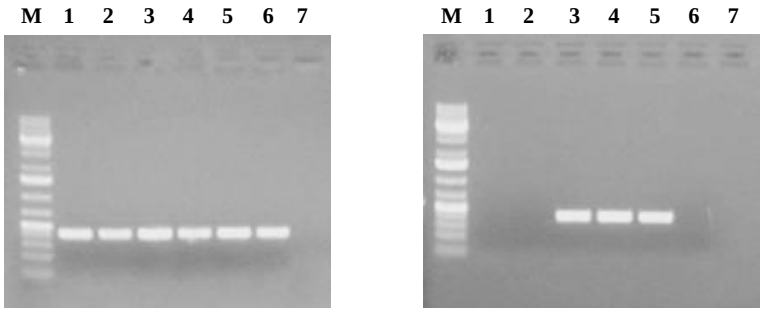


Fig. 2. PCR selection of *F. graminearum* Δpg gene disruption mutant strains. Transformant colonies resistant to hygromicin were screened by PCR using the primer pair HygPRBf and HygPRBr **(A)** and the primer pair 11011INTf and 11011INTr for the specific *pg1* gene **(B)**. The internal fragment of the *pg1* gene (B) was amplified in Δpg 2.9 (lane 3), Δpg 2.10 (lane 4) and Δpg 2.11 (lane 5), but not in Δpg 2.3 (lane 1), Δpg 2.8 (lane 2) and Δpg 2.13 (lane 6) mutant strains. A negative control was loaded in lane 7. GeneRuler DNA Ladder Mix (Fermentas, Milano, Italy) was used as molecular size marker (M).

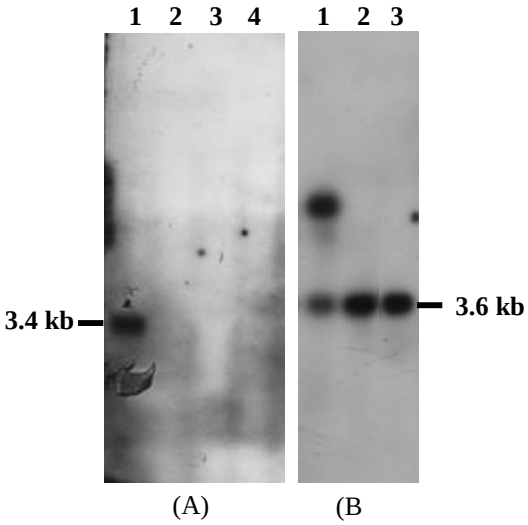
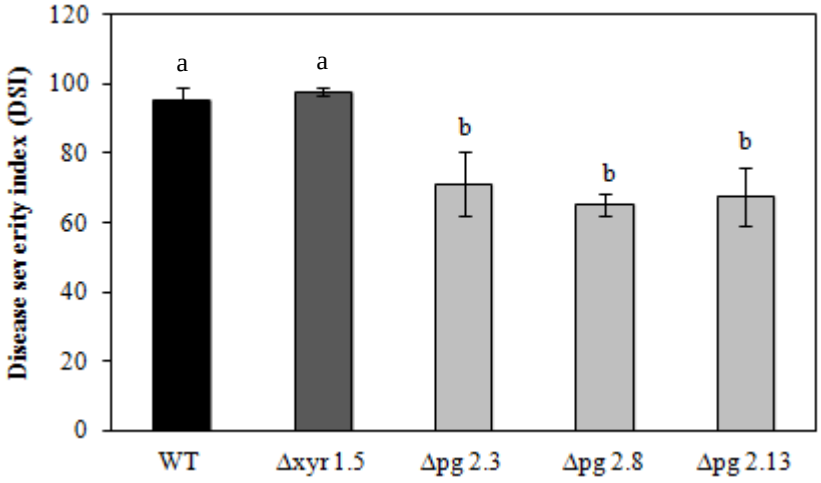


Fig. 3. Southern blot analysis of genomic DNA from *F. graminearum* WT and mutant strains. Genomic DNA was digested with *Xba*I (Promega). **(A)** A fragment of the *pg1* gene was used as specific probe. The WT (lane 1) showed an hybridization signal of 3.4 kb, while the Δpg 2.3 (lane 2), Δpg 2.8 (lane 3) and Δpg 2.13 (lane 4) mutant strains did not show any hybridization signal. **(B)** A fragment of the *hyg* resistance gene was used as probe. All the Δpg mutant strains (Δpg 2.3, Δpg 2.8, Δpg 2.13, lanes 1-3, respectively) showed an hybridization signal at 3.6 kb corresponding to the targeted insertion of the *hyg* gene in the *pg1* locus. For the Δpg 2.3 strain a second ectopic integration was present; the WT strain gave no hybridization signal (data not shown). GeneRuler DNA Ladder Mix (Fermentas, Milano, Italy) was used as molecular size marker (M).

(A)



(B)

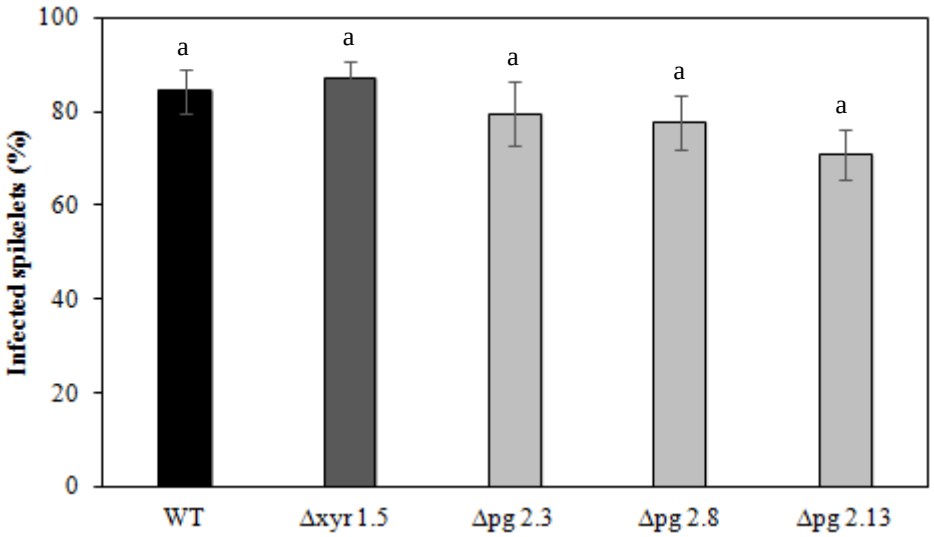
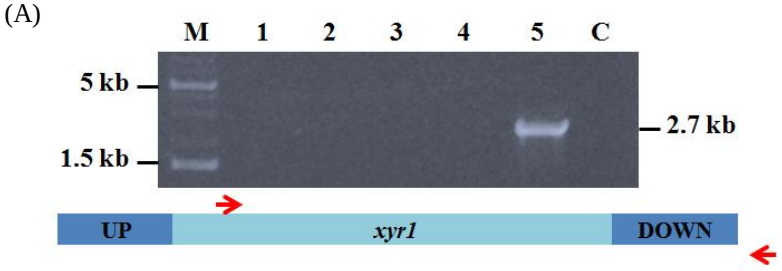


Fig. 4. Infection of soybean seedlings and wheat spikes with *F. graminearum* WT and single mutant strains. (A) Two hundred µl of spore suspension containing 2 × 10^4 conidia of the *F. graminearum* WT or mutant strains were dropped on each soybean seed (cv. Demetra) and disease symptoms expressed as DSI were assessed on seedlings at 6 dpi. Bars represent the mean ± standard error of at least two independent infection experiments performed with the rolled towel method. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $P < 0.05$. **(B)** Ten µl of spore suspension containing 1 × 10^3 conidia of the *F. graminearum* WT or mutant strains were pipetted between the glumes of two florets of two opposite central spikelets. Disease symptoms were assessed at 21 dpi by counting the number of visually diseased spikelets (cv. Bobwhite). Infected spikelets are expressed as percent of symptomatic spikelets on total number of spikelets of the respective head. Data represent the average ± mean standard error (indicated by bars) of at least four independent experiments performed inoculating at least 8 plants with each strain. Data were statistically analyzed by applying the Tukey-Kramer's test.

Production of the *F. graminearum xyr1* disruption mutant

A *xyr1* deletion mutant was produced on the WT 3827 strain with the same construct used by Sella et al. (2016) for obtaining the Δxyr mutant on the PH1 strain background. Several strains were obtained and screened by PCR for the absence of the *xyr1* gene and the disruption was confirmed in three of them (Fig. 5). Enzymatic assays and infection experiments confirmed the results previously obtained with the Δxyr mutant in the PH1 strain background (Sella et al., 2016), with about 90% reduction of xylanase activity (Fig. 9) and dry weight on xylan containing medium (Fig. 11B) and a virulence comparable with WT on both soybean and wheat (Fig. 4).

(A)



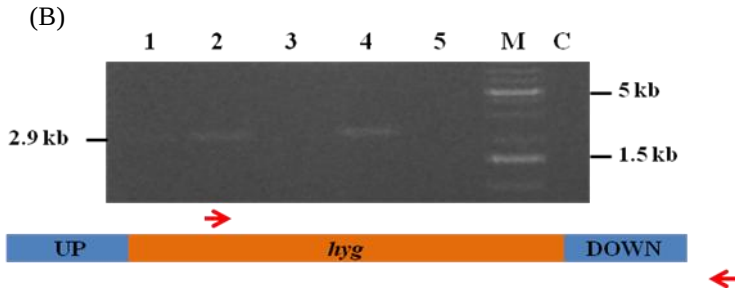
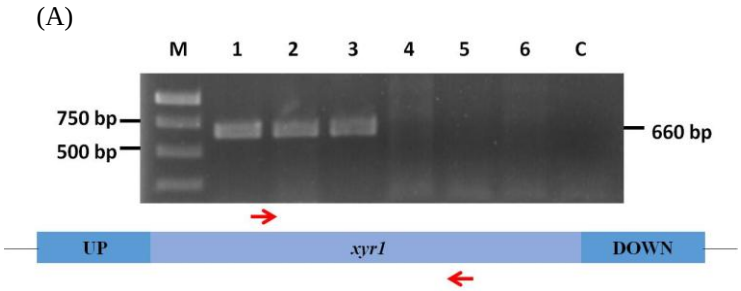


Fig. 5. PCR selection of *F. graminearum xyr1* gene disruption mutant strains. Transformant colonies resistant to hygromycin were screened by PCR. **(A)** A 2.7 Kb fragment of the *xyr1* gene was amplified with the primer pair Fg17662intF and Fg17662downR in WT (lane 5), but not in the mutant strains Δxyr 1.1 (lane 1), Δxyr 1.2 (lane 2), Δxyr 1.3 (lane 3), Δxyr 1.5 (lane 4). **(B)** A 2.9 kb fragment of the *hyg* gene was amplified with the primer pair Hyg for and Fg17662downR in the mutant strains Δxyr 1.1 (lane 1), Δxyr 1.2 (lane 2) and Δxyr 1.5 (lane 4) and was absent in WT (lane 5) and in the mutant strain Δxyr 1.3 (lane 3). M: molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy) are shown on the left (A) and right (B). A negative control was loaded in lane C.

Fungal transformation to obtain the ΔΔxyr/pg mutant

Protoplasts of the *F. graminearum* Δpg 2.13 strain were transformed with a construct containing geneticin (*gen*) as selectable marker to replace the *xyr1* gene. In total forty geneticin resistant colonies were selected and four were confirmed by PCR for the disruption of the gene of interest (Fig. 6). After single conidiation of these transformants, Southern blot analysis was performed using a probe specific for the *gen* gene (Fig. 7). The geneticin probe gave no hybridization signal for the WT and Δpg strains, while the double knock-out mutant strains tested (ΔΔxyr/pg 1.18, 1.22 and 1.31) showed a single hybridization signal at the expected size of 8.3 kb. In the ect 1.1 strain the *gen* hybridization signal was higher than the expected size, indicating an ectopic integration of the construct (Fig. 7).



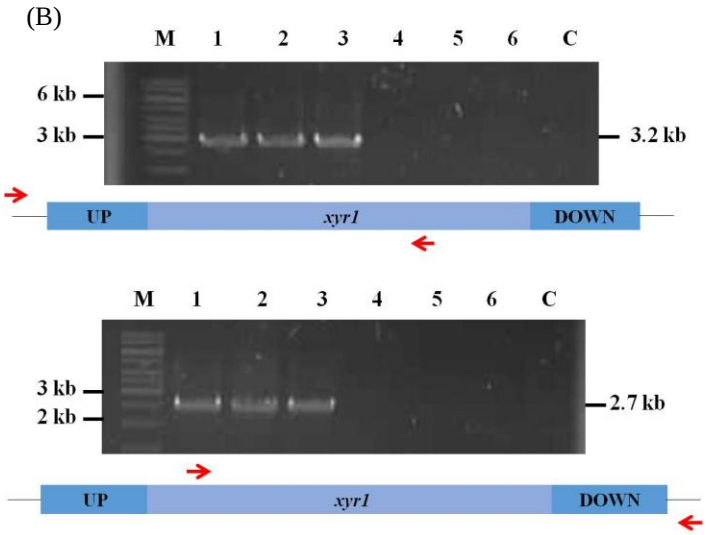


Fig. 6. PCR selection of *F. graminearum* ΔΔxyr/pg gene disruption mutants. Transformant colonies resistant to geneticin were screened by PCR. **(A)** The 661 bp internal fragment of the *xyr1* gene was amplified with the primer pair Fg17662intF and Fg17662intR in WT (lane 1), Δpg 2.13 single mutant (lane 2) and ΔΔxyr/pg 1.1 (ect 1.1) mutant strain (lane 3), but not in ΔΔxyr/pg 1.18 (lane 4), ΔΔxyr/pg 1.22 (lane 5) and ΔΔxyr/pg 1.31 (lane 6) mutant strains. **(B)** The 3.2 kb and 2.7 kb fragments of the *xyr1* gene were amplified with the primer pair Fg17662upF/Fg17662intR and Fg17662intF/Fg17662downR respectively in WT (lane 1), Δpg 2.13 single mutant (lane 2) and ect 1.1 mutant strain (lane 3), but not in the double mutant strains ΔΔxyr/pg 1.18 (lane 4), ΔΔxyr/pg 1.22 (lane 5), and ΔΔxyr/pg 1.31 (lane 6). A negative control was loaded in lane C. GeneRuler DNA Ladder Mix (Fermentas, Milano, Italy) was used as molecular size marker (M) and sizes are shown on the left.

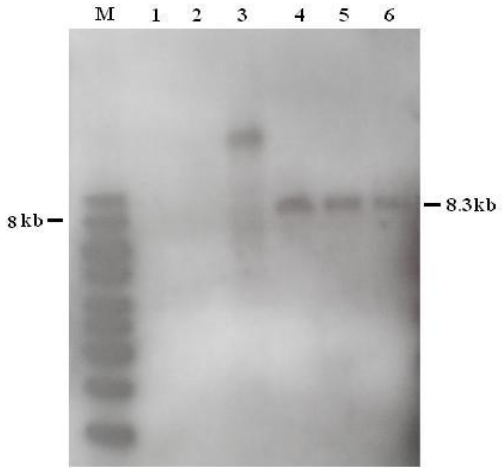


Fig. 7. Southern blot analysis of genomic DNA from *F. graminearum* WT and mutant strains. Genomic DNA was digested with *Mfe*I and *Kpn*I . A fragment of the *gen* resistance gene was used as probe. The WT (lane 1) and Δpg 2.13 (lane 2) strains gave no hybridization signal. ΔΔxyr/pg 1.18 (lane 4), ΔΔxyr/pg 1.22 (lane 5), and ΔΔxyr/pg 1.31 (lane 6) mutant strains showed a single hybridization signal at 8.3 Kb, while in the ect 1.1 mutant strain (lane 3) the single signal was higher than the expected size. GeneRuler DNA Ladder Mix (Fermentas, Milano, Italy) was used as molecular size marker (M).

In vitro characterization of WT and mutant strains

Double mutant strains confirmed by PCR and Southern blot were transferred to CM and SNA agar plates to verify growth and conidia production, respectively. While growth was similar for all the strains (data not shown), conidia production was significantly reduced in Δpg and double mutant on SNA medium (Fig. 8).

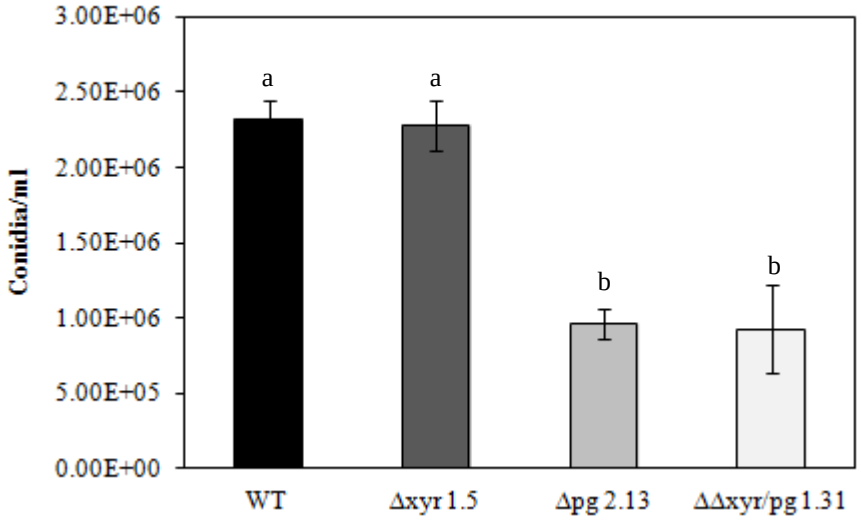


Fig. 8. Conidiation of WT and mutant strains in SNA medium. Histograms are in exponential scale. Each data represents the mean of two biological replicates counted three times. Average values were significantly different according to the Tukey-Kramer's test. Different letters indicate significant differences at $p<0.05$.

To confirm the effective disruption of the *xyr1* transcriptional regulator gene in the double mutant strains, total xylanase activity produced after 4 days of culture in a liquid medium containing xylan as sole carbon source was determined on 0.5% (w/v) beechwood xylan substrate according to the DNS method. As expected, the xylanase activity produced by the double mutant strains as well as by the Δxyr strain was 90% lower than that produced by the WT and Δpg strains (Fig. 9). The difference observed between mutants and WT strain was also confirmed at 7 dpi (data not shown).

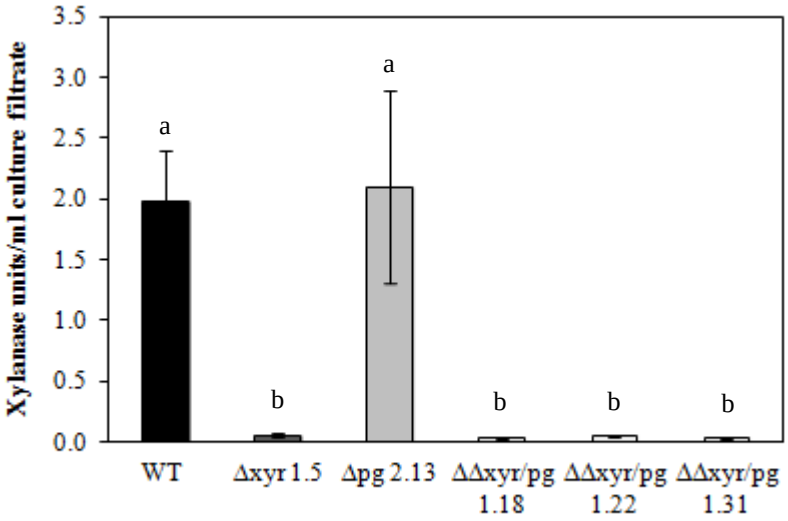


Fig. 9. Xylanase activity produced by *F. graminearum* WT and mutant strains. Fifty µl of culture filtrates collected after 4 days of culture in Szécsi medium with 0.5% xylan as the sole carbon source were incubated in 1 ml of reaction mixture containing 0.5% (w/v) beechwood xylan. Xylanase activity, measured by DNS method, was expressed as xylanase units/ml of culture filtrate, defining one xylanase unit as the amount of enzyme required to release 1 µmol of xylose in 1 min. Data represent the average ± standard error (indicated by bars) of two independent experiments each one performed using two flasks per strain. Average values were significantly different according to the Tukey-Kramer's test. Different letters (a, b) indicate significant differences at $p<0.05$.

PG activity produced by WT and mutant strains in liquid cultures with pectin as sole carbon source was determined with the radial gel diffusion assay. While the WT and the Δxyr mutant strain produced an halo of PG activity corresponding to about 1.3 agarose diffusion units (U), Δpg and double mutant strains did not produce a visible halo, confirming the loss of PG activity due to the disruption of the *pg1* gene (Fig. 10).

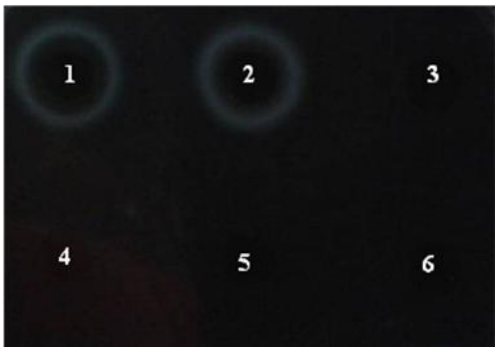


Fig. 10. Radial gel diffusion assay for determining the PG activity. Thirty µl of each fungal culture, produced by *F. graminearum* WT and mutants grown for 4 days in liquid cultures containing pectin as the only carbon source, were loaded in the correspondent wells (5 mm of diameter). Only the WT and the Δxyr mutant strain (wells 1 and 2, respectively) produced an halo corresponding to about 1.3 U. The absence of the halo for Δpg 2.13 and double mutant strains ΔΔxyr/pg 1.18, ΔΔxyr/pg 1.22, ΔΔxyr/pg 1.31 (wells 3-6 respectively) confirmed the loss of PG activity.

Dry weight experiments were also performed inoculating 1×10^4 conidia ml^{-1} of WT and mutant strains in 20 ml of Szécsi medium supplemented with 0.5% (w/v) pectin or xylan. In the first medium no significant difference between WT and mutant strains was observed (Fig. 11A), while there was a significant dry weight reduction of about 90% when the double knock-out mutant strains were grown on xylan (Fig. 11B), similarly to what observed with the single Δxyr mutant.

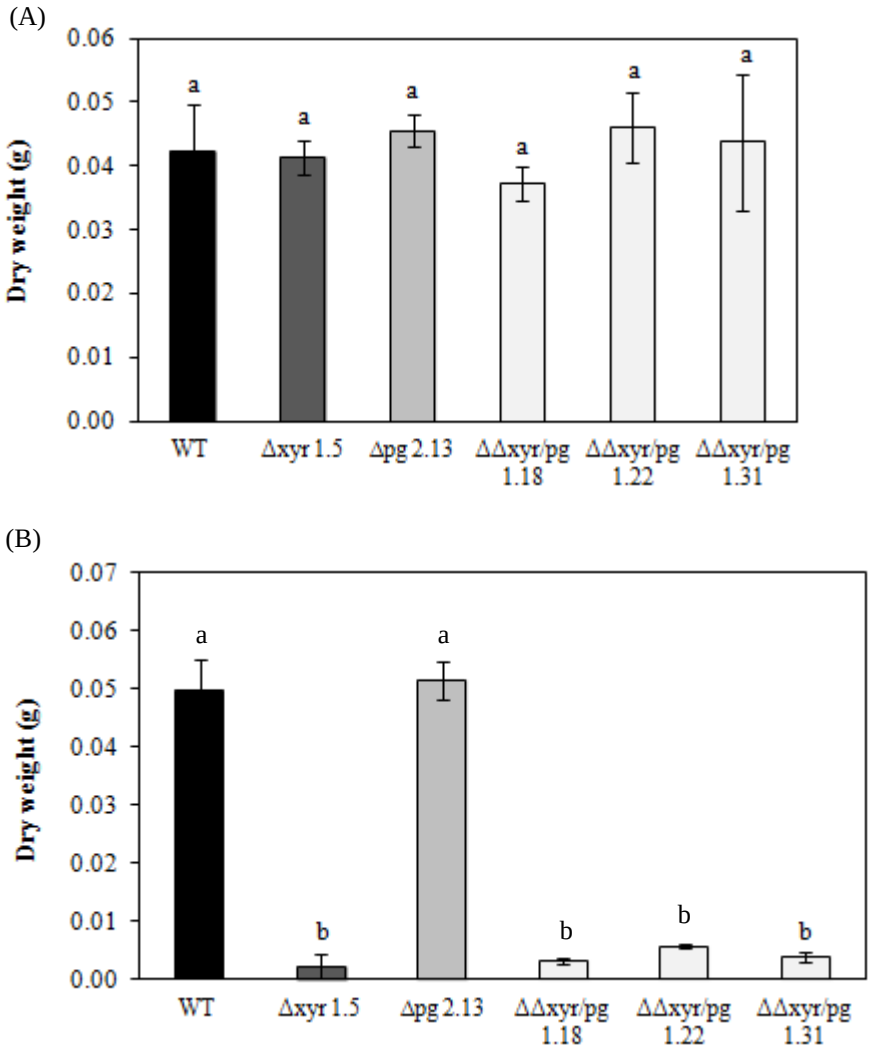


Fig. 11. Dry weight of WT and mutant strains grown for 7 days in a liquid culture containing pectin (A) or xylan (B) as the sole carbon source. Data represent the average ± standard error (indicated by bars) of three independent experiments each one performed testing three flasks per strain. Average values were significantly different only on xylan medium according to the Tukey-Kramer's test. Different letters (a, b) indicate significant differences at $p<0.05$.

Soybean and wheat infections experiments

Infection experiments were initially performed inoculating soybean seeds with 2×10^4 conidia and symptoms were measured on seedlings at 6 dpi as disease severity index (DSI). Compared to the Δpg mutant, the DSI caused by the ΔΔxyr/pg strains was significantly reduced by about 40% (Fig. 12), with the WT being more virulent than the Δpg mutant as previously observed (Fig. 4A). As expected, the virulence of the ectopic transformant (ect 1.1) was similar to that of the Δpg mutant.

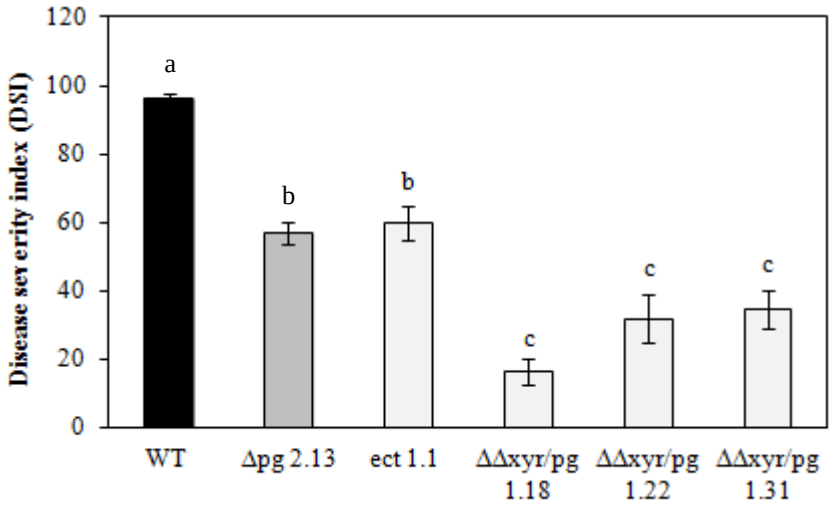


Fig. 12. Infection of soybean seedlings with *F. graminearum* WT and mutant strains. Two hundred µl of spore suspension containing 2×10^4 conidia of the *F. graminearum* WT or mutant strains were dropped on each seed (cv. Demetra) and disease symptoms were assessed at 6 dpi. Data represent the mean ± standard error of at least six independent infection experiments performed with the rolled towel method. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $p<0.05$.

Wheat spikes of *T. aestivum* were inoculated at anthesis with a spore suspension containing 2,000 conidia. The virulence of the double mutant strains was significantly reduced compared to the Δpg strain by about 50% while the virulence of the Δpg mutant was not significantly different compared to the WT (Fig. 13).

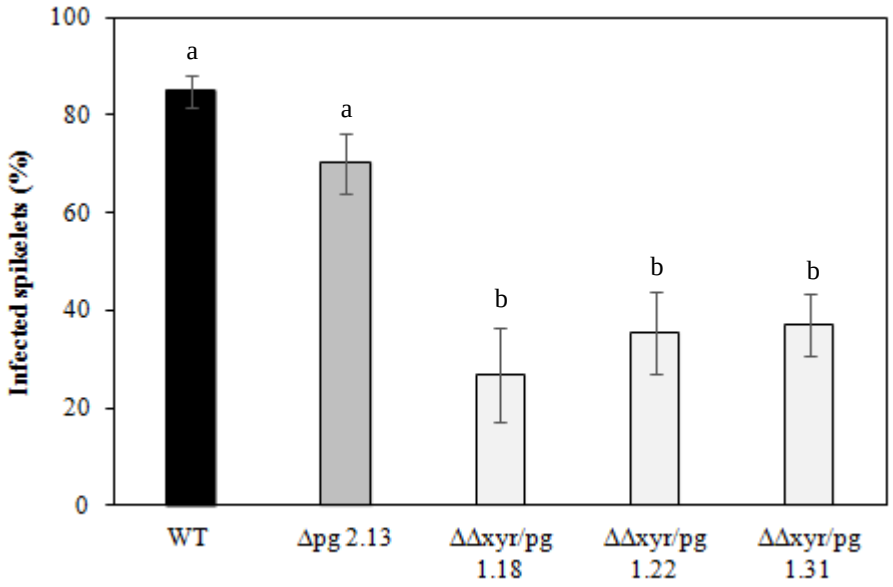


Fig. 13. Wheat spikelets infection with *F. graminearum* WT and mutant strains. Ten µl of spore suspension containing 1×10^3 conidia of the *F. graminearum* WT or mutant strains were pipetted between the glumes of two florets of two opposite central spikelets. Disease symptoms were assessed at 21 dpi by counting the number of visually diseased spikelets. Infected spikelets are expressed as percent of symptomatic spikelets on total number of spikelets of the respective head. Data represent the average ± mean standard error (indicated by bars) of at least six independent experiments performed infecting both 'Bobwhite' and 'Nandu' cultivars. Data were statistically analyzed by applying the Tukey-Kramer's test. Different letters indicate significant differences at $p<0.05$.

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

To verify if the *F. graminearum* xylanase and PG activities have a synergistic effect in degrading the plant cell walls, the main PG isoform (PG1) expressed by *F. graminearum* and the two most expressed xylanases (FGSG_10999 and FGSG_03624) were incubated separately and together in presence of *T. aestivum* cell walls. Results showed that the amount of uronides released by mixing together the two enzymatic activities was about 35% higher than the sum of the uronides released by the two enzymatic activities tested separately (Fig.14).

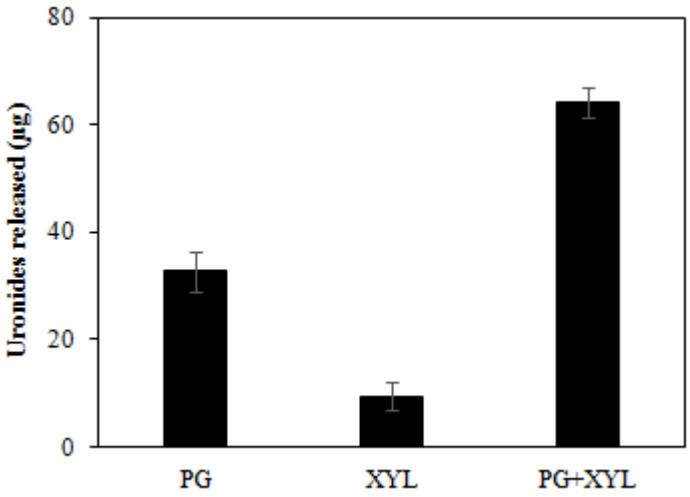


Fig. 14. Uronides released incubating *F. graminearum* polygalacturonase and xylanase activities with *T. aestivum* cell walls. The polygalacturonase PG1 and the xylanases FGSG_10999 and FGSG_03624 were purified and used alone or in combination in presence of 1% wheat cell walls. Data, expressed as µg equivalents of uronides released from a 300 µl reaction mixture, were obtained with the uronic acid assay (Blumenkrantz and Asboe-Hansen, 1973) and represent the mean ± standard error (indicated by bars) of two independent experiments.

GFP expression in WT, Δpg and ΔΔxyr/pg mutant and wheat infection histology

The *F. graminearum* WT, Δpg 2.13 and ΔΔxyr/pg 1.22 mutant strains were transformed to constitutively express the GFP in order to localize the fungus in the wheat tissue. Transformants were selected by PCR and analyzed by fluorescence microscopy to confirm the expression of the green fluorescent protein (data not shown). The GFP mutants were then used in wheat infection experiments (cv. Nandu): in particular, paleas were inoculated with 250 conidia and incubated in Petri dishes at 23°C. After 5 days, the mutant strains were analyzed by fluorescence microscopy and still retained the ability to produce infection cushions and lobate appressoria like the WT strain (Fig. 15).

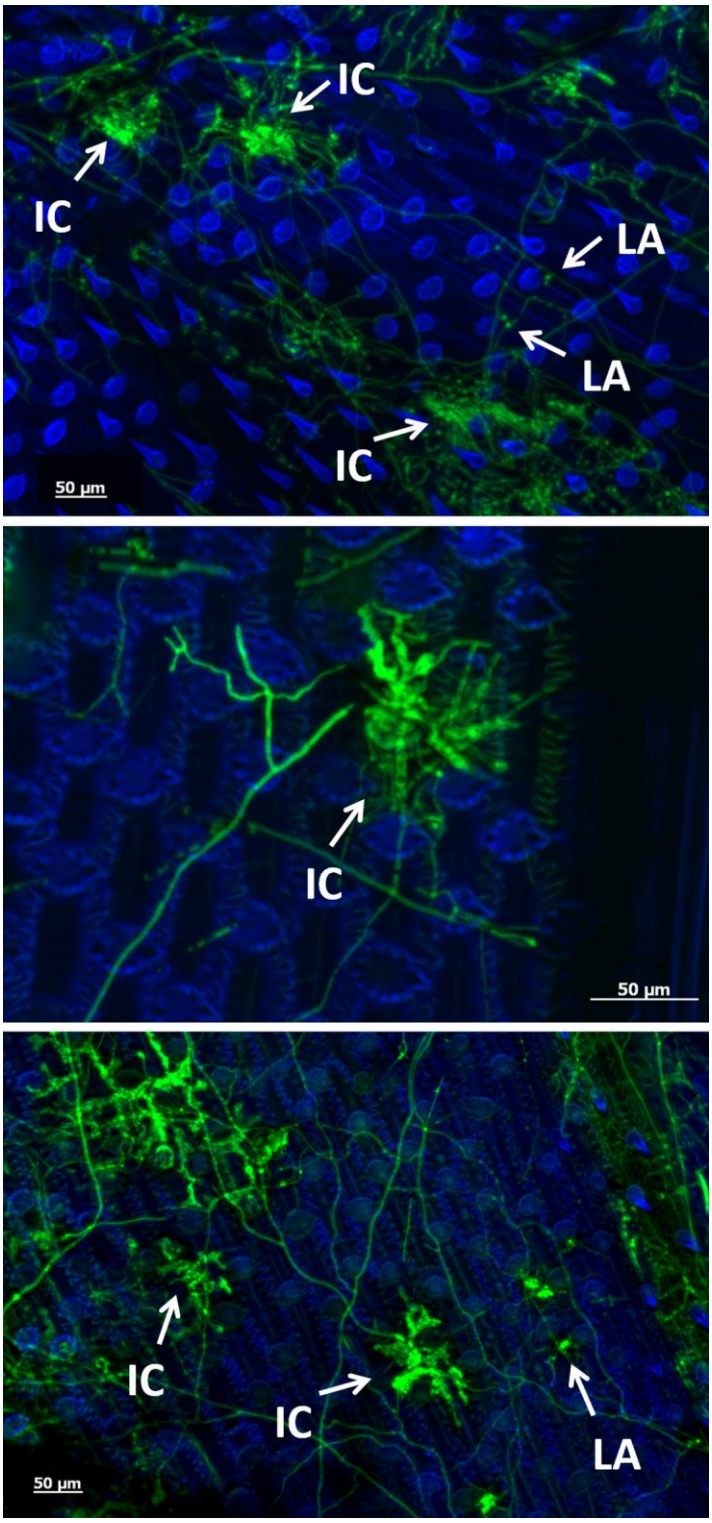


Fig. 15. Infection cushions and lobate appressoria of GFP mutant strains. Five µl of spore suspension containing 250 conidia of the *F. graminearum* WT or mutant strains constitutively expressing the GFP were dropped on the adaxial side of paleas (cv. Nandu). The mutant strains analyzed after 5 days by fluorescence microscopy showed no differences in infection cushions (IC) and lobate appressoria (LA) formation compared to WT. WT-GFP (A), Δpg-GFP (B) and ΔΔxyr/pg-GFP (C). Blu color indicates the plant autofluorescence.

DISCUSSION

Gene disruption is a genetic technique useful to investigate the role of specific factors produced by pathogenic organisms during plant infection and to determine their contribution to the development of disease symptoms. This work focused on the cell wall degrading enzymes endo-PGs and endo-xylanases produced by *F. graminearum* and their contribution to FHB in wheat and seedlings rot in soybean (Goswami and Kistler, 2004; Pioli et al., 2004).

In culture supplemented with pectin this fungus produces high amount of PG activity, and particularly the two endo-PG isoforms PG1, the most abundantly secreted, and PG2 (Tomassini et al., 2009). During wheat spike infection, both PG1 and PG2 encoding genes, *Fgpg1* and *Fgpg2*, are expressed, but the PG1 activity detected in the infected ovary was much higher than that of PG2 (Tomassini et al., 2009). Indirect evidence about a role of *F. graminearum* PG activity in plant infection comes from experiments with transgenic wheat plants expressing a bean polygalacturonase inhibiting protein (PGIP). In fact this PGIP inhibits the *F. graminearum* PG activity and the transgenic PGIP plants are less susceptible to *F. graminearum* infection (Ferrari et al. 2012). In order to directly establish a role of the *F. graminearum* PG activity during plant infection, in the present work three Δpg1 mutant strains were characterized. Infection experiments showed a different behavior of the mutant according to the host used in the infection assay. In fact, although these mutant strains were drastically reduced in their capacity to produce PG activity compared to the WT, their virulence was reduced only in soybean seedlings. The dispensability of the *F. graminearum* PG activity for fungal virulence on wheat and its significant contribution to soybean symptoms is in agreement with the different content in pectin of these tissues; in fact, pectin is known to be more abundant in the cell wall of dicots such as soybean than in graminaceous monocots such as wheat (Vogel, 2008). The results obtained in wheat with the Δpg1 mutant apparently contrast with the improvement of resistance to FHB obtained with the transgenic PGIP plants (Ferrari et al. 2012). However, it should be noticed that the residual PG activity of PG2 may be sufficient to support fungal infection within the wheat spike. Alternatively, the effect of PGIP in decreasing the rate of fungal infection may be ascribed to a generic reinforcement of the plant cell wall barrier as reported by several authors (Joubert et al., 2006; Tundo et al., 2016).

During infection of wheat spikes, *F. graminearum* expresses at high levels also several endo-xylanase encoding genes (Sella et al., 2016) and wheat transgenic plants constitutively expressing TAXI-III, a *T. aestivum* xylanase inhibitor, show a delay of FHB symptoms

(Moscetti et al., 2013). These observations suggest that the *F. graminearum* xylanase activity could be involved in fungal virulence on wheat spikes. Indeed a recent paper showed that the deletion of the *F. graminearum* FGSG_10999 xylanase, one of the most expressed during wheat spikes infection (Sella et al., 2016), produced a mutant strongly reduced in virulence (Sperschneider et al., 2015). In contrast, the targeted gene replacement of a *F. graminearum* transcriptional regulator of the xylanase genes (XYR1) in the fungal PH1 strain produced a mutant strongly impaired in xylanase activity but with a virulence comparable with the WT strain on both soybean seedlings and wheat spikes (Sella et al., 2016). This result was unexpected in wheat because the cell wall of this plant is particularly rich of xyloglucan. The failure of xyr1 disruption to alter fungal virulence is confirmed here also with a Δxyr1 mutant of the *F. graminearum* 3827 strain, i.e. the same isolate used for the targeted disruption of the *pg1* gene. The apparently contrasting results obtained with the Δxyr1 mutants and the ΔFg10999 mutant need further investigation. However, it should be noticed that the complementation experiment of the Fg10999 knock-out was not performed by Sperschneider and co-workers (Sperschneider et al., 2015) and the lacking of this experimental verification cannot exclude collateral effects of the mutation on other non target genes.

Overall, our results seem to indicate that the *F. graminearum* PG and xylanase activities do not play an important role during wheat spike infection, while PG activity is required for full virulence on soybean. However, pectin and xylan are polymers strictly interwoven one other in the primary plant cell wall (Kikot et al., 2009) and disruption experiments of PG1 and XYR1 do not exclude that, at least during wheat spike infection, PG and xylanase may have an overlapping role, whereby one or the other enzymatic activity may be sufficient for loosening the cell wall allowing the advancement of fungal hyphae into the plant tissue. In addition, PG and xylanase activities could work synergistically making the cell wall structure more easily accessible to the activity of other CWDEs. Assuming that the simultaneous reduction of both PG and xylanase activity could be detrimental to the progress of infection, in this work ΔΔxyr/pg double mutant strains were obtained and characterized both *in vitro* and *in planta*.

The three ΔΔxyr/pg mutant strains obtained showed a dramatic reduction of both xylanase and PG activities in liquid media with xylan or pectin as sole carbon sources. Dry weight experiments performed on the same media showed that a significant reduction of growth of the double knock-out mutant strains was evident only in xylan containing medium, similarly to what observed with the *xyr1* deletion mutant (Sella et al., 2016 and this paper). The

observation that the lack of PG activity does not seem to affect the ability of the Δpg and ΔΔxyr/pg mutants to grow in pectin containing medium could depend on the contribution to fungal growth of other pectinase activities such as pectate lyase or pectin lyase. However, the presence in the medium of other carbon sources, which are usually contained in the commercial pectins used for our growth experiments, could have also affected this result (Sella et al., 2016).

Infection experiments with the ΔΔxyr/pg mutant strains, compared to the Δpg mutant, showed symptoms significantly reduced by about 40% in soybean and 50% in wheat, thus indicating that the contemporary lack of PG and xylanase activities affects the virulence of *F. graminearum*; this result is particularly interesting in wheat spikes, where, as above described, the single Δxyr and Δpg mutants were as virulent as the WT. The synergistic effect of xylanase and PG activities was confirmed by incubating together two endo-xylanases (FGSG_10999 and FGSG_03624; Sella et al., 2013; Tundo et al., 2015) and PG1 with wheat cell walls. In fact, the uronides released by xylanases and PG1 together were about 35% more than the sum of uronides obtained by the two enzymes separately incubated with the cell walls.

In order to verify if the lack of *pg1* and *xyr1* genes affects the capacity of *F. graminearum* to differentiate its penetration structures, WT, Δpg and ΔΔxyr/pg mutants were also transformed to constitutively express the GFP in order to localize the fungus in the wheat tissue by fluorescence microscopy. The inoculation of wheat glumes and paleas showed that the mutants were still able to produce infection cushions and lobate appressoria like the WT, thus indicating that the reduced virulence of the double mutant is not related to a different ability to produce infection structures but it is most likely due to a different ability to progress inside the infected tissues probably for its altered capacity to degrade the cell wall polysaccharides.

In conclusion, our results show that the *F. graminearum* PG activity is directly involved in fungal virulence on soybean, while there is a synergistic effect between *F. graminearum* PG and xylanase activities, mostly during wheat spikes infection. This result is in accordance with the further improvement of wheat resistance to Fusarium Head Blight when the PGIP and TAXI were pyramided together in the same line (Janni et al., 2008; Moscetti et al., 2013; Tundo et al., 2016). Our results confirm that this effect is likely due to a combined inhibitory effect against the *F. graminearum* PG and endo-xylanase activities.

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Chapter II

Exploiting a wheat xylanase inhibitor and a *Fusarium graminearum* xylanase to increase plant resistance to pathogens

INTRODUCTION

Plant cell wall constitutes a barrier to microbial invasion. It is composed mainly of polysaccharides (i.e. cellulose, hemicelluloses and pectins) and different classes of proteins (Carpita et al., 1993; Kikot et al., 2009). During plant infection, a great variety of pathogenic organisms including bacteria and fungi produce many cell wall degrading enzymes (CWDE), such as cellulases, xylanases, pectinases, cutinases, lipases and proteases, in order to colonize the host tissue and also to obtain nutrients (Prins et al., 2000). In particular endo-β-1,4-xylanases (EC 3.2.1.8, Collins et al., 2005; Wong et al., 1988) are glycoside hydrolase enzymes able to catalyze the hydrolysis of β-1,4-xylan, an abundant structural polysaccharide of plant cell wall particularly present in the primary cell wall of monocot plants (Cooper et al., 1988).

Endo-β-1,4-xylanases (endo-xylanases) have been grouped in the glycoside hydrolase families 10 (GH10) and 11 (GH11) according to their catalytic activities and their tertiary structure. GH10 xylanases have higher molecular weight than GH11 xylanases (Collins et al., 2005) and, due to their more flexible structure, are also catalytically more versatile than GH11 xylanases (Biely et al., 1997). Moreover GH10 family includes plant, fungal, and bacterial enzymes while the structurally unrelated GH11 family includes only fungal and bacterial enzymes (Bourne et al., 2001; Henrissat et al., 1991).

Botrytis cinerea is a necrotrophic fungus causing grey mould disease on several dicotyledonous plants. During infection, *B. cinerea* secretes several CWDEs to penetrate host tissue, like pectinases, cellulases, xylanases and arabinases (Kars and van Kan, 2007). A key role during the infection process of *B. cinerea* is played by the Xyn11A endo-xylanase, which belongs to the GH11 family and is essential for virulence in tomato leaves and grape berries (Brito et al., 2006). This xylanase has been found to induce necrosis in plants independently from its enzymatic activity: in particular Xyn11A kills the plant tissue around the infected area, allowing the fungus to grow faster on dead tissue and its role in fungal virulence seems related to this ability to elicit necrosis rather than to its enzymatic activity (Noda et al., 2010). The necrotizing activity is shared by many fungal endo-β-1,4 xylanases and is associated to the capacity to activate defence responses in plants. For example, the endo-xylanases of *Trichoderma reesei* and *Trichoderma viride* were the first shown to induce defence responses in planta (Bailey et al., 1990; Avni et al., 1994) through oxidative burst and ethylene

biosynthesis (Felix et al., 1991) and also this activity was independent from the enzymatic activity, as shown by site directed mutagenesis (Enkerli et al., 1999).

Also the fungal pathogen *Fusarium graminearum*, the causal agent of Fusarium Head Blight (FHB, Goswami and Kistler, 2004) of cereals, during wheat infection secretes several xylanases (of both GH11 and GH10 families; Sella et al., 20013, Tundo et al., 2015) which are necrotizing factors. In particular the FGSG_03624 of the GH11 family has a sequence very similar to the *B. cinerea* Xyn11A and shares with this xylanase a stretch of amino acids essential for the necrosis-inducing ability (Sella et al., 2013). Besides, the FGSG_10999 xylanase, which is another *F. graminearum* endo-xylanase of the GH11 family, and the FGSG_11487, which belongs to the GH10 family, induce cell death unrelated to their enzymatic activity (Tundo et al., 2015). The role of *F. graminearum* xylanase activity in pathogenicity was investigated by disrupting the FGSG_03624 encoding gene and the *xyr1* gene, which encodes the major regulator of xylanase gene expression. In spite of the strong reduction of xylanolytic activity, by 40% and 90%, in the *FGSG_03624* and *xyr1* disruption mutants, respectively, the virulence of both mutants was unaffected (Sella et al., 2013 and 2016).

Among the defense mechanisms used by plants to counteract microbial pathogens, xylanase inhibitor proteins (XIs) are able to reduce or completely block the fungal endoxylanolytic activity and are usually localized in the plant cell wall. Three inhibitor families with different inhibitory capacities have been identified in wheat: *Triticum aestivum* XI (TAXI) (Debyser et al.,1999), xylanase inhibitor protein (XIP) (McLauchlan et al., 1999) and thaumatin-like XI (TLXI) (Fierens et al., 2007). TAXI type endo-xylanase inhibitors have a molecular mass of about 40 kDa and occur in two molecular forms, A and B (Debyser et al.,1999). Form A consists of a single 40 kDa polypeptide chain, while form B is composed by two disulfide-linked subunits of 29 and 11 kDa. The *Taxi-I* gene encodes a 381 amino acids non glycosylated protein of 38.8 kDa (Fierens et al., 2003); TAXI-I inhibits fungal and bacterial GH11 family endo-xylanases, but is inactive against GH10 family xylanases (Debyser et al., 1999; Gebruers et al., 2001). Two members of the TAXI family (TAXI-I and TAXI-III) are expressed in wounded leaves but only TAXI-III is strongly induced after microbial infection (Igawa et al., 2004; Moscetti et al., 2013). The overexpression of the TAXI-III in transgenic plants (Moscetti et al., 2013) has caused a delay of FHB symptoms, indicating the possibility to increase plant resistance to fungal disease by engineering this xylanase inhibitor, even

though a contribution of the *F. graminearum* xylanase activity to virulence is not clearly demonstrated considering the previous gene disruption experiments.

Since, differently from *F. graminearum*, the *B. cinerea* Xyn11A xylanase is clearly required for virulence (Brito et al., 2006) and the wheat xylanase inhibitor TAXI-I inhibits this xylanase (Brutus et al., 2005), we considered the possibility to increase plant resistance to *B. cinerea* by expressing the wheat TAXI inhibitors. To this aim we transiently expressed TAXI-I and TAXI-III (which has similar inhibitory capability) in tobacco leaves by agroinfiltration and we also produced by floral dip transgenic *Arabidopsis* plants constitutively expressing TAXIs to test the ability of these inhibitors to counteract infection.

Moreover, since the *F. graminearum* xylanase FGSG_03624 has been shown to induce necrosis and defense responses in wheat tissues (Sella et al., 2013), we verified if this xylanase can be exploited to increase plant resistance to pathogens. To this aim, we first sprayed this xylanase on *Arabidopsis* leaves to test its ability to induce effective defense responses against the bacterium *Pseudomonas syringae pv. maculicola* and the fungus *B. cinerea*. Since enzymatic activity of the FGSG_03624 can affect negatively the plant cell wall, we performed a site-directed mutagenesis on one of the two Glu residues that is essential for enzyme activity but is not necessary for elicitor activity (Enkerli et al., 1999; Sella et al., 2013). This modified xylanase was transiently expressed in tobacco plants through agroinfiltration and was used for transformation of *Arabidopsis* plants by floral dip in order to test its effect in inducing plant resistance to *P. syringae* and *B. cinerea*.

MATERIALS AND METHODS

Strains, plants and growth conditions

Agrobacterium tumefaciens (strain GV3101) was used to express *Taxi-I* and *Taxi-III* genes and a mutated form of the *F. graminearum* FGSG_03624 xylanase. The strain was resistant to rifampicin and to gentamicin. Recombinant plasmids were previously transferred to *Escherichia coli* strain DH5α grown in Lysogeny broth (LB) at 37 °C.

Arabidopsis thaliana (ecotype Col-0) and *Nicotiana tabacum* (ecotype SR1) plants were grown in a controlled environment at 20°C-22°C with a 16 hours photoperiod.

Infection experiments were performed with the fungal pathogen *Botrytis cinerea* (strain B05.10) grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) and with the bacterial pathogen *Pseudomonas syringae pv. maculicola* grown in King's B medium (King et al., 1954) at 200 rpm and 28°C.

For the enzymatic assay, the mycelium of *B. cinerea*, grown first in minimal medium supplemented with glucose 1% for 3 days, was washed three times with sterile water and then transferred in Szecsi medium (Szecsi, 1990) with xylan 1% as the only carbon source for two days to induce xylanases expression as reported in Brito et al. (2006).

Xylanase treatment of *Arabidopsis* leaves

Leaves of *A. thaliana* plants (8–12 leaves stage) were treated spraying abaxial leaf surfaces with the *F. graminearum* xylanase FGSG_03624 (100 $\mu g\, ml^{-1}$) or water as control both supplemented with pinolene 0.04% (v/v). Treated leaves were collected after 24, 48, 72 and 96 hours for gene expression analysis. The heterologous expressed FGSG_03624 xylanase used in the experiments was produced and purified as described by Sella et al. (2013).

RNA extraction and reverse transcription

RNA was extracted from 100 mg of frozen *Arabidopsis* leaves (treated or transformed) and infiltrated tobacco leaves by using the "RNeasy Plant Mini Kit" (Qiagen, Milano, Italy) following the manufacturer's instructions. RNA was treated with DNaseI (Promega, Milano, Italy) following manufacturer's instructions and quantified both spectrophotometrically and by a denaturing gel.

Reverse transcription was performed by mixing 500 ng of an oligo-dT (15/18 thymine) reverse primer with 0.5 µg target RNA and by using the ImPromII reverse transcriptase (Promega), following manufacturer's instructions.

Gene expression analysis

Gene expression analysis of the *Arabidopsis* PR1 (AY117187.1) and PDF 1.2 (AY063779.1) genes was performed by quantitative polymerase chain reaction (qPCR, Rotor-Gene Q 2plex, Qiagen GmbH) using specific primers (Table 1). The 20 µl reaction mixture contained 10 µl of 2X Rotor-Gene SYBR Green PCR Master Mix (Qiagen GmbH), 0.4 mM of each specific primer and 3 µl of cDNA as template. The qPCR was performed by repeating 40 times the following cycle: 30 s at 94°C; 30 s at 56°C; 30 s at 72°C. Each transcript was normalized with the *Arabidopsis* ubiquitin gene (AY139810.1) used as housekeeping and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen GmbH) setting to 1 the expression of the housekeeping gene. At least two independent qPCR experiments were performed with different RNA preparations.

Gene expression analysis of *Arabidopsis* and tobacco leaves expressing *Xyl*, *Taxi-I* and *Taxi-III* genes was carried out by qPCR with the Rotor-Gene Q 2plex. The 20 µl reaction mixture was performed as reported above with forward and reverse primers mentioned in Table 1. qPCR conditions were 20 sec at 94°C, 20 sec at 54°C, 30 sec at 72°C for 40 cycles. Results were analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen GmbH). The reference genes used in qPCR analysis were ubiquitin and actin respectively for *Arabidopsis* and tobacco. Two independent qPCR experiments were performed with different RNA preparations.

Production of the constructs and *Agrobacterium* transformation

The *Taxi-I* gene was amplified from the plasmid kindly provided by prof. D'Ovidio (Tuscia University) with the primers pair TAXI-I1F_BamHI/TAXI-I1259R_SacI (Table 1) by using the "REDTaq ReadyMix PCR Reaction Mix" (Sigma-Aldrich). The PCR was performed by repeating for 35 times the following cycle: 30 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C. The amplified DNA fragment was purified, cloned into pGEM-T Easy Vector (Promega), cut with *Sac*I and *BamH*I and then introduced into the pBI121 plant vector.

The *Taxi-III* gene was isolated from the pAHC17-TaxiIII vector provided by prof. D'Ovidio and was inserted into the *BamH*I and *Sac*I sites of the pBI121 vector, after a previous cloning

in the pGEM-T Easy Vector. A “Splicing by Overlap Extension” (SOE) has been performed to eliminate a cutting site (Fig. 1), using modified forward and reverse primers (Table 1) and this conditions: 1 min at 94°C, 1 min 60°C and 1.5 min at 72°C for 30 cycles. PCR condition to amplify the gene were the same described above and primer used are mentioned in Table 1.

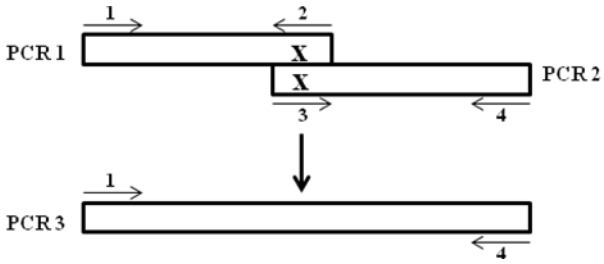

Fig. 1. **Splicing by overlap extension (SOE).** PCR products from PCR 1 and 2 were purified by columns and served as templates for the PCR 3. The final PCR product was purified and then ligated to *BamH*I and *Sac*I digested pBI121 vector. 2 and 3 represent the modified reverse and forward primers used to eliminate a cutting site, while 1 and 4 the primers used to amplify *Taxi-III* gene.

The *F. graminearum FGSG_03624* gene encoding an endo-1,4-beta-xylanase of 228-amino acids (Fig. 2A) was first amplified using cDNA from wheat spikes infected by *F. graminearum* by using the primers 03624Fc and 03624Rc (Table 1) and repeating for 35 times the following cycle: 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C. The DNA fragment was purified and cloned into pGEM-T Easy Vector (Promega). Since the catalytic activity of the protein is due to two Glu residues (conserved in all the xylanases of the GH11 family) at position 122 and 214, to obtain the FGSG_03624 protein without enzymatic activity a glutamic acid codon (GAG) coding for the Glu (E) residue at position 214 was changed to serine (S) codon (TCG) (Fig 2B). The site directed mutagenesis was performed using PCR amplification with primers pair XYL-F XbaI and XYL-R SacI (Table 1) containing a mutation in the reverse primer. PCR conditions were 30 sec at 94°C and 2 min at 72°C (annealing and extension steps) for 35 cycles. The mutated gene (*Xyl*) was then digested with *Sac*I and *Xba*I and ligated into the pBI121expression vector cut with the same restriction enzymes. Each expression cassette is under control of the constitutive CaMV 35S promoter and NOS terminator and the vector contains the NPTII gene for selection with kanamycin. After verification of the correctness of all the cloned sequences by sequencing, plasmids were electroporated at 2.5 KV into the *A. tumefaciens* strains as described by Mozo and Hooykaans (1991).

(A)

```
  1 MVSFTYLLAA VSAVTGAVAA PNPTKVDAQP PSGLLEKRTS PTTGVNNGFY FSFWTDTPSA
 61 VTYTNGNGGQ FSMNWNGNRG NHVGGKGWNP GAARTIKYSG DYRPNGNSYL AVYGWTRNPL
121 VEYYIVENFG TYNPSSGAQK KGEINIDGSI YDIAVSTRNC APSIEGDCKT FQQYWSVRRN
181 KRSSGSVNTG AHFNAWAQAG LRLGSHDYQI LAVEGYQSSG QATMTVSG
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(B)

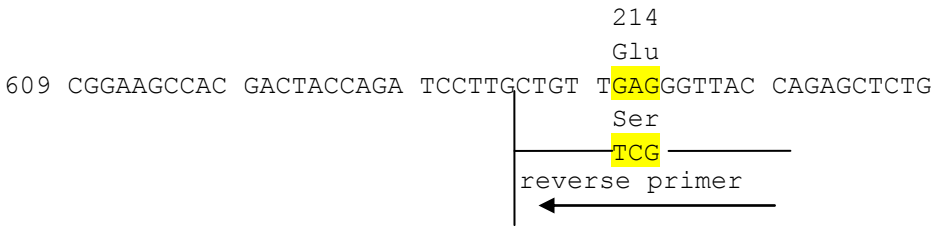

Fig. 2. Amino acid sequence of the *Fusarium graminearum* xylanase FGSG_03624 and the site directed mutagenesis performed. (**A)** The first 19 underlined amino acids represent the signal peptide and the two highlighted Glu residues are essential for enzyme activity. (**B)** Site directed mutagenesis performed to change the Glu codon at position 214 to Ser codon using a mutated reverse primer represented by the arrow.

Tobacco agroinfiltration and *Arabidopsis* transformation

Agrobacterium-mediated transformation of *Nicotiana tabacum* and *Arabidopsis thaliana* were performed by agroinfiltration and floral dip methods, respectively.

Agroinfiltration: *A. tumefaciens* strain harboring *Taxi-I*, *Taxi-III*, *xyl* genes and the pBI-GUS vector as control were grown overnight in a shaker at 28°C and 200 rpm in LB supplemented with 50 μg ml^{-1} rifampicin, kanamycin and gentamicin. For agroinfiltration of *N. tabacum* leaves, the overnight cultures were collected by centrifugation at 5000 *g* for 15 min at room temperature and resuspended in infiltration buffer (10 mM $MgCl_2$ and 150 μg ml^{-1} acetosyringone) to a final OD_{600} of 0.8-1 (Voinnet, 2003). Cell solution was loaded into a 1 ml plastic syringe without needle and infiltrated into the abaxial leaf surface of 6 week-old *N. tabacum* plants. In particular, four leaves were infiltrated for each plant and each leaf was infiltrated in one side of the mid-vein with *Taxi* or *Xyl* genes and in the other side with the *A. tumefaciens* strain harboring pBI-GUS as control. Infiltration spots were outlined with a black marker for tissue sampling and infection experiments. Tobacco plants were covered with transparent plastic bags and maintained at the growth conditions above reported. After 1, 2, 3 and 4 days from agroinfiltration, infiltrated leaf tissues were collected and stored at -80°C for RNA extraction.

Floral dip: Each *Agrobacterium* strain containing the recombinant vector was grown overnight in LB medium supplemented with kanamycine (50 μg ml^{-1}) at 28°C and 250 rpm. At a final OD_{600} of 0.8-1, 5 ml of each culture were added to 40 ml of dipping solution [5% (w/v) sucrose and 0.05% (v/v) Silwet L-77 (Ghedira et al., 2013)]. Secondary flowering stems of *Arabidopsis* plants (10-15 cm in length) were dipped for 10 s by bending the inflorescences

into the Falcon tube containing the *Agrobacterium*. Then, plants were covered for 24 h and were allowed to grow under normal conditions described above. Recovered T0 seeds were sterilized by immersion in 70% (v/v) ethanol for 2 min and 1% (v/v) sodium hypochlorite for 10 min, washed three times with sterile water and plated on Murashige and Skoog medium containing 50 μg ml^{-1} kanamycin, 1X vitamins (Sigma-Aldrich), 3% w/v sucrose and 0.9% w/v bacto agar (Difco Laboratories).

After 15 days, kanamycin-resistant seedlings were transferred to soil. A total of two TAXI-I, one TAXI-III and two XYL transgenic plants were obtained and tested by PCR using the primer pairs indicated in Table 1. The genomic DNAs of the kanamycin-resistant transformed *Arabidopsis* plants were extracted by using the "DNeasy Plant MiniKit" (Qiagen GmbH Italy) according to the manufacturer's instructions. The PCR reaction, performed in a 25 μl volume, consisted of 3 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 3 min.

Protein extraction and enzymatic assay

For protein extraction, frozen *Taxi-I, Taxi-III* and *Xyl* tobacco infiltrated leaves and transgenic *Arabidopsis* leaves samples were ground in McIlvaine's buffer pH 5.0 (0.2 M Na_2HPO_4, 0.1 M citric acid), shaked 1 hour at 4°C and centrifuged twice at 8000x g for 10 min. Supernatants were tested in radial diffusion assay (Emami and Ethan, 2001) to verify the capacity of TAXI-I and TAXI-III to inhibit the *F. graminearum* FGSG_03624 xylanase, purified as described by Sella et al. (2013), and the *B. cinerea* xylanase activity produced in liquid culture as above reported.

Plates containing agarose (1% w/v) and birchwood xylan (1%, Sigma-Aldrich) dissolved in McIlvaine's buffer (pH 5) were prepared with 0.5 cm diameter wells. Different amounts of total protein extracts from infiltrated tobacco leaves or transgenic *Arabidopsis* plants were loaded in the wells with or without 1.5 U of the *F. graminearum* FGSG_03624 xylanase or 1 U of the *B. cinerea* culture filtrate. The protein extracts containing the mutated XYL were used to confirm the loss of enzymatic activity of the mutated form. The final reaction volume was adjusted to 50 μl by adding McIlvaine's buffer and plates were incubated at 37°C for 16 h. The halo caused by enzymatic activity was visualized by adding 95% ethanol to the plates. Xylanase activity was expressed as agarose diffusion units where 0.5 cm radius correspond to one agarose diffusion unit.

Infection of *Arabidopsis* exogenously treated leaves

P. syringae pv. maculicola bacterial cells were collected by centrifugation at 5000 *g* for 15 min at room temperature and resuspended in $MgSO_4$ 0.01M to an OD_{600} of 0.2. Cells solution was loaded into a 1 ml plastic syringe without needle and infiltrated into the abaxial *Arabidopsis* leaf surface 3 days after exogenous xylanase treatment. Control leaves were treated with water supplemented with pinolene 0.04% (v/v).

For *B. cinerea* (strain B05.10) infection, 5.0 mm diameter fresh mycelial disks grown on PDA were placed on the adaxial leaf surface 3 and 4 days after xylanase treatment.

Infected leaves were then placed in Petri dishes containing sterilized humid paper and infection was monitored up to 6 days. Infection results were analyzed by using the "Fiji Is Just ImageJ" software (licensed under the GNU General Public License).

Infection of tobacco and *Arabidopsis* transformed plants

Infection experiments of agroinfiltrated tobacco leaves were performed by inoculating the marked infiltrated area with actively growing mycelium disks (5 mm diameter) of *B.cinerea* (strain B05.10). Infections were performed 3 days after infiltration (dai) for *Taxi* and 4 dai for *Xyl* infiltrated leaves. Lesions were measured after three days with the graphic software "Fiji imageJ".

Arabidopsis transgenic leaves were infected with fresh *B. cinerea* mycelium (0.3 cm diameter disks) and the size of necrotic lesions was measured after 24 hours by calculating the average area of lesions with the graphic software "Fiji imageJ".

Table 1. List of primers used in this work.

PRIMER NAME	PRIMER SEQUENCE (5'-3')
Primers for gene expression analysis and transgenic plant screening	
PR1-F	ACTAAGAGGCAACTGCAGACTCAT
PR1-R	ATGGCTTCTCGTTCACATAATTCC
PDF1.2-F	GGGTTTGCGGAAACAGTAATG
PDF1.2-R	TGTAACAACAACGGGAAAATAAAG
03624RTfor	GTCTCCTTCACCTACCTTCTC
03624RTrev	TCCATCCCTTACCACCGA
TAXI1 774F	CTACCCTACGTCTTGCTCCG
TAXI1 1002R	TCCACCATCGAGTTCTTCCC
TAXI3 170F	GTCCACGTGCGAGGGTAGT
TAXI3 398R	CGACGTTCACCTCGCTAACC
UBQ5-F	GTGGTGCTAAGAAGAGGAAGA
UBQ5-R	TCAAGCTTCAACTCCTTCTTT
Actin Tob103-For	TAGGCTGGATTTGCTGGTGA
Actin Tob103-Rev	TCCATGTCATCCCAGTTGCT
Primers for cloning the *Xyl*, *Taxi-I* and *Taxi-III* genes	
03624Fc	ATGGTCTCCTTCACCTACCT
03624Rc	TATCCAGAGACAGTCATGGT
XYL-F XbaI	AATAATCTAGAATGGTCTCCTTCACCTACCTTCTCGCCGCTGTCTCGGCC
XYL-R SacI	AATAAGAGCTCTTATCCAGAGACAGTCATGGTAGCCTGGCCACTGCTCTGGTAACCCG AAAC
TAXI-I1F_BamHI	AATAGGATCCATGCCACCAGTGCTCCTCCTC
TAXI-I-1259R SacI	AATAGAGCTCTTACAGGCCGCCGCAACC
SOE_TAXI3_F	CCGTTCGAGCTATGCTACG
SOE_TAXI3_R	CGTAGCATAGCTCGAACGG
Taxi3_BamHI1F	AATAGGATCCATGGCACGGGTCCTCCTCCTG
TAXI-III 1206R_SacI	AATAGAGCTCCTAGCTGCCGCAACCCGTAAAG

RESULTS

Exogenous treatments of *Arabidopsis* leaves with the FGSG_03624 xylanase

Leaves of *Arabidopsis* plants were exogenously treated with the *F. graminearum* FGSG_03624 xylanase to test its ability to induce defence responses. In particular, the expression pattern of two selected *A. thaliana* genes, PR1 and PDF1.2, respectively markers of salicylate and jasmonate/ethylene pathways, was analyzed by RT-qPCR. While PR1 showed a low relative expression in treated compared to control leaves at all the time point analyzed, the expression of PDF1.2 was strongly induced in treated leaves especially at 96 h after treatment (Fig. 3).

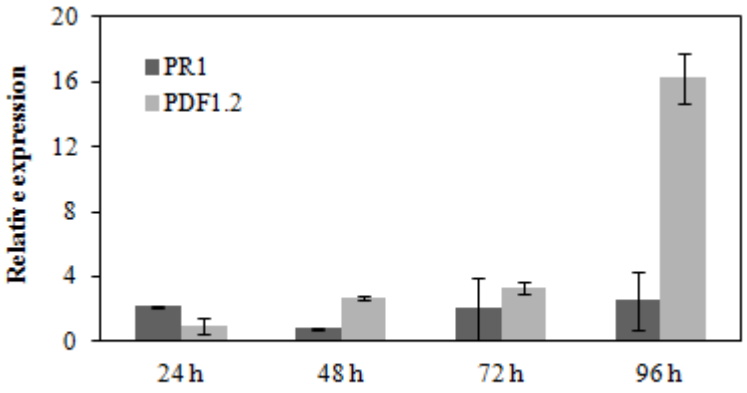

Fig. 3. Relative expression level of *A. thaliana* PR1 and PDF1.2 genes in leaves treated with FGSG_03624. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the ubiquitin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen, Italy). Data represent the average ± mean standard error (SE, indicated by bars) of the relative expression of at least 2 independent qPCR experiments.

Three days after exogenous treatment with the *F. graminearum* xylanase, *Arabidopsis* leaves were infected with the bacterium *P. syringae pv. maculicola* and the fungus *B. cinerea*. Symptoms caused by the bacterial pathogen were slowed down in xylanase treated leaves, with a significant 25% reduction of symptoms at 3 dpi and 20% at 6 dpi compared to control leaves (Fig. 4).

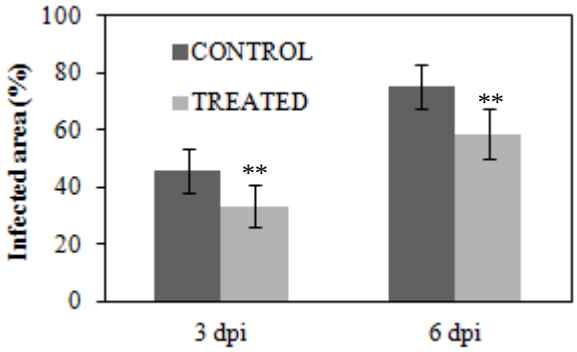

Fig. 4. Infection of *Arabidopsis* leaves with *P. syringae pv. maculicola* after exogenous treatment with the FGSG_03624 xylanase. Disease symptoms were assessed at 3 and 6 dpi by measuring lesion area with the graphic software "Fiji ImageJ". Data (percentage of infected area on total leaf area) represent the average ± mean standard error (SE, indicated by bars) of at least 6 independent infection experiments. Leaves treated with water were used as control. Xylanase treated leaves were statistically more resistant than control leaves by applying Student's t test. ** indicate significant differences at $p < 0.01$.

Differently, symptoms caused by *B. cinerea* infections performed three and four days after the xylanase treatment were similar in treated and control leaves (data not shown).

Transient expression of the *F. graminearum* xylanase in tobacco leaves by agroinfiltration

In order to obtain a mutated form (Xyl) of the *F. graminearum* FGSG_03624 xylanase without enzymatic activity but retaining the ability to induce HR, we modified one of the two amino acids of the catalytic site by site-directed mutagenesis. *A. tumefaciens* strains harboring the mutated Xyl or pBI-GUS as control were infiltrated in tobacco leaves for transient gene expression. A RT-qPCR analysis to verify the presence of the transcript of interest and quantify its expression level was initially performed. The *Xyl* gene showed an almost stable expression level between two and four days post infiltration, with a 6 folds increase compared to tobacco actin gene (Fig. 5).

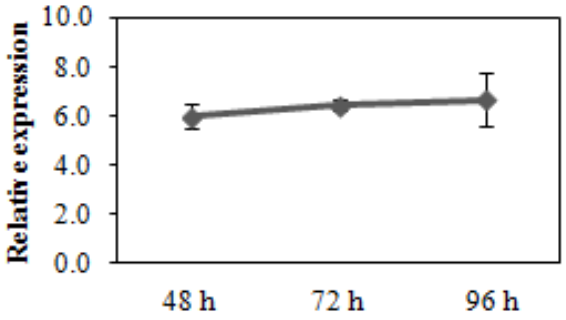

Fig. 5. Relative expression of the *Xyl* transcript in tobacco agroinfiltrated leaves at different timing post agroinfiltration. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Relative expression of the mutated *Xyl* gene was normalized with the tobacco actin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen). Data represent the average ± mean standard error (SE, indicated by bars) of two independent experiments.

A radial gel diffusion assay performed with the proteins extracted from tobacco plants expressing the mutated Xyl confirmed the loss of its enzymatic activity (data not shown). Infiltrated tobacco leaves transiently expressing Xyl were therefore tested for their resistance towards *B. cinerea* but resulted as susceptible as the control leaves agroinfiltrated with the pBI-GUS plasmid (Fig. 6).

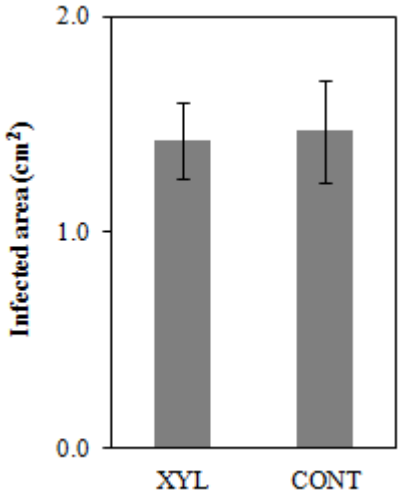

Fig. 6. Lesion area produced by *B. cinerea* (strain B05.10) on tobacco leaves expressing XYL at 3 dpi. Histograms show the lesions area (expressed in cm^2) calculated with the graphic software "Fiji ImageJ". Bars indicate the standard error (SE) calculated from three independent infection experiments. No difference was observed between XYL expressing leaves and leaves transformed with the pBI-GUS plasmid (CONT).

Production of *Arabidopsis* transgenic plants expressing the *F. graminearum* xylanase

Seeds obtained from *Arabidopsis* plants transformed by floral dip with the *Agrobacterium* strain containing the mutated Xyl encoding gene were selected by plating on Murashige and Skoog medium containing kanamycin. A total of two Xyl putative

transgenic plants showed antibiotic resistance. A PCR performed by using genomic DNA from the selected plants confirmed the successful transformation (Fig. 7, lanes 1-2).

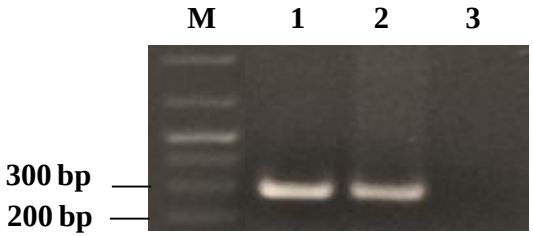

Fig. 7. PCR amplification performed using gene specific primers and total DNA of T0 plants of *A. thaliana* transformed with the Xyl construct. Amplicons were separated on 1% (w/v) agarose gel. Lanes 1 and 2: two transgenic lines of Xyl. A negative control was loaded on lanes 3. Lane M represent molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy).

Transgenic plants of the T1 generation showed no macroscopic phenotypic differences compared to the WT line (not shown). RT-qPCR analysis confirmed the presence of the *Xyl* transcript, with a peak of expression of about one fold in both lines compared to *Arabidopsis* ubiquitin gene, respectively (Fig. 8).

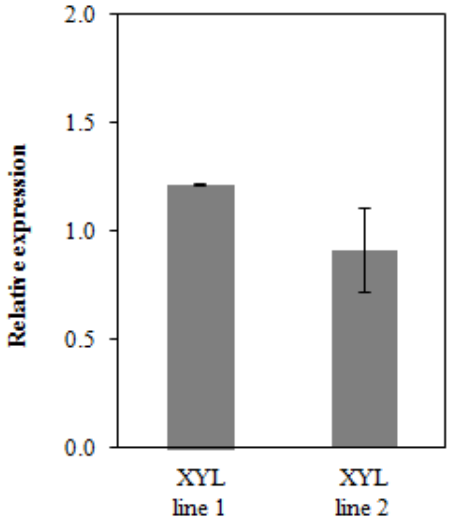

Fig. 8. Relative expression level of the *Xyl* gene in *A. thaliana* transgenic plants. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the *Arabidopsis* ubiquitin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen). Data represent the average ± mean standard error (SE, indicated by bars) of two qPCR experiments.

The transgenic *Arabidopsis* lines constitutively expressing the mutated Xyl were also characterized for the expression of PR1 and PDF1.2 defence genes. RT-qPCR analysis showed no difference in their expression level comparing transgenic and control lines (Fig. 9).

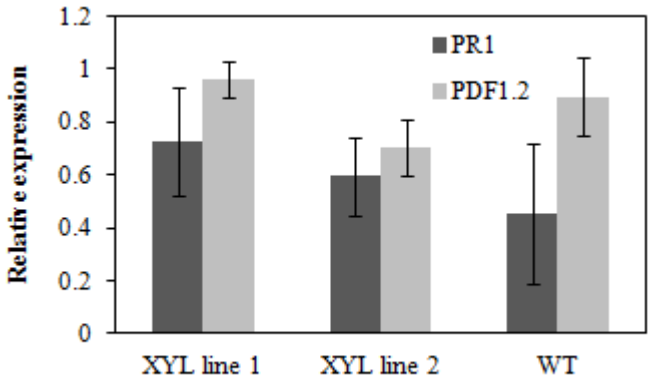

Fig. 9. Relative expression level of *A. thaliana* PR1 and PDF1.2 genes in leaves constitutively expressing the mutated XYL. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the ubiquitin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen, Italy). Data represent the average ± standard error (SE, indicated by bars) of the relative expression of at least 2 independent qPCR experiments.

Transgenic XYL lines were then characterized in infection experiments with *B. cinerea* and *P. syringae pv. maculicola*. When infected with the fungus, no significant reduction of symptoms was observed compared to WT plants (Table 2) and the same result was observed infecting with the bacterium (Table 3).

Lines	Infected area
XYL line 1	$0.23^a \pm 0.04$
WT	$0.23^a \pm 0.06$
XYL line 2	$0.23^a \pm 0.02$
WT	$0.30^a \pm 0.05$

Table 2. Lesion area produced by *B. cinerea* (strain B05.10) on *Arabidopsis* XYL transgenic lines at 1 dpi. Leaves were inoculated with disks (0.3 cm diameter) containing actively growing mycelium and lesion area was calculated with the graphic software "Fiji imageJ". Lesion areas are expressed in cm^2 ± standard error (SE) calculated from at least three independent infection experiments.

Lines	Infected area
XYL line 1	$0.69^a \pm 0.13$
WT	$0.62^a \pm 0.10$
XYL line 2	$0.52^a \pm 0.06$
WT	$0.67^a \pm 0.11$

Table 3. Lesion area produced by *P. maculicola* on *Arabidopsis* XYL transgenic lines at 3 dpi. Leaves were inoculated with *P. syringae pv. maculicola* and lesion area was calculated with the graphic software "Fiji imageJ". Lesion areas are expressed in cm^2 ± standard error (SE) calculated from at least three independent infection experiments.

Transient expression of wheat TAXIs in tobacco leaves by agroinfiltration

A. tumefaciens strains harboring the wheat *Taxi-I* and *Taxi-III* genes or pBI-GUS as control were infiltrated in tobacco leaves for transient gene expression. To verify the presence of the corresponding transcripts and quantify their expression levels, RT-qPCR was performed by using specific primers. TAXI-I and TAXI-III encoding genes showed a peak of expression two days after agroinfiltration, with about 3 folds increase for *Taxi-I* and 1.5 folds for *Taxi-III* compared to the tobacco actin gene used as housekeeping (Fig. 10).

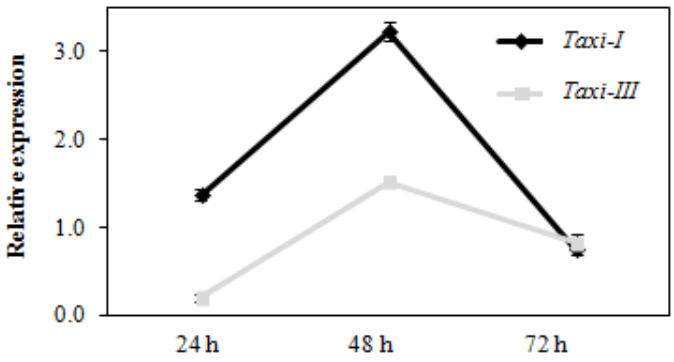

Fig. 10. Relative expression of *Taxi* transcripts in tobacco agroinfiltrated leaves at different timing post agroinfiltration. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Relative expression of TAXI-I and TAXI-III encoding genes was normalized with the tobacco actin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen). Data represent the average ± mean standard error of two independent experiments.

Total protein extracts from tobacco plants transiently expressing TAXIs were analyzed for their inhibition activity against the purified *F. graminearum* xylanase FGSG_03624 by radial gel diffusion assay. Compared to the halo produced by the xylanase (corresponding to 1.5 U of xylanase activity), TAXI-I inhibited by about 35% the xylanase activity while TAXI-III by about 20% (Fig. 11).

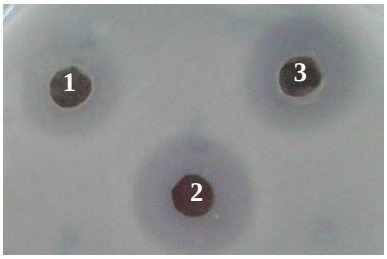

Fig. 11. Radial gel diffusion assay to quantify the xylanase activity. Halo produced by 1.5 U of the *F. graminearum* FGSG_03624 alone (2) or in presence of 30 µl of protein extract obtained from tobacco plants agro-infiltrated with TAXI-I (1) and TAXI-III (3).

Three days after agroinfiltration, tobacco plants transiently expressing TAXI-I and TAXI-III were tested in infection experiments with *B. cinerea*. At three dpi, tobacco leaves showed a significant symptoms reduction by about 20% for TAXI-I and 25% for TAXI-III compared to control leaves agroinfiltrated with the pBI-GUS plasmid (Fig. 12).

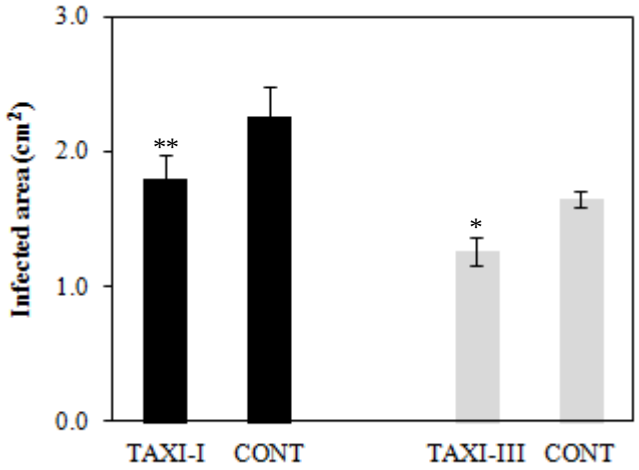

Fig. 12. Lesion area produced by *B. cinerea* (strain B05.10) on tobacco leaves expressing TAXI-I and TAXI-III at 3 dpi. Histograms show the lesions area (expressed in cm^2) calculated with the graphic software "Fiji ImageJ". Bars indicate the standard error (SE) calculated from three independent infection experiments. TAXI-I and TAXI-III leaves were statistically more resistant to *B. cinerea* infections than leaves transformed with the pBI-GUS plasmid (CONT) by applying Student's t test. ** indicates significant differences at $p < 0.01$, while * at $p < 0.05$.

Production of *Arabidopsis* transgenic plants expressing TAXI-I and TAXI-III

Seeds from *Arabidopsis* plants transformed by floral dip with *Agrobacterium* strains containing TAXI-I and TAXI-III encoding genes were selected by plating on Murashige and Skoog medium with kanamycin. Two TAXI-I and one TAXI-III putative transgenic lines showing antibiotic resistance were obtained. A PCR performed by using genomic DNA from the selected plants confirmed the successful transformation (Fig. 13, lanes 1, 2 and 4).

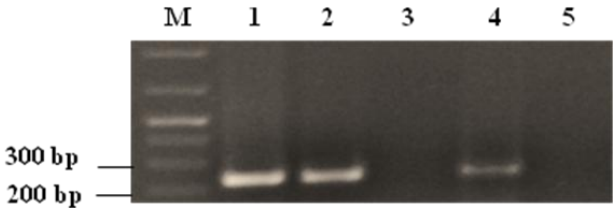

Fig. 13. PCR amplification performed using gene specific primers and total DNA of T0 plants of *A. thaliana* transformed with the Taxi constructs. Amplicons were separated on 1% (w/v) agarose gel. Lanes 1 and 2: two transgenic lines of Taxi-I; lane 4: a Taxi-III transgenic line. A negative control (one for each gene) was loaded on lanes 3 and 5. Lane M represent molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy).

The obtained transgenic lines were phenotypically identical to untransformed plants (not shown). The expression of the *Taxi-I* and *Taxi-III* genes was tested on T1 generation transgenic lines by RT-qPCR using *Arabidopsis* ubiquitin gene as housekeeping. The analysis confirmed the presence of both *Taxi* transcripts, with a peak of expression of about 1 fold for the *Taxi-I* gene and 3 folds for the *Taxi-III* gene (Fig. 14).

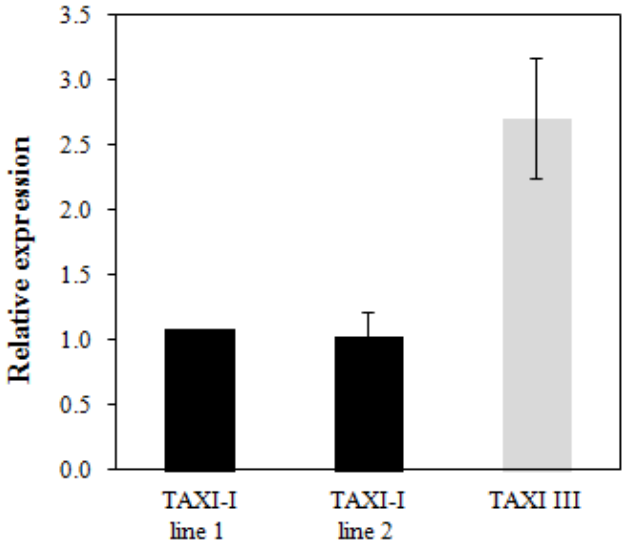

Fig. 14. Relative expression level of *Taxi-I* and *Taxi-III* genes in *A. thaliana* transgenic plants. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the *Arabidopsis* ubiquitin gene set to 1 and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen). Data represent the average ± mean standard error (SE, indicated by bars) of two qPCR experiments.

Total protein extracts from *Arabidopsis* plants constitutively expressing TAXIs were first analyzed for their inhibitory activity against the *F. graminearum* FGSG_03624 xylanase through radial gel diffusion assay. Compared to the halo produced by the xylanase (corresponding to 1.5 U), both TAXI-I and the TAXI-III lines almost completely inhibited the xylanase activity (Fig. 15).

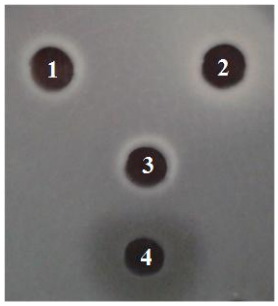

Fig. 15. Radial gel diffusion assay to quantify the *F. graminearum* xylanase inhibition by TAXIs. Experiment was performed by using 1.5 U of *F. graminearum* FGSG_03624 xylanase (4) and 30 µl of total protein extract obtained from *Arabidopsis* transgenic plants. 1 and 2: lines 1 and 2 of TAXI-I; 3: TAXI-III line 1.

Total protein extracts were also tested against the xylanase activity produced by *B. cinerea* in a liquid culture containing xylan as sole carbon source. Compared to the halo produced by the *B. cinerea* culture filtrate (corresponding to about 1 U), a slight reduction of the intensity of the halo was observed in presence of both TAXI-I and TAXI-III lines (Fig. 16).

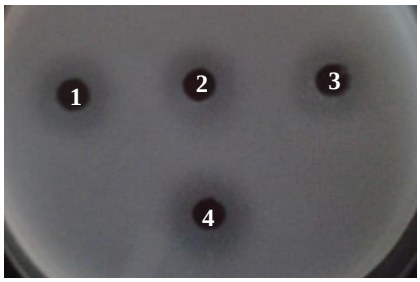

Fig. 16. Radial gel diffusion assay to quantify the *B. cinerea* xylanase inhibition by TAXIs. Experiment was performed by using 1 U of *B. cinerea* xylanase (4) and 30 µl of total protein extract obtained from *Arabidopsis* transgenic plants. 1 and 2: TAXI-I lines 1 and 2; 3: TAXI-III line 1.

Basal *Arabidopsis* leaves of the transgenic and WT plants were inoculated with agar disks colonized by actively growing *B. cinerea* mycelium. Only the TAXI-I line 2 was slightly but not significantly less susceptible compared to WT plants (Table 4), and no difference was observed for TAXI-I line 1 and TAXI-III lines.

Lines	Infected area
TAXI-I line 1	$0.21^{a} \pm 0.04$
WT	$0.22^{a} \pm 0.04$
TAXI-I line 2	$0.23^{a} \pm 0.03$
WT	$0.28^{a} \pm 0.04$
TAXI-III	$0.24^{a} \pm 0.05$
WT	$0.24^{a} \pm 0.04$

Table 4. Lesion area produced by *B. cinerea* (strain B05.10) on *Arabidopsis* TAXI transgenic lines at 1 dpi. Leaves were inoculated with disks (0.3 cm diameter) containing actively growing mycelium and lesion area was calculated with the graphic software "Fiji imageJ". Lesion areas are expressed in cm^2 ± standard error (SE) calculated from at least three independent infection experiments.

DISCUSSION

Xylanases are CWDE belonging to the family of hemicellulase and are secreted by pathogenic fungi during plant infection. Although some of these enzymes could play an important role for the success of the infection process, as shown for the Xyl11A xylanase of *B. cinerea* (Brito et al., 2006), some endo-β-1,4-xylanases have been shown to induce hypersensitive response in plants regardless of their enzymatic activity (Enkerli et al., 1999; Sella et al., 2013; Tundo et al., 2015) thus they could be potentially exploited to induce plant resistance to pathogens. To this aim, in this work we tested the ability of the *F. graminearum* FGSG_03624 xylanase to induce defence responses and resistance in *A. thaliana* and tobacco plants against the hemibiotrophic bacterium *P. syringae pv. maculicola* and the necrotrophic fungus *B. cinerea.*

Exogenous treatments of *A. thaliana* leaves with the *F. graminearum* xylanase strongly induced the expression of the PDF1.2 gene, especially at 96 hours after treatment, thus demonstrating that this protein mostly induces the jasmonate/ethylene pathway. *A. thaliana* leaves treated with the FGSG_03624 xylanase were therefore infected after 48 hours with *P. syringae pv. maculicola* and *B. cinerea*, but the treatment induced a partial resistance only against the bacterial infection. On the contrary, symptoms caused by *B. cinerea* were similar in treated and control leaves, also infecting the plant 96 hours post treatment, when PDF1.2 gene expression was strongly induced. Therefore, the jasmonate and ethylene pathway, that is generally considered to be involved in the response to necrotrophic pathogens, in this case seems ineffective in reducing the infection rate of *B. cinerea*. The lack of effect against this necrotrophic fungus could depend on the timing or the level of defense responses mediated by the jasmonate/ethylene signaling pathway, that might not be timely or adequate to counteract the speed of a fast colonizing fungus as *B. cinerea*.

As an alternative to spray, the FGSG_03624 xylanase was transiently expressed in the plant. This experiment was performed in tobacco plants that is a useful model for agroinfiltration experiments. To avoid possible undesirable effects to plant tissue due to the enzymatic activity of the xylanase, we performed a site-directed mutagenesis to produce a mutated xylanase. In fact, enzymatic activity of many fungal xylanases including the FGSG_03624 xylanase (Sella et al., 2013) is unnecessary for their eliciting activity. The transient relative expression of the mutated *Xyl* in tobacco leaves showed a stable level between two and four days post infiltration, thus tobacco leaves were infected with *B. cinerea* four days after agroinfiltration. Results did not show any effect in reducing *B. cinerea* symptoms, thus confirming what

previously observed with the exogenous treatments of *Arabidopsis*. Also *Arabidopsis* transgenic plants constitutively expressing the mutated xylanase did not show any significant difference in *B. cinerea* symptoms reduction. Besides, the transgenic plants were also as susceptible as the control to *P. syringae pv. maculicola*. The observation that the level of PR1 and PDF1.2 genes in the transgenic *Arabidopsis* was similar to that of control plants could indicate that defence responses are not induced in the transgenic plants.

Xylanase inhibitors (XI) like wheat TAXI-I and TAXI-III proteins are considered part of the plant defense mechanisms able to inhibit microbial xylanases and counteract pathogens infection as shown in durum wheat transgenic plants where the constitutive expression of the xylanase inhibitor TAXI-III delays FHB symptoms (Moscetti et al., 2013). Since *B. cinerea*, during the infection process, produces a xylanase (Xyn11A) necessary for full virulence (Brito et al., 2006) and wheat TAXI-I has been shown to be effective against this xylanase (Brutus et al., 2005), this xylanase inhibitor could be exploited to increase tobacco and *Arabidopsis* plants to *B. cinerea*. To verify this hypothesis, TAXI-I and TAXI-III were transiently and constitutively expressed in tobacco leaves and in transgenic *Arabidopsis* plants, respectively. In tobacco the transcript levels of TAXI-I and TAXI-III showed a peak of expression two days after agroinfiltration and the inhibitory activity was detected in leaf protein extracts. When infected with *B. cinerea*, TAXI expressing leaves showed a significant symptoms reduction compared to control leaves, suggesting an effective role of TAXIs in counteracting the *B. cinerea* xylanase. This result was not confirmed in *A. thaliana* constitutively expressing TAXI-I and TAXI-III, because no reduction of *B. cinerea* symptoms were observed even though the plant extract contained inhibitory activity. It is difficult to explain the lack of resistance to *B. cinerea* of the transgenic *Arabidopsis* plants compared to the positive effect displayed by TAXIs in agroinfiltrated tobacco. However, as above reported, *B. cinerea* is much more aggressive and fast in colonizing *Arabidopsis* leaves, which are more thin and fragile than tobacco leaves. These differences might account for the lack of resistance of the TAXI transgenic plants in comparison to the reduced virulence observed in TAXI agroinfiltrated tobacco leaves.

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CONCLUSIONS

Polygalacturonases (PGs) and xylanases are considered important factors of fungal pathogenesis, but only few studies established a significant contribution to virulence and little it is known about the combined effect of these enzymatic activities. To better define the role of PG and xylanases during the diseases caused by the fungal pathogen *F. graminearum*, we produced single and double gene disruption mutants of the *xyr1* and *pg1* genes, encoding the major regulator of xylanase genes expression and the main PG isoform of the fungus, respectively. Results showed that PG1 moderately contributes to fungal virulence against the soybean dicot plant but not towards the monocot wheat, whilst XYR1 was dispensable for infection on both these hosts. Instead, the combined disruption of *xyr1* and *pg1* genes, that determines a dramatic reduction of xylanase and PG activities in the mutant strains, reduced further the virulence of the pathogen on soybean and decreased significantly its pathogenicity on wheat. The synergistic effect of *F. graminearum* PG and xylanase activities was confirmed by demonstrating that purified xylanases increase the enzymatic activity of PG1 on wheat cell walls. These results suggest that *F. graminearum* PG and xylanases have an overlapping role, so that one of the two enzymatic activities may be sufficient to assist the infection process, and clearly demonstrate the importance of the cooperative action of two different type of CWDEs in the degradation of the plant cell wall polymers and the necessity of their combined activity for enhancing virulence. This likely happens because the two enzymatic activities together loosen more efficiently the plant cell wall allowing a faster advancement of fungal hyphae into the plant tissue.

The second part of my research was aimed at increasing plant resistance to pathogens' infection by using two different approaches. The first was a "pathogen's derived resistance" approach, exploiting a *F. graminearum* xylanase able to induce defense responses to provide resistance against *P. syringae* pv. *maculicola* in *Arabidopsis* and against *B. cinerea* in tobacco and *Arabidopsis*. The second approach for inducing resistance was to transiently express in tobacco and constitutively in *Arabidopsis* a wheat xylanase inhibitor (TAXI) that is able to inhibit a *B. cinerea* xylanase considered essential for the virulence of this fungus.

Different experiments were tried with the *F. graminearum* xylanase: a xylanase preparation was used to exogenously treat *Arabidopsis* leaves before inoculating the challenging pathogens; the xylanase gene was transiently expressed in tobacco or constitutively expressed in *Arabidopsis*. Results showed that the exogenous treatment induced the jasmonate/ethylene

pathway and was partially effective only against the hemibiotrophic bacterial pathogen but not against the necrotrophic fungus *B. cinerea*. The *Arabidopsis* transgenic plants constitutively expressing this protein did not show any enhancement of defense responses and, probably for this reason, they were as susceptible as the control also against the bacterial infection. The ineffectiveness of the xylanase in protecting the plant from *B. cinerea* infection was confirmed in tobacco transiently expressing the xylanase. It is likely that the timing and level of defense responses are not appropriate to contrast this fungus, that is much more aggressive and rapid in colonizing the host tissue than the bacterial pathogen.

The transient expression of TAXI in tobacco leaves increased slightly but significantly the resistance to *B. cinerea* infection, whilst the constitutive expression of this inhibitor in *Arabidopsis* did not show any positive effect against this fungus. However, it is worth noting that *Arabidopsis* leaves are more easily and quickly infected by *B. cinerea* than tobacco leaves, and this might explain the unchanged susceptibility of the transgenic *Arabidopsis* plants to this pathogen. Therefore, the effectiveness of a defense strategy based on exploiting a CWDE inhibitor may depend on the host tissue and/or on the aggressiveness of the pathogen in a specific plant tissue.

Overall, the results obtained in this PhD thesis suggest that significant improvement of plant resistance to pathogens could be pursued by expressing or overexpressing in transgenic plants a suitable combination of plant inhibitors capable to counteract the activity of different type of CWDEs produced by pathogens.

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