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**INVOLVEMENT AND CONTRIBUTION TO VIRULENCE OF ENDO- β -1,4-XILANASES OF
FUSARIUM GRAMINEARUM DURING HOST INFECTION**

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Declaration

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Index

Index	4
Riassunto	7
Summary	9
Introduction	11
Chapter I	16
Study of <i>Fusarium graminearum</i> endo-β-1,4-xylanase genes expression during wheat infection and necrotizing activity of the recombinant FGSG_03624 xylanase	16
1.1 Introduction	17
1.2 Materials and method	19
1.2.1 Fungal strains and culture conditions	19
1.2.2 Selection of <i>F. graminearum</i> endoxylanase genes and primer design for expression analysis.....	19
1.2.3 Cloning and expression of the FGSG_03624 xylanase in <i>P. pastoris</i>	20
1.2.4 SDS-Page analysis and xylanase assay	21
1.2.5 Purification of the recombinant xylanase	21
1.2.6 <i>In vivo</i> inoculation with the heterologous xylanase	22
1.2.7 Histo-cytochemistry of wheat tissue treated with FGSG_03624 xylanase	22
1.2.7 Nucleic acid extraction	23
1.2.8 Xylanase genes expression <i>in vitro</i> and <i>in planta</i>	23
1.2.9 Construction of the gene replacement vector and fungal transformation mediated gene disruption	24
1.2.10 Southern blot analysis.....	26
1.2.11 Growth and infection of wheat plants.....	26
1.3 Results	30
1.3.1 Sequence comparison and expression analysis of <i>Fusarium graminearum</i> xylanase genes	30
1.3.2 Heterologous expression of the FGSG_03624 xylanase in <i>P. pastoris</i>	34
1.3.3 Effects of treatments with the heterologous xylanase on wheat tissues.....	35
1.3.4 Targeted knock-out of the FGSG_03624 xylanase encoding gene.....	38

1.3.5 Characterization of the <i>FGSG_03624</i> knock-out mutant strains in culture and during wheat infection.....	40
1.4 Discussion.....	44
Chapter II	48
Gene disruption approach to investigate the role of <i>Fusarium graminearum</i> Xyr1 transcriptional regulator during host infection	48
2.1 Introduction.....	49
2.2 Materials and methods.....	52
2.2.1 Fungal cultures, media and growth condition.....	52
2.2.2 DNA manipulation and construction of the gene replacement vector.....	52
2.2.3 Fungal transformation-mediated gene disruption and preliminary screening of mutant lines.....	54
2.2.4 Southern blot analysis	55
2.2.5 Total xylanase activity assay	55
2.3 Results.....	57
2.3.1 Targeted disruption of <i>F. graminearum</i> Xyr1 encoding gene.....	57
2.3.2 <i>In vitro</i> characterization of the Fg Δ xyr mutants.....	60
2.4 Discussion.....	62
Conclusions and perspectives	64
References	66

Riassunto

Fusarium graminearum è un fungo filamentoso conosciuto come principale agente della fusariosi della spiga (FHB), una importante malattia che colpisce principalmente cereali da granella. Nel frumento il patogeno attacca la spiga in fase di fioritura, causando considerevoli perdite di raccolto e riducendo la qualità delle cariossidi a causa della contaminazione da micotossine tricoteceni, prodotte dal fungo durante il processo infettivo.

Diversi studi citologici hanno dimostrato una correlazione tra l'infezione della spiga e la produzione da parte di *F. graminearum* di enzimi degradativi della parete cellulare vegetale. I tessuti del frumento, come quelli di altre piante monocotiledoni commelinoidi, sono particolarmente ricchi in xilano, il quale può essere idrolizzato dalle endo- β -1,4-xilanasi fungine.

FGSG_03624 è uno dei geni codificanti endo-xilanasi più espressi da *F. graminearum* in spiga di frumento al terzo giorno di infezione, ed è stato espresso eterologamente nel lievito *Pichia pastoris*. La proteina ricombinante ottenuta (22.7 kDa) possedeva attività xilanasica e induceva morte cellulare e accumulo di perossido di idrogeno in tessuti di frumento quali foglie infiltrate con 10 ng/ μ l o glume trattate con 20 ng/ μ l. Questo effetto riflette quanto osservato per altre xilanasi fungine di *Trichoderma reesei*, *T. viride* and *Botrytis cinerea*, con cui *FGSG_03624* condivide uno stretch di aminoacidi riportato in letteratura come essenziale per l'elicitazione della risposta necrotica.

Sono stati ottenuti diversi mutanti con il gene *FGSG_03624* deletato, i quali mostravano circa il 40% di riduzione dell'attività xilanasica totale rispetto al ceppo wild type se cresciuti in una coltura contenente xilano come unica fonte di carbonio.

Anche se la xilanasi *FGSG_03624* è in grado di indurre sintomi simili a quelli osservati nella risposta ipersensibile in tessuti di monocotiledone, i mutanti ottenuti dalla delezione del gene corrispondente sembravano possedere completa virulenza quando inoculati in spighe di frumento tenero delle cultivar Nandu e Bobwhite, probabilmente a causa della ridondanza dei geni codificanti xilanasi. Il ruolo della xilanasi *FGSG_03624* durante l'infezione del frumento rimane quindi elusivo.

Per ottenere informazioni circa l'importanza dell'attività xilanasica prodotta da *F. graminearum* durante l'infezione della pianta ospite, il fattore trascrizionale Xyr1, che

regola putativamente l'espressione di diversi geni codificanti per enzimi xilanolitici, è stato deletato tramite ricombinazione omologa sito-specifica.

I mutanti derivati dalla delezione del gene *FGSG_17662*, codificante Xyr1, sono stati cresciuti in coltura liquida con xilano come unica fonte di carbonio e hanno mostrato una ridotta capacità di produrre biomassa ed una consistente diminuzione dell'attività xilanasica totale rilevata nel mezzo di coltura: in particolare, l'attività xilanasica prodotta dai mutanti era solo il 10% dell'attività secreta dal ceppo WT, e il peso secco dei mutanti risultava ridotto del 40-70%.

I mutanti di *F. graminearum* con gene *xyr1* inattivato sono ora disponibili per l'analisi trascrizionale *in vivo*, che consentirà di studiare la regolazione mediata da Xyr1 dell'espressione di geni codificanti enzimi xilanolitici durante l'infezione della pianta ospite.

I mutanti saranno inoltre caratterizzati per la loro virulenza mediante inoculo di spighe di *Triticum aestivum*. Se la virulenza di questi mutanti sarà dimostrata essere compromessa dalla delezione di *xyr1*, la sovrpressione di inibitori delle xilanasi in pianta potrà essere considerata come strumento per il controllo della fusariosi della spiga.

Summary

Fusarium graminearum is the fungal pathogen mainly responsible for Fusarium head blight (FHB) of cereal crops, which attacks wheat spikes, reducing crop production and quality of grain by producing trichothecene mycotoxins.

Several cytohistological studies showed that spike infection is associated with the production of cell wall degrading enzymes. Wheat tissue, as in other commelinoid monocot plants, is particularly rich in xylan which can be hydrolyzed by fungal endo- β -1,4-xylanase.

The FGSG_03624 is one of the most expressed xylanase genes in wheat spikes 3 days after inoculation and was heterologously expressed in the yeast *Pichia pastoris*. The recombinant protein (22.7 kDa) possessed xylanase activity and induced cell death and hydrogen peroxide accumulation in wheat leaves infiltrated with 10 ng/ μ l or in wheat glume surface treated with 20 ng/ μ l. This effect reflects that observed with other described fungal xylanases (from *Trichoderma reesei*, *T. viride* and *Botrytis cinerea*) with which the FGSG_03624 protein shares a stretch of amino acids reported as essential for elicitation of necrotic responses.

Several *F. graminearum* mutants with the FGSG_03624 gene disrupted were obtained, and showed about 40% reduction of total xylanase activity in comparison to the wild type when grown in culture with xylan as carbon source.

Even if the FGSG_03624 xylanase is able to induce hypersensitive-like symptoms on a monocot plant, FGSG_03624 gene deletion mutants were fully virulent on wheat cvs. Bobwhite and Nandu, probably because of xylanase gene redundancy. Therefore the role of this xylanase during wheat infection remains elusive.

To get insight about the importance of xylanase activities produced by the pathogen during infection of host plants, Xyr1, a transcriptional regulator factor putatively regulating the expression of several xylanase genes has been deleted by targeted homologous recombination.

Deleted mutants of the FGSG_17662 gene, encoding Xyr1, were heavily impaired both in total secreted xylanase activity and in fungal biomass formation when grown in liquid culture with xylan as sole carbon source. In comparison to WT, the fungal dry weight was reduced, by 40% to 70% and the total xylanase activity was reduced by about 90%.

F. graminearum Xyr1 deletion mutants are now available to be submitted to expression analysis *in vivo* to verify the Xyr1 mediated regulation of the expression of genes encoding for xylanolytic enzymes during plant infection.

The Xyr1 disrupted mutants will be characterized for their virulence by inoculating *Triticum aestivum* spikes. If these mutants will be verified as impaired in virulence, *in planta* overexpression of xylanase inhibitors may be considered as a new tool to control FHB.

Introduction

The filamentous Ascomycete *Fusarium graminearum* [teleomorph *Gibberella zeae* (Schwein.) Petch] is an ubiquitous plant pathogen (O'Donnell et al., 2004) with a wide range of hosts. The fungus is the most common responsible for Fusarium Head Blight (FHB), a serious plant disease which affects monocotyledonous plants of huge agronomical interest as well wheat, barley and other small grains leading to large economic losses (Goswami et al., 2004). However this pathogen can infect dicotyledonous plants as *Arabidopsis*, tobacco, tomato and soybean (Urban et al., 2002). FHB reduces production, caryopsis weight and germination, and affects composition in carbohydrates and proteins and quality of seeds, which result discolored, wilted and contaminated with trichothecene mycotoxins.

During wheat grain infection *F. graminearum* produces several mycotoxins, including deoxynivalenol (DON) and derivatives, zearalenone, fusarin C and aurofusarin. The limits of DON and zearalenone in food and feed are regulated by law in many countries (Trail, 2009). Zearalenone causes estrogenic effects in animals, including humans. The Deoxynevalenol, a potent protein biosynthesis inhibitor, affects the digestive system by decreasing intestinal barrier permeability, and the major organ function in animals and humans (Trail, 2009; Pinton et al., 2009). DON is the most important contaminant of wheat grain and has been shown to be a virulence factor of the fungus, during colonization of the spike (Proctor et al., 1995; Bai et al., 2001).

F. graminearum exhibits a hemibiotrophic behavior in the *Triticum aestivum* pathosystem (Brown et al., 2010). This fungus enters the host through natural openings (Pritsch et al., 2000) or penetrates the epidermal cell walls directly with short infection hyphae (Wanyoike et al., 2002) and complex infection structures, such as lobate appressoria and infection cushions (Boenisch et al., 2011). Following spikelet inoculation, the primary penetration sites of the fungus are mainly the ovary and the inner surfaces of the lemma and palea (Wanyoike et al., 2002). Once inside the tissue, the fungal hyphae grow inter- and intra-cellularly and spread systemically along the rachis.

In plant-microbe interactions, the plant cell wall plays several roles: first physical barrier to pathogen infection, source of nutrients for invaders, and origin of signaling molecules. The plant cell wall is composed of cellulose and matrix material. Cellulose, a β -1,4-glucan, is one of the most abundant polysaccharides in nature and constitutes the fibrillar component of the primary and secondary cell walls. The matrix material is composed of pectin, hemicelluloses and structural proteins (Carpita et al., 1993; Kikot et al., 2009). Pectin is the main component of the middle lamella and of the primary cell wall of dicotyledons and non-graminaceous monocotyledons, but is less abundant in the cell walls of grasses (Ridley et al., 2001; Vogel J., 2008). Hemicelluloses are a group of complex polymers abundant in primary cell walls and composed of different backbone sugars, such as xylose, glucose and mannose.

To overcome the plant cell wall, *F. graminearum*, as most fungal pathogens, secretes during the pathogenesis a wide array of cell wall-degrading enzymes with different substrate specificity, which facilitate the penetration of the pathogens into the host (Kang et al., 2000 a and b; Wanyoike et al., 2002). Cytological studies performed with enzyme-gold labeling techniques (Wanyoike et al., 2002) showed that during the early stages of wheat spike infection *F. graminearum* secretes xylanase, pectinase and cellulase activities, but little is known about the role actually played by these enzymes.

The role of cell wall degrading enzymes in fungal virulence has been examined by targeted gene disruption in dicot and monocot plant pathogens leading to controversial results. With regard to xylanolytic enzymes, the deletion of the gene encoding for the *Botrytis cinerea* xylanase Xyn11A caused a moderate reduction of growth on xylan medium but a pronounced effect on virulence (Brito et al., 2006). In contrast, the inactivation of individual xylanase encoding genes in the cereal pathogens *Cochliobolus carbonum*, *Magnaporthe grisea* and *Fusarium oxysporum* did not affect the fungal virulence (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gomez-Gomez et al., 2002; Wu et al., 2006). Besides, enzymes degrading hemicellulose are suspected to play an important role in the pathogenesis of the necrotrophic cereal pathogen *Mycosphaerella graminicola* (Douaier et al., 2007).

Fungal xylanolytic enzymes, and in particular endo- β -1,4-xylanases, may play an important role during plant infection not only by degrading the cell wall xylan but also by inducing

necrosis and activating defense responses in the host tissues independently from their enzymatic activity, as shown by the products of *Xyn11A*, *Xyn2* and *Eix* genes of *B. cinerea*, *Trichoderma reesei* and *Trichoderma viride*, respectively.

Recently, a putative transcriptional regulator of genes encoding for xylanolytic enzymes (Xyr1) has been identified in the *F. graminearum* genome (Brunner et al., 2007).

During my work of thesis I followed two main research lines. Firstly I characterized the activity and the role of the FGSG_03624 endo- β -1,4-xylanase during *Triticum aestivum* infection process. Secondly, I produced deletion mutants of the *F. graminearum* encoding for the transcriptional activator of xylanase genes Xyr1, which were characterized *in vitro* and are actually available for the future investigation about the involvement of xylanase activity during host plants colonization.

Chapter I

**Study of *Fusarium graminearum* endo- β -1,4-xylanase
genes expression during wheat infection and
necrotizing activity of the recombinant FGSG_03624
xylanase**

1.1 Introduction

Xylan is a hemicellulosic polysaccharide composed of monomers of D-xylose linked by β -1,4-bridges, more or less substituted by various groups (Collins et al., 2005; Hatsch et al., 2006). Xylan is particularly abundant in the primary cell walls of commelinoid monocot plants (Cooper et al., 1988; Vogel et al., 2008), and its complete hydrolysis requires the action of several enzymes, among which endo-1,4- β -D-xylanases (EC 3.2.1.8; further referred to as endoxylanase) randomly cleave the internal glycosidic β -1,4 bonds in the xylose backbone (Collins et al., 2005; Wong et al., 1988). Endoxylanases have been grouped in the glycoside hydrolase families 10 and 11 corresponding, respectively, to high and low molecular weight xylanases (Bailey et al., 1997; Collins et al., 2005; Wong et al., 1988). Several endoxylanase encoding genes have been identified in the genome of most phytopathogenic microorganisms (Wong et al., 1988). Many of these genes are translated and the corresponding enzymes are secreted in the infected plant tissues (Wu et al., 1997).

The contribution of endoxylanase to virulence has been established in the necrotrophic pathogen *Botrytis cinerea* where the *Xyn11A* gene is required to determine full virulence during the infection of tomato leaves and grape berries (Brito et al., 2006). Fungal endoxylanases could also induce necrosis and activate defense responses in plants independently from their enzymatic activity, as shown by the product of *Xyn2* and *Eix* genes of *Trichoderma reesei* and *T. viride*, respectively, which induce necrosis and defense responses in plants (Bailey et al., 1990; Enkerly et al., 1999; Fuchs et al., 1989; Furman-Matarasso et al., 1999). In tomato, a short amino acid stretch (TKLGE) in the EIX primary structure is essential for recognition by a LRR receptor-like protein (Ron et al., 2004) and for elicitation of defense responses (Rotblat et al., 2002). This short region is partially conserved in XYN11A of *B. cinerea* (TEIGS) and together with the successive conserved six amino acids (VTSDGS) is thought to be responsible of the necrotizing activity and virulence of this fungus (Noda et al., 2010).

Fusarium graminearum is thought to penetrate the host tissue through the activity of secreted cell wall degrading enzymes (CWDE) such as xylanases, pectinases and cellulases (Kang et al., 2000 a and b; Wanyoike et al., 2002); in fact, the fungus has been shown to secrete xylanase, pectinase and cellulose activities during the early stages of

wheat spike infection (Wanyoike et al., 2002), although little is known about the role actually played by these enzymes.

With regard to xylanolytic enzymes, *in silico* analysis of the genome of *F. graminearum* (<http://mips.helmholtz-muenchen.de/genre/proj/FGDB/> and http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/) allowed us to identify 10 genes putatively encoding endo- β -1,4-xylanases.

A transcriptomic analysis of *F. graminearum* performed in different growing conditions demonstrated that six endoxylanase encoding genes (*FGSG_03624*, *FGSG_11487*, *FGSG_06445*, *FGSG_10999*, *FGSG_11304* and *FGSG_11258*) were expressed when the fungus was grown on hop plant cell wall (Hatsch et al., 2006). These genes are also transcribed during infection of barley (Güldener et al., 2006) and, except for *FGSG_11258*, of wheat spikes (Lyøe et al., 2011). The results obtained in wheat are also consistent with a proteomic analysis (Paper et al., 2007).

Among the *F. graminearum* endoxylanase genes expressed during wheat infection, the *FGSG_03624* endoxylanase has been biochemically characterized following heterologous expression in *Escherichia coli* (named XylB) (Beliën et al., 2005; Pollet et al., 2009) or purification from cultures of *F. graminearum* grown on wheat bran (named Xyl2) (Dong et al., 2012). In a sequence comparison analysis, we observed that the *FGSG_03624* possessed a high level of similarity to the fungal xylanases XYN11A, EIX and XYN2 reported as necrosis-inducing factors in other plant–fungal interactions (Bailey et al., 1990; Enkerli et al., 1999; Fuchs et al., 1989; Furman-Matarasso et al., 1999; Noda et al., 2010). Since necrosis is a typical symptom of FHB, this observation prompted us to investigate the role of the *FGSG_03624* xylanase during wheat infection. Here we report the transcript accumulation of *FGSG_03624* compared to five other endoxylanase genes after spike inoculation with *F. graminearum*. Additionally, we tested the necrotizing capacity of *FGSG_03624* on wheat tissue, by using this protein produced heterologously in *Pichia pastoris*, and the virulence of *F. graminearum* mutant strains with the *FGSG_03624* gene disrupted.

1.2 Materials and method

1.2.1 Fungal strains and culture conditions

The *Fusarium graminearum* strain PH1 was cultured at 25 °C on complete medium (CM) (Leach et al; 1982) supplemented with 1.5% (w/v) granulated agar (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) or on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA).

Conidia were obtained by culturing *F. graminearum* WT and mutant strains in 50 ml of carboxymethyl cellulose (CMC) liquid medium (Cappellini et al; 1965) at 25 °C and 100 rpm.

Fungal growth was determined on a liquid culture (Szécsi et al; 1990) supplemented with 0.5% (w/v) larchwood xylan (Sigma-Aldrich, Milano, Italy) as the sole carbon source by inoculating 20 ml of medium with 500 conidia ml⁻¹. After 7 days at 25 °C on an orbital shaker at 100 rpm the flasks were transferred into 50 ml pre-weighed tubes and centrifuged at 8500 x g for 20 min. The mycelium was collected, washed twice with deionized water, oven dried at 80 °C for 3 d and weighed.

Xylanase activity was determined at 4 and 7 dpi on 50 ml cultures inoculated with 1x10⁴ conidia ml⁻¹ and grown at 25 °C in the xylan medium. Alternatively, 2.5 ml of the medium were inoculated with one agar plug (5 mm diameter) taken from the edge of actively growing colonies.

1.2.2 Selection of *F. graminearum* endoxylanase genes and primer design for expression analysis

Six endoxylanase encoding genes previously shown to be transcribed and/or secreted during wheat infection (MIPS database entries: *FGSG_03624*, *FGSG_11487*, *FGSG_06445*, *FGSG_10999*, *FGSG_11304*, *FGSG_11258*) and the β -tubulin gene (MIPS database entry *FGSG_06611*) as reference gene were selected.

Primers for cloning the *FGSG_03624* gene in *Pichia pastoris* and for the Real Time expression analysis *in vitro* and *in planta* were designed by using DNAMAN (Lynnon Biosoft) and PerlPrimer v.1.1.17 programs (Table 1).

1.2.3 Cloning and expression of the FGSG_03624 xylanase in *P. pastoris*

The cDNA of the entire coding sequence of the FGSG_03624 gene was obtained from total RNA extracted from infected wheat spikelets at 3 dpi by using the reverse specific primer 03624Rc (Table 1) and the ImPromII reverse transcriptase (Promega, Milano, Italy), following manufacturer's instructions. The gene was amplified from the obtained cDNA with the primers pair 03624Fc/03624Rc by using the "REDTaq ReadyMix PCR Reation Mix" (Sigma, Milano, Italy). The PCR was performed by repeating for 35 times the following cycle: 1 min at 94 °C; 30 sec at 55 °C; 1 min at 72 °C.

The amplification product of the expected size (686 bp) was purified using the "Wizard SV Gel and PCR Clean-Up System kit" (Promega, Milano, Italy) and was then cloned into the pGEM-T Easy vector (Promega, Milano, Italy) following manufacturer's instructions. The cloned cDNA was sequenced in order to check the correctness of the nucleotide gene sequence and then amplified with *Pfu* DNA polymerase (Promega, Milano, Italy) by using two specific primers (03624ecorIF/03624xbaIR, Table 1) containing adaptors for *EcoRI* and *XbaI* recognition sequences. The amplification was performed by repeating for 35 times the following cycle: 1 minute at 94 °C, 30 seconds at 55 °C, 1 minute at 72 °C. The PCR amplicon, purified as above reported, was ligated into the *EcoRI* and *XbaI* sites of the pPICZ α A expression vector and the ligation mixture was then used to transform *E. coli* competent cells, selected in Low Salt LB medium (Invitrogen Life Technologies, Milano, Italy) supplemented with 25 μ g/ml zeocin. The recombinant plasmid pPICZ α A/Fg03624 was extracted and purified from one PCR positive colony using the "Plasmid Maxi Kit Spin" protocol (EZNA), linearized with *PmeI*, precipitated with 2 volumes of absolute ethanol and 1/10 volume of sodium acetate 3 M pH 5.2 and the pellet was resuspended in 20 μ l of water. Five μ g of the linearized plasmid were added to 100 μ l of *P. pastoris* competent cells prepared following the "EasySelect™ *Pichia* expression kit manual" (Invitrogen Life Technologies, Milano, Italy). Electroporation was performed into a pre-cooled sterile cuvette (Electroporation Cuvettes Plus™, BTX Harvard Apparatus, USA) by an ECM® 630 Electro Cell Manipulator (BTX Harvard Apparatus, USA) applying 9.4 msec electric pulse at 1500 V and 400 Ω . Transformed cells were plated according to the

expression kit manual above reported. Some positive colonies were tested by PCR using the specific primers 03624-FOR and 03624-REV (Table 1) and the “REDTaq ReadyMix PCR Reaction Mix” (Sigma, Milano, Italy), repeating for 35 times the following cycle: 30 sec at 94 °C, 30 sec at 53 °C, 1 min at 72 °C. The colonies showing the expected 560 bp band were grown and induced with methanol according to the Invitrogen Life Technologies manual. After 96 h liquid cultures were centrifuged at 10,000g for 10 min and supernatants were assayed for xylanase activity and subjected to SDS-PAGE.

1.2.4 SDS-Page analysis and xylanase assay

Forty μ l of the *P. pastoris* culture medium were analyzed by SDS-PAGE according to Laemmli (Laemmli et al., 1970) by a Mini Protean II unit (Bio-Rad). The gel was stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich, Milano, Italy).

Xylanase activities of *F. graminearum* and *P. pastoris* cultures were determined by measuring the reducing sugars released from 0.5% (w/v) larchwood xylan (Sigma-Aldrich, Milano, Italy) dissolved in 50 mM sodium citrate buffer at pH 5 according to the dinitrosalicylic acid (DNSA) method described by Miller et al. (Miller et al; 1959) and modified by Bailey et al. (Bailey et al., 1992). D-xylose (Merck Chemicals) was used as a standard. Xylanase activity of *F. graminearum* was assayed by incubating 50 μ l of fungal culture in a 200 μ l reaction mixture, while xylanase activity secreted by *P. pastoris* was performed by incubating 100 μ l of culture filtrates in a 1 ml reaction mixture. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μ mol of xylose in 1 min under the assay conditions.

1.2.5 Purification of the recombinant xylanase

P. pastoris cultures were centrifuged at 10000xg for 5 min and the supernatant was filtered through GFA cellulose acetate membranes (Sartorius) of decreasing pore sizes (0.8, 0.45 and 0.2 μ m), concentrated using the Vivaflow 200 (10,000 MWC PES) system (Sartorius) and then diluted and concentrated three times against sodium acetate buffer 50 mM pH 5.0 to a final volume of 20 mL. The concentrated sample was purified by an AKTA system (GE Healthcare, Sweden) equipped with a cation exchange S-Sepharose

column (1.6 x 10 cm). Bound protein was eluted with a linear gradient of NaCl 0-0.5 M in 20 column volumes. The activity of the fractions were assayed for xylanase activity, as above reported. The protein concentration of the most active fractions was determined by A_{280} using BSA as a standard.

1.2.6 *In vivo* inoculation with the heterologous xylanase

Ten μ l of the culture supernatants of two selected *P. pastoris* transformed colonies producing xylanase (*Ppxyl5* and *Ppxyl6*) were pipetted into wheat (cv. Bobwhite) florets between palea and lemma at anthesis. After treatment the spikes were covered for 3 days with a plastic bag and plants maintained as described below for fungal infection. The development of symptoms was monitored from 3 to 7 days after inoculation and compared with those obtained by treating florets with 10 μ l of an untransformed *P. pastoris* liquid culture.

1.2.7 Histo-cytochemistry of wheat tissue treated with FGSG_03624 xylanase

The second or third emerging leaf from 3-weeks old plantlets were infiltrated with the purified FGSG_03624 xylanase diluted at 100 ng/ μ l or 10 ng/ μ l in phosphate buffer saline (PBS) 0.01 M pH 7.4, or with PBS alone as control, or with xylanase boiled for 20 or 30 min to destroy its enzymatic activity. The inactivation of the boiled FGSG_03624 was evaluated by using both the DNSA method and the Nelson-Somogyi assay (NSA) with copper and arsenomolybdate reagents, since the DNSA method has been recently shown to give 3- to 6-fold overestimations of xylanase activity compared to NSA (Gusakov et al., 2011).

Infiltration was carried out with 100 μ L of solution, using a needleless insulin syringe. After 24 h, infiltrated tissues were excised and some of them infiltrated overnight with 1 mg/L 3,3'-diaminobenzidine (DAB)-HCl to detect H_2O_2 while the others were stained with Evans blue to detect cell death, as detailed in Faoro and Iriti (Faoro et al., 2005). In some experiments also lemma tissues excised from spikelets were treated with the FGSG_03624 xylanase diluted at 20 ng/ μ l, by pipetting a 10 μ l drop onto the surface of

a detached lemma and incubating for 24 h in a humid chamber. Staining was performed with Evans blue as above reported for detecting dead cells.

All samples were examined with an Olympus BX50 light microscope (Olympus, Tokyo, Japan), equipped with differential interference contrast (DIC) and epi-polarization filters.

1.2.7 Nucleic acid extraction

Genomic DNA from *F. graminearum* WT and mutant strains was extracted as reported by Henrion et al. (Henrion et al., 1994) from 200-400 mg of mycelium recovered from 50 ml CM liquid culture inoculated with 5 mycelium plugs (5 mm diameter) and grown at 24 °C for 4 days at 150 rpm on a orbital shaker.

RNA was extracted from 100 mg of frozen infected wheat spikes or fungal mycelium collected after 4 days of liquid culture in a medium containing xylan as the sole carbon source by using the “RNeasy Plant Mini Kit” (Qiagen) following the manufacturer’s instructions. RNA was then treated with DNaseI (Promega, Milano, Italy) following manufacturer’s instructions and quantified both spectrophotometrically and by a denaturing gel.

1.2.8 Xylanase genes expression *in vitro* and *in planta*

Reverse transcription was performed by mixing 0.5 µg of an oligo-dT (15/18 thymine) reverse primer with about 1 µg of target RNA and by using the ImPromII reverse transcriptase (Promega, Milano, Italy), following manufacturer’s instructions.

The amplification of the xylanase and reference genes was performed by qPCR (Rotor-Gene Q 2plex, Qiagen GmbH) using specific primers (Table 1). The 20 µl reaction mixture contained 10 µl of 2X Brilliant III Ultra-Fast SYBR Green QPCR MasterMix (Agilent Technologies), 0.4 µM of each specific primer and 3 µl of cDNA as template.

The qPCR was performed by repeating 40 times the following cycle: 15 sec at 95 °C; 15 sec at 56 °C; 40 sec at 72 °C. Relative expression results were analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen GmbH). Two independent qPCR experiments were performed with different RNA preparations.

1.2.9 Construction of the gene replacement vector and fungal transformation mediated gene disruption

To generate the construct for disrupting the *F. graminearum* *FGSG_03624* gene, its flanking homologous regions, necessary for targeted homologous recombination, were amplified by PCR using *F. graminearum* genomic DNA as template. Specific oligonucleotides were designed to amplify the upstream (primers fg_03624-5UP-for and fg_03624-3UP-rev) and downstream (primers fg_03624-5DOWN-for and fg_03624-3DOWN-rev) flanking regions (Figure 1 and Table 1) of about 800 bp. The amplification was performed by using the “REDTaq ReadyMix PCR Reaction Mix” (Sigma, Milano, Italy) in a 50 µl volume. PCR conditions were as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 1 min. The amplicons obtained were purified and used in a second PCR to fuse the homologous flanking regions with the hygromycin resistance gene (hygromycin B phosphotransferase, *hyg*), used as selection marker (Punt et al; 1987). The fusion PCR reaction was performed with the “REDTaq ReadyMix PCR Reaction Mix” (Sigma, Milano, Italy) in a 25 µl volume using 200 ng of the purified flanking regions containing tails homologous to the 5’ and 3’ region of the *hyg* gene (Figure 1) and 400 ng of the *hyg* gene cut with *Sma* I (Fermentas, Milano, Italy) from pAN7-1 vector (Voigt et al; 2005). The fusion PCR conditions were as follows: 94 °C for 4 min, followed by 20 cycles of 94 °C for 1 min, 56 °C and 72 °C for 4 min. The fusion PCR product was then used as template in a nested PCR reaction with the primers Nested-For and Nested-Rev (Table 1 and Figure 1). Nested PCR conditions were as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 30 sec and 72 °C for 4 min. The amplicon obtained was cut from agarose gel, purified and then cloned into the pGEM-T easy vector (Promega, Milano, Italy) following the manufacturer’s instruction. An *E. coli* positively transformed colony was grown in LB-broth high salt culture (Fluka, BioChemika) and the plasmid DNA was extracted with the “MIDI Nucleobond® Ax” (Macherey-Nagel GmbH & Co.) kit following manufacturer’s instructions. The construct of 3328 bp was then cleaved from the pGEM-T vector by double digestion using *Bst*XI and *Sfi*I (Fermentas, Milano, Italy) and used to transform protoplasts of *F. graminearum* WT

strain. Protoplast formation and fungal transformation were performed according to Van Nguyen et al. (Nguyen et al., 2012).

Hygromycin-resistant colonies were collected and transferred to 30-mm CM plates supplemented with 250 µg/ml of hygromycin B (Duchefa Biochemie, Haarlem, The Netherlands). Resistant mutants were single-conidiated and preliminarily screened by PCR using the primer pair fg03624int-For and fg03624-int-Rev (Table 1 and Figure 1). Transformants without the *FGSG_03624* gene were then tested by Southern blot hybridization for single insertion of the disruption construct.

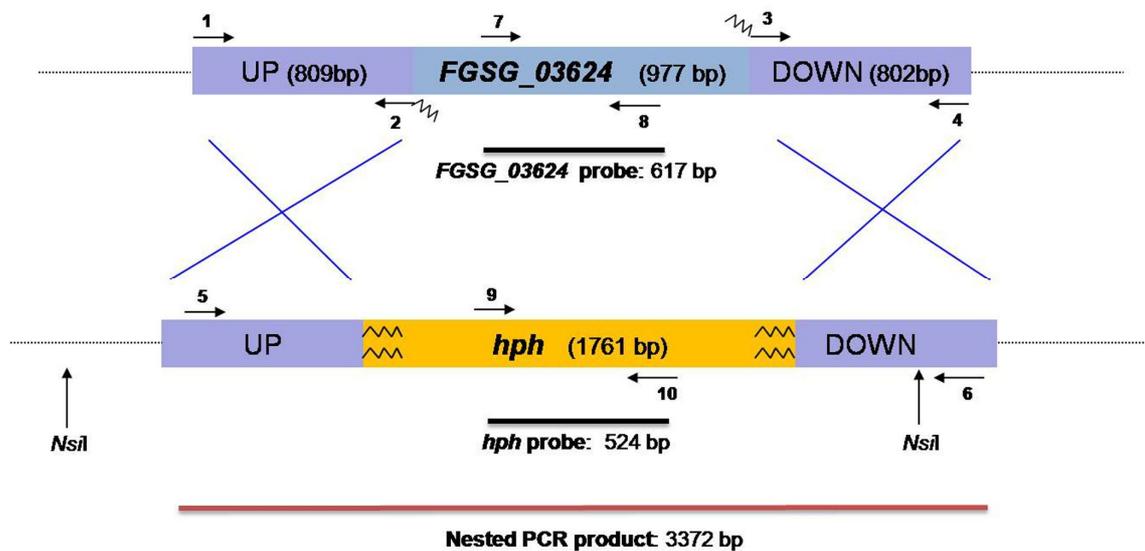


Figure 1 – Schematic illustration of the PCR-based construction of the gene replacement vector. Flanking homology regions of the *F. graminearum* *FGSG_03624* gene were amplified by PCR using specific primers for each gene: primers 1 (fg_03624-5UP-For) and 2 (fg_03624-3UP-Rev) were used for the amplification of the upstream region (UP), and primers 3 (fg_03624-5DOWN-For) and 4 (fg_03624-3DOWN-Rev) for the downstream region (DOWN). UP and DOWN amplicons were fused with the hygromycin resistance *hph* gene by the “Fusion PCR” technique, using as primers the tails (∩∩) of primers 2 and 3, complementary to the 5’ and 3’ *hph* regions, respectively. The fusion PCR product was used as template in a subsequent nested PCR reaction, where primers 5 (Nested-For) and 6 (Nested-Rev) were used to obtain the full construct of 3372 bp. The disruption of the target gene was obtained by homologous recombination via two crossing-over events. Primers pairs 7-8 (fg03624int-For and fg03624int-Rev) and 9-10 (Hyg-For

and Hyg-Rev) were used to obtain the *FGSG_03624* and *hph* probes for Southern blot analysis, respectively. Sites recognized by *Nsi*I, used for DNA digestion, are also indicated.

1.2.10 Southern blot analysis

Genomic DNA samples of 5-7 µg were digested with *Nsi*I (Fermentas, Milano, Italy), separated on a 1% (w/v) agarose/TBE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Italy). Digoxigenin (DIG)-labeled (Roche, Mannheim, Germany) specific probes were generated with primers specific for the *FGSG_03624* or *hyg* genes (Table 1 and Figure 1) by using genomic or plasmid DNA as template, and were used for overnight hybridization at 65 °C. The PCR reaction, performed in a 50 µl volume using DIG-11-dUTP (Roche, Mannheim, Germany), consisted of 5 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min.

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

1.2.11 Growth and infection of wheat plants

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 before transplanting in soil. Plants were grown in a climatic chamber with a 14 h photoperiod and a 19/17 °C day/night temperature for approximately 1 month, and with a 14 h photoperiod and 22/20 °C day/night temperature until anthesis for an additional month.

Wheat spikes were inoculated at anthesis (Zadocks stage 65-67) (Zadocks et al., 1974) with *F. graminearum* WT or mutant strains by dropping 10 µl of a fresh conidial suspension (approximately 2,000 conidia for cv. Bobwhite infections and 500 conidia for cv. Nandu infections) between the bracts of two florets of two opposite spikelets. After inoculation, spikes were covered for 3 dpi with a plastic bag to maintain a moist environment. Plants were then moved into a growth chamber with 85% relative humidity under a 16 h photoperiod and at a 22/20 °C day/night temperature.

For RNA extraction, wheat spikelets infected with the *F. graminearum* WT and mutant strains were collected 3 and 5 dpi. Symptom development on inoculated spikes was monitored up to 3 weeks post-inoculation. Data were statistically analyzed by applying the two-tailed Student's test.

Table1 – List of used primers.

Primer name	Sequence (5'-3')
Primers for cloning the <i>FGSG_03624</i> gene in <i>Pichia pastoris</i>	
03624Fc	ATGGTCTCCTTCACCTACCT
03624Rc	TTATCCAGAGACAGTCATGGT
03624ecorIF	ATGCAGAATTCGCTCCCAACCCTACCA
03624xbaIR	ATGCATCTAGATTATCCAGAGACAGTCATGGTA
FG03624-FOR	CCTACAACCTGGTGTCAACAAT
FG03624-REV	CAGAGACAGTCATGGTAGCC
Primers for qPCR expression analysis	
03624RTfor	GTCTCCTTCACCTACCTTCTC
03624RTrev	TCCATCCCTTACCACCGA
06445RTfor	GGTGGCTAATGGTAACTGGA
06445RTrev	AGGTTGTAGTCGTTGTAGTAGAG
10999RTfor	GTCTCGTTCAAATCCCTTCTC
10999RTrev	TTCCACCGACAAAGTTACCA
11258RTfor	GTTTCTGACAAGACACCTCC
11258RTrev	TTTGTGCCAAGCCAATCC
11304RTfor	CAAGTGGGTTTCTCAGGGT
11304RTrev	CAATGTCAAGCTCGGTAATGG
11487RTfor	GATTACAGCATTGACGACCC
11487RTrev	GCAACCTCCTTTACACCAG
BetatubF1	ACTTCCGTCTGTTCCGTG
BetatubR1	TTCCATCTCGTCCATACCCT
Primers to produce the disruption construct	
fg_03624-5UP-For	GAGAGCGGTCAGACTCTCAG
fg_03624-3UP-Rev	AGATGCCGACCGAACAAGAGCTGTCCCCCTTGGACTTGTGGCGATAAT
fg_03624-5DOWN-For	TCAATGCTACATCACCCACCTCGCTCCCCCAATAAAGACGGCTCAGTCCA
fg_03624-3DOWN-Rev	ACAGTTTCGTGCTTGCCTAC
Nested-For	GTGCGACCTTTCGGTTATTA
Nested-Rev	TACATGGAGATGGTGGTTCG
Primers for preliminary screening of ΔXyl mutants and for DIG-labeled probes	
fg03624int-For	CCTACAACCTGGTGTCAACAAT

fg03624int-Rev	CAGAGACAGTCATGGTAGCC
Hyg-For	CTCGATGAGCTGATGCTTTG
Hyg-Rev	GGACAGCTCTTGTTTCGGTCGG

1.3 Results

1.3.1 Sequence comparison and expression analysis of *Fusarium graminearum* xylanase genes

Sequence comparison between *F. graminearum* xylanases and the *B. cinerea* XYN11A, *T. reesei* XYN2 and *T. viride* EIX xylanases (known as necrosis-inducing factors) showed that FGSG_03624 possessed the highest sequence similarity (Figure 2) and seven of 11 conserved amino acids regarded as essential for eliciting necrosis in plant tissues (Noda et al., 2010).

Relative expression level of *FGSG_03624* was determined by qPCR in comparison with five other selected xylanase genes both *in vitro* and during *F. graminearum* wheat spike infection.

After 4 days of culture in a liquid medium containing xylan as sole carbon source, the most expressed gene was *FGSG_10999*; in particular, this gene was about 2.6 folds more expressed than the reference gene *β -tubulin* (set to 1) (Figure 3). The other xylanase genes were expressed at levels comprised between 0.1 and 0.5 folds that of the *β -tubulin* (Figure 3).

At 3 days post inoculation (dpi), *FGSG_03624* showed the strongest expression together with *FGSG_10999*. The transcript level of these two genes was similar to that of the *β -tubulin* reference gene (set to 1), while those of the other four genes ranged between 0.2-fold (*FGSG_11487*, *FGSG_11304* and *FGSG_11258*) and 0.4-fold (*FGSG_06445*) (Figure 4A). At 5 dpi, transcript accumulation declined for all xylanase genes except *FGSG_11487*. However, *FGSG_03624* was still the most expressed one (0.3-fold) together with *FGSG_11487*, which showed steady expression (Figure 4B).

FGSG_03624	MVSFTYLLAAVSAVTGAVAAPNPTKVDAQPPSGLLEKRIS	40
BcXYN11A	MVSASSLLLAASAIAGVFSAPAAAPVSENLNVLQERALTS	40
TrXYN2	MVSFTSLLAGVAAISGVLAAPAAEVEPVAVEKRQTIQPGT	40
TvEIX	MVSFTTLLAGFVAVTGVL SAPTETVEVVDVEKRQTIIGPGT	40
FGSG_03624	PTTGVNNGFYFSFWIDTFSAVTYTNGNGGQFSMNWNGNRG	80
BcXYN11A	SATGTSGGYYSFWIDGSGGVTYSNANGQYAVSWTGNKG	80
TrXYN2	GY...NNGYFHSYWNDDGHGGVTYTNGPGGQFSVNWSNSGN	77
TvEIX	GF...NNGYYSYWNDDGHSV TYTNGAGGSFSVNWANSNGN	77
FGSG_03624	NHVGKKGWNPGAARTIKYSGDYRPNNGNSYLA VYGWTRNPL	120
BcXYN11A	NFVGKKGWAVGSERSISYTGSKPNNGNSYLSVYGWTT SPL	120
TrXYN2	FVGKKGWQPGTKNKVINFGSYNPNNGNSYLSVYGWSRNPL	117
TvEIX	FVGKKGWNP GSSSRVINFGSYNPNNGNSYLSVYGWSKNPL	117
FGSG_03624	VEYYIVENFGTYNPSSGAQKKGEINIDGSIYDIAVSTRNC	160
BcXYN11A	IEYYIVEDFGTYDPSSAATEIGSVTSDGSTYKILETTRIN	160
TrXYN2	IEYYIVGNFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVN	157
TvEIX	IEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVN	157
FGSG_03624	APSIIEGDKTFQYQYWSVRRNKRSSGSVNTGAHFNAWAQAG	200
BcXYN11A	QPSVQGTATFKQYWSVRTSKRTSGIVTTANHF AAWKKLGL	200
TrXYN2	QPSIIIGTATFY.QYWSVRRNHRSSGSVNTANHFNAWAQQG	196
TvEIX	QPSIIIGTATFY.QYWSVRRNHAPAARSRLRTTSNAWRNLG	196
FGSG_03624	LRLGSHDYQILAVEGYQSSGQATMTV	226
BcXYN11A	TLGSTYNYQIVAVEGYQSSGSASITV	226
TrXYN2	LTLGTMDYQIVAVEGYFSSGSASITV	222
TvEIX	LTLGTLDYQIIAVEGYFSSGNANINV	222

Figure 2. –Amino acidic alignment of the sequence deduced from the *F. graminearum* FGSG_03624 gene with the sequences of *B. cinerea* XYN11A (Noda et al., 2010), *T. reesei* XYN2 (Enkerli et al., 1999) and *T. viride* EIX (Furman-Matarasso et al., 1999) endoxylanases. Red box indicates the amino acid region putatively involved in the induction of necrosis (Noda et al., 2010). Green, blue and yellow backgrounds indicate respectively 100%, 75% and 50% sequence similarity.

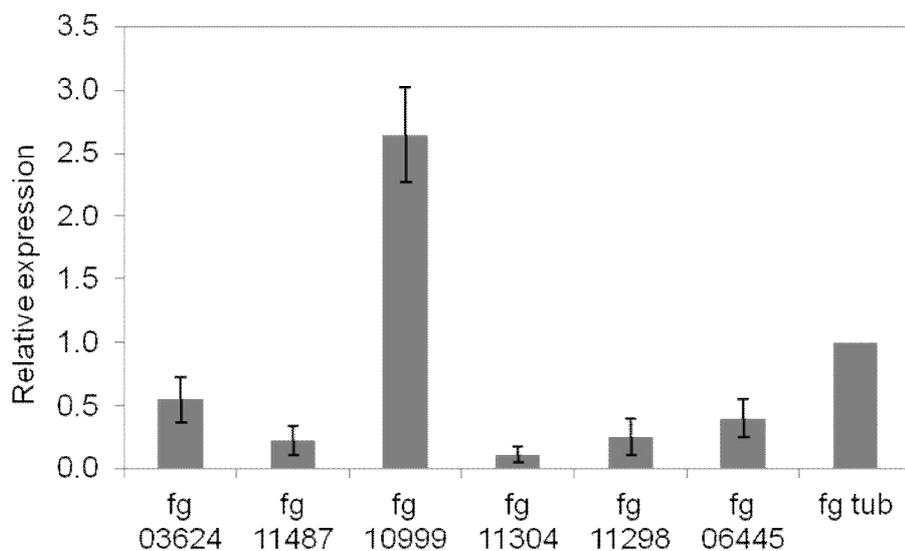


Figure 3 – Relative expression level of six selected *F. graminearum* xylanase genes after 4 days of liquid culture in a medium containing xylan as the sole carbon source. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the *F. graminearum* β -tubulin gene as reference (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 \pm mean standard error (indicated by bars) of the relative expression of two independent qPCR experiments.

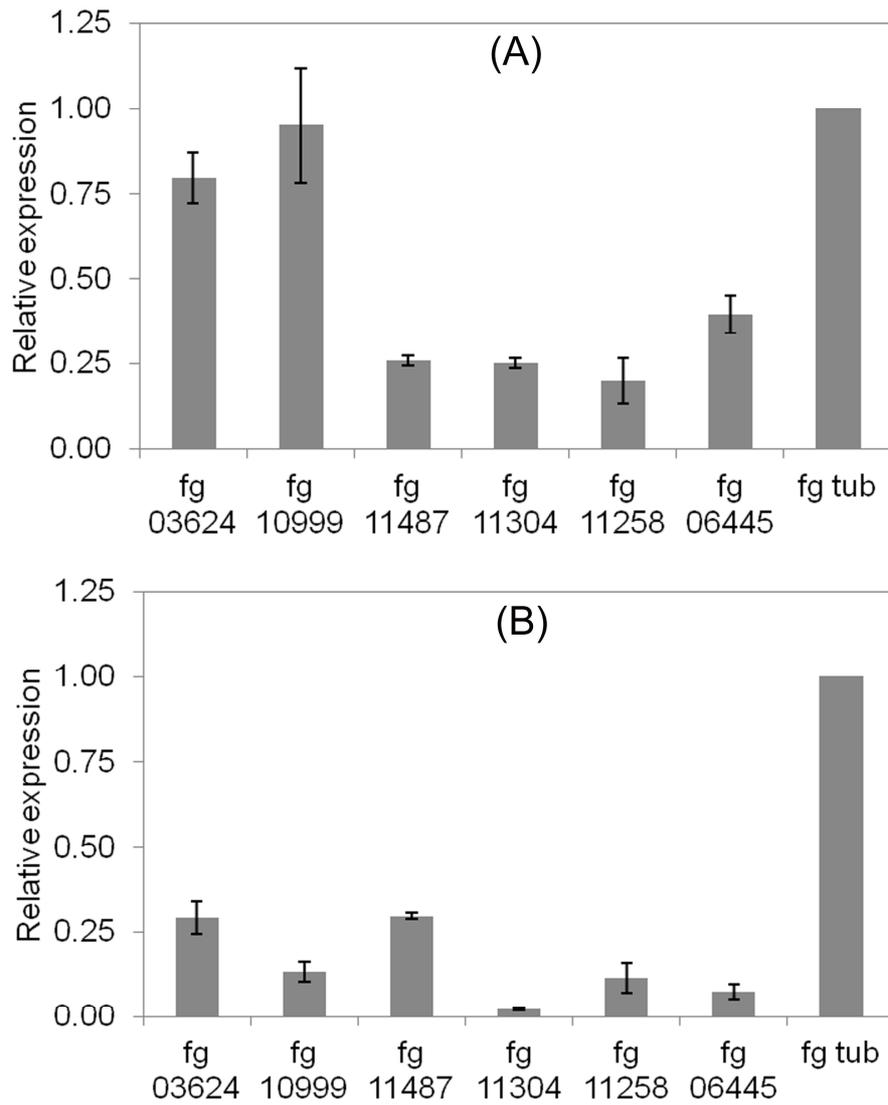


Figure 4 – Relative expression level of six selected *F. graminearum* xylanase genes during wheat spike infection. qPCR was performed with Rotor-Gene Q 2plex (Qiagen GmbH). Each transcript was normalized with the *F. graminearum* β -tubulin gene as reference (set to 1) and the relative expression was analyzed by using the Rotor-Gene 2.0.3.2 Software version (Qiagen). Data represent the average \pm mean standard error (indicated by bars) of the relative expression of two independent qPCR experiments. Relative expression at (A) 3 dpi and (B) 5 dpi.

1.3.2 Heterologous expression of the *FGSG_03624* xylanase in *P. pastoris*

The mature coding region of the *FGSG_03624* xylanase gene was cloned into the pPICZ α A vector to obtain the pPICZ α A/*Fg03624* plasmid which was used to transform *P. pastoris* cells. Twenty transformed colonies were obtained, and 7 of them were tested by PCR for the integration of the construct into the genome of *P. pastoris*: the expected 560-bp amplicon was present in all selected colonies (data not shown). The presence of the recombinant protein in the secretome of *P. pastoris* was verified by subjecting to SDS-PAGE analysis aliquots of culture broths of four colonies (Ppxyl1, Ppxyl2, Ppxyl5 and Ppxyl6) grown for 96 h in presence of methanol. A band of the expected size corresponding to a protein with a molecular mass of approximately 22.7 kDa was particularly expressed by the Ppxyl5 and Ppxyl6 colonies (Figure 5). There was a second faint band of about 25 kDa, possibly corresponding to a glycosylated form of *FGSG_03624*, which possesses four putative O-glycosylation and two N-glycosylation sites in its sequence (predicted by NetNGlyc 1.0 and NetOGlyc 3.1 programs, available online at <http://www.cbs.dtu.dk/services/>).

Ppxyl5 and Ppxyl6 cultures showed high xylanase activities (data not shown) and the culture filtrate of Ppxyl6 was used to purify the recombinant enzyme by cation exchange chromatography (S-sepharose). Bound protein eluted in a single peak with xylanase activity (data not shown).

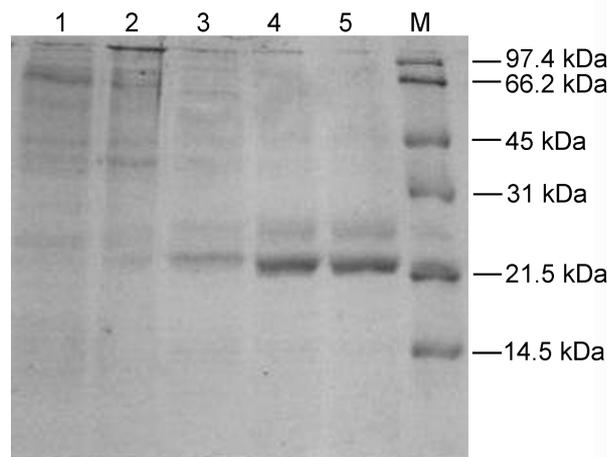


Figure 5. – SDS-PAGE analysis of the secretome of a WT (lane 1) and four *P. pastoris* colonies (Ppxyl1, Ppxyl2, Ppxyl5 and Ppxyl6, lanes 2–5, respectively) transformed to express the *F. graminearum* FGSG_03624 xylanase. Forty μ l of *P. pastoris* cultures induced with methanol for 96 h were loaded on a 12% polyacrylamide gel then stained with Coomassie Brilliant Blue R250. M: molecular weight standards (Low Range; Bio-Rad Laboratories); molecular masses are shown on the right.

1.3.3 Effects of treatments with the heterologous xylanase on wheat tissues

An aliquot of Ppxyl5 and Ppxyl6 cultures, containing the xylanase of interest, was used to perform a preliminary treatment of wheat spikelets (*Triticum aestivum* cv. Bobwhite). After 5–7 dpi, lemmas of spikelets treated with culture supernatants showed necrotic symptoms, while no symptoms appeared on spikelets treated with the culture supernatant of an untransformed *P. pastoris* colony (data not shown).

To investigate the possible necrotizing activity of FGSG_03624, both wheat leaves and spikes (cv. Bobwhite) were treated with the purified xylanase. Infiltration of young wheat leaves with a solution containing 100 ng/ μ l of purified FGSG_03624 caused the formation of chlorotic lesions due to extensive death of mesophyll cells within 24 h, as revealed by Evans blue staining (Figure 6A). DAB staining showed the elicitation of large hydrogen peroxide (H_2O_2) deposits in treated tissues (Figure 6B). Since the above xylanase concentration was so destructive, infiltration experiments were carried out with a more diluted enzyme (10 ng/ μ l), obtaining similar results both in terms of cell death

and H₂O₂ induction (Figure 7A and B). To verify whether the induction of H₂O₂ and cell death were related to enzymatic activity, leaves were infiltrated with FGSG_03624 (10 ng/μl) boiled for 20 min. The boiled enzyme produced microscopic symptoms similar to those obtained with non-boiled xylanase, with numerous dead mesophyll cells corresponding with large H₂O₂ deposits (Fig. 7C and D). However, neither macroscopic nor microscopic alterations were present in leaves infiltrated with buffer only (Fig. 7E and F). Since a residual minimal enzymatic activity (about 10% of the initial activity) was measured after boiling the xylanase for 20 min, we tested the retained capability of inducing cell death also in spikelets, which are the preferred penetration sites of *F. graminearum* (Yang et al; 2012), by boiling the FGSG_03624 xylanase for 30 min; after this treatment no residual enzymatic activity was detected. In this case a 10-μl drop of xylanase either boiled or not and diluted to 20 ng/μl was laid between lemma and palea for 24 h. Evans blue staining of lemma showed extensive cell death in both cases (Fig. 8A and B), while control spikelets treated with buffer appeared unaltered (Fig. 8C). Interestingly, and as in infiltrated leaves, dead cells were those of the tissues underneath the epidermis, both in boiled and non-boiled xylanase treatments.

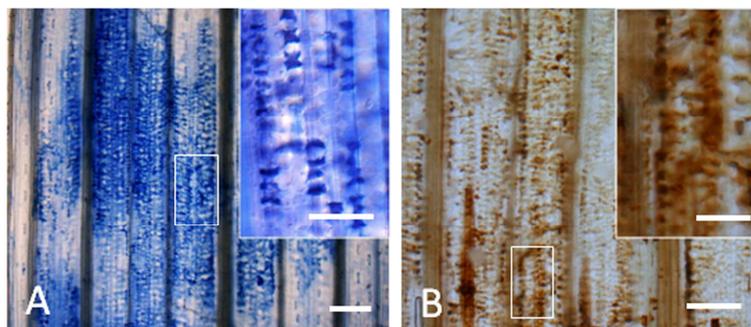


Figure 6 – Wheat leaf tissues infiltrated with 100 ng/μl of FGSG_03624 xylanase and stained after 24 h with Evans blue (**A**) or DAB (**B**); insets are enlargements of the corresponding framed areas. (**A**) The mesophyll tissue, but not the epidermis, shows numerous dead cells (stained blue) in the infiltrated area; the intensity of staining indicates different degrees of cell membrane degradation and/or different stages of cell death. (**B**) Extensive H₂O₂ deposits revealed by brownish DAB staining are co-localized with dead and neighboring cells shown in (**A**); all bars = 200 μm; bars in the insets = 100 μm.

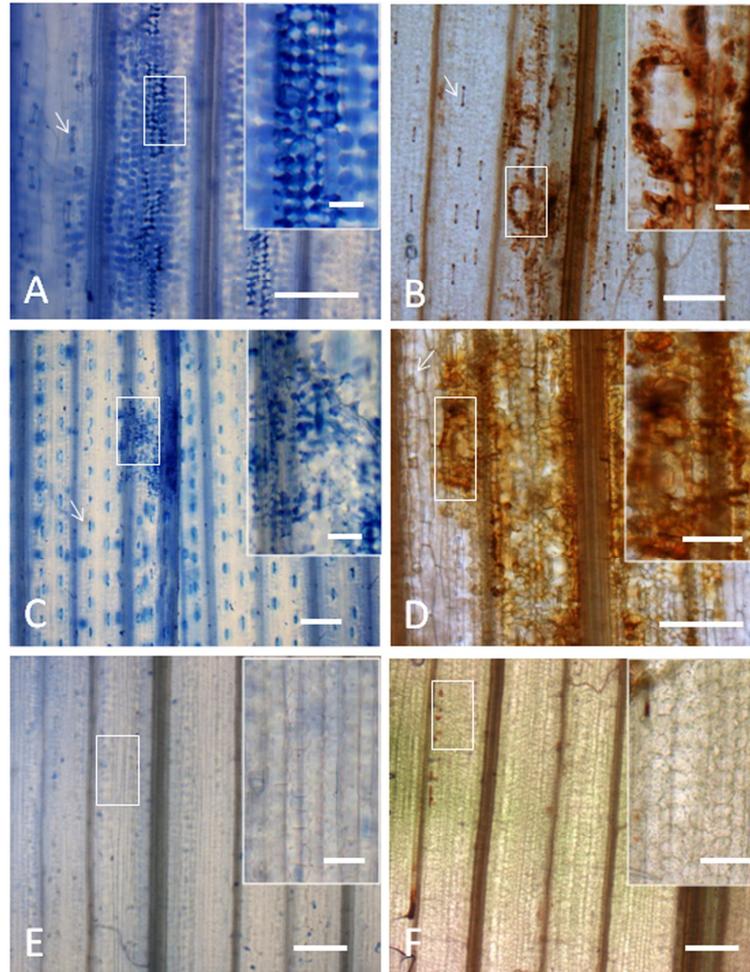


Figure 7 – Wheat leaf tissues infiltrated with 10 ng/μl of FGSG_03624 xylanase (**A, B**) or with the same amount of xylanase boiled for 20 min (**C, D**), or with buffer as control (**E, F**) and stained after 24 h with Evans blue (**A, C, E**) or DAB (**B, D, F**); insets are enlargements of the corresponding framed areas; arrows indicate stomata. Numerous dead cells (stained blue) are present in the mesophyll tissue infiltrated with both non boiled (**A**) and boiled (**C**) xylanase, but not in the tissue infiltrated with buffer only (**E**); the intensity of staining indicates different degrees of cell membrane degradation and/or different stages of cell death; extensive H₂O₂ deposits, revealed by brownish DAB staining are co-localized with dead and neighboring cells in both cases (**B, D**), but not in buffer infiltrated tissue (**F**); all bars = 200 μm; bars in the insets = 50 μm.

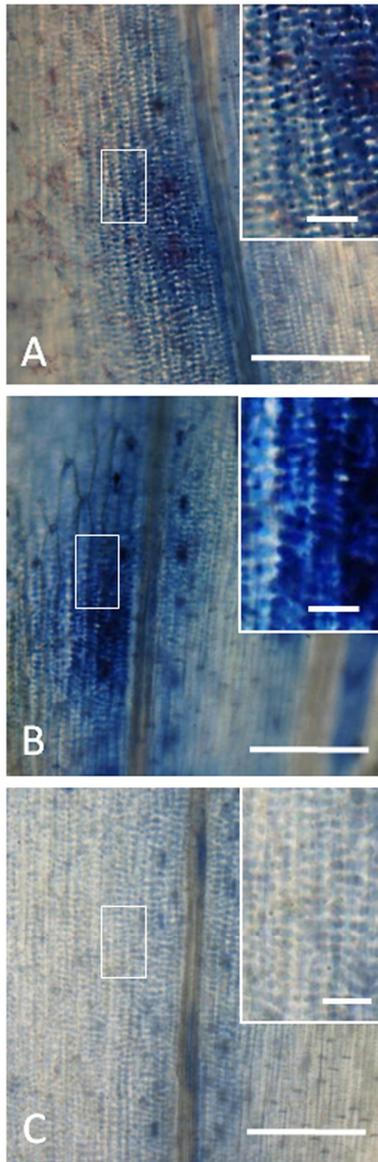


Figure 8 – Glume tissues of wheat spikelets treated with 20 ng/ μ l of FGSG_03624 xylanase (**A**) or with the same amount of xylanase boiled for 30 min (**B**) or with buffer as control (**C**) and stained after 24 h with Evans blue. All mesophyll cells under the areas treated with both non-boiled (**A**) and boiled (**B**) xylanase are dead (stained in blue), while those treated with buffer only appear unaltered (**C**); all bars = 500 μ m; bars in the insets = 50 μ m.

1.3.4 Targeted knock-out of the *FGSG_03624* xylanase encoding gene

The *F. graminearum* wild type (WT) protoplasts were transformed with a construct containing the hygromycin resistance gene in order to replace the *FGSG_03624* gene. Among the 30 hygromycin resistant regenerated colonies, 24 transformants were single-conidiated and checked by PCR for the presence/absence of the 617-bp internal fragment of the *FGSG_03624* gene. Three PCR negative mutants (Δ Xyl5.1, Δ Xyl11 and Δ Xyl5.2, Figure 9) were further analyzed by high-stringency Southern blot using the *FGSG_03624* and *Hyg* specific probes.

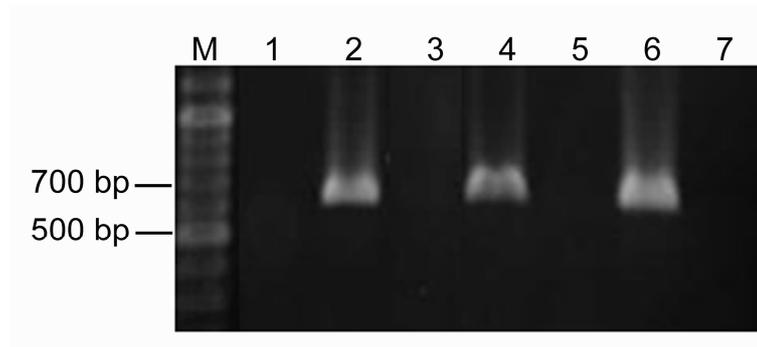


Figure 9 – PCR selection of *F. graminearum* *FGSG_03624* gene disruption mutants. Transformed fungal colonies resistant to hygromycin were screened by PCR using the primer pair fg03624int-For and fg03624-int-Rev. The 617 bp internal fragment of the *FGSG_03624* gene was amplified in WT (lane 6), Δ Xyl6 (lane 2) and Δ Xyl20 (lane 4) strains, but not in the mutant strains Δ Xyl5.1 (lane 1), Δ Xyl11 (lane 3) and Δ Xyl5.2 (lane 5). A negative control was loaded on lane 7. M: molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy) are shown on the left.

Hybridization results showed a single homologous integration of the hygromycin resistance gene and the disruption of the gene of interest (Figure 10A and B).

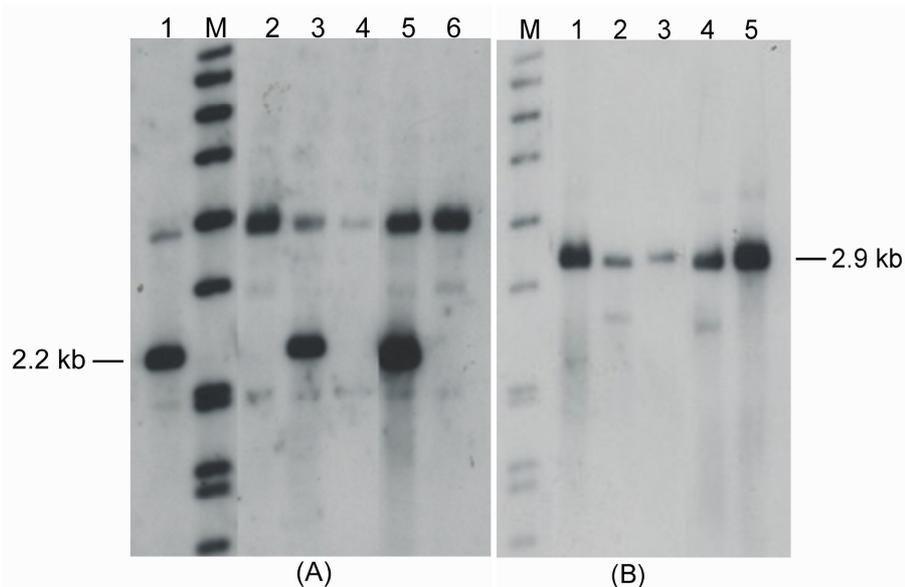


Figure 10 – High-stringency Southern blot analysis of genomic DNA from *F. graminearum* WT and mutant strains digested with *Nsi*I (Promega, Milano, Italy). **(A)** A fragment of the *FGSG_03624* gene was used as specific probe. The WT strain (lane 1) and the Δ Xyl6 (lane 3) and Δ Xyl20 (lane 5) mutant strains showed an hybridization signal of 2.2 kb corresponding to the *FGSG_03624* gene, while the Δ Xyl5.1 (lane 2), Δ Xyl11 (lane 4) and Δ Xyl5.2 (lane 6) mutant strains did not show this hybridization signal. A second hybridization signal of about 3.6 kb was also evident in all the WT and mutant strains; this band was due to the cross-hybridization of the *FGSG_03624* probe with the *FGSG_10999* xylanase gene, which shows 70% nucleotide similarity with the *FGSG_03624* probe. **(B)** A fragment of the *Hyg* resistance gene was used as probe. All the Δ Xyl mutant strains (Δ Xyl5.1, Δ Xyl6, Δ Xyl11, Δ Xyl20 and Δ Xyl5.2, lanes 1-5, respectively) showed a single hybridization signal at 2.9 kb; the WT strain gave no hybridization signal (data not shown). M: molecular size marker (Dig Marker VII, Fermentas, Milano, Italy).

1.3.5 Characterization of the *FGSG_03624* knock-out mutant strains in culture and during wheat infection

Total xylanase activity secreted by the Δ Xyl5.1, Δ Xyl11 and Δ Xyl5.2 mutant strains in Szécsi liquid culture containing xylan as sole carbon source was measured at 4 and 7 dpi. At 4 dpi the xylanase activity of all three mutant strains was significantly reduced by about 40% compared to WT (Figure 11), without significant differences among the mutant strains. This difference was confirmed at 7 dpi (data not shown).

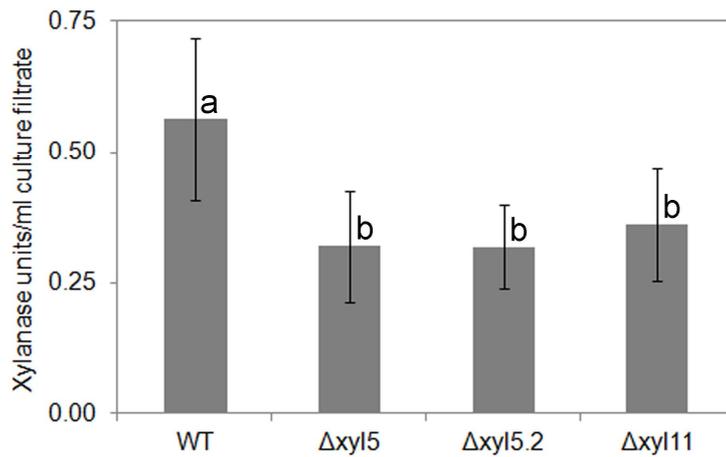


Figure 11 – Total xylanase activity secreted by WT and Δ Xyl mutant strains. Fifty μ l of culture filtrates, collected after 4 d of liquid culture in Szécsi medium with xylan as carbon source, were incubated in 1 ml of reaction mixture containing 0.5% (w/v) larchwood xylan. Xylanase activity, measured with the DNSA method, was expressed as xylanase units/ml of culture filtrate. One xylanase unit was defined as the amount of enzyme required to release 1 μ mol of xylose in 1 min under the assay conditions. Data represent the average \pm mean standard error (indicated by bars) of four independent experiments. Treatments were statistically different ($p < 0.05$, $F = 5.655$) by applying randomized complete blocks ANOVA. Different letters (a, b) indicate significant differences at $p < 0.05$ (Student–Newman–Keuls test).

The dry weight of WT and of the three mutant strains show no significant differences after 7 d of growth in a liquid culture containing xylan as sole carbon source (Figure 12).

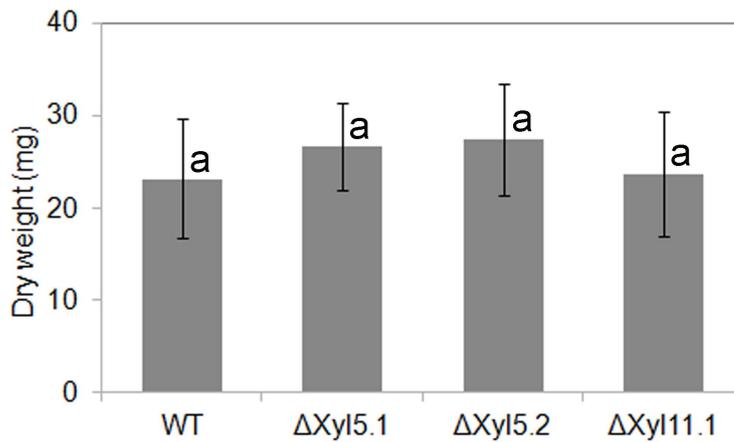


Figure 12 – Dry weight of WT and *FGSG_03624* knock-out mutant strains grown for 7 days in a liquid culture containing xylan as the sole carbon source. 1×10^4 conidia ml^{-1} of *F. graminearum* WT and mutant strains were grown in 20 ml of Szècsi liquid medium supplemented with 0.5% (w/v) larchwood xylan at 100 rpm and 25 °C. After 7 days mycelia were collected, filtered, washed twice with sterile water, oven dried at 80 °C for 3 days and then weighed. Dry weights are expressed in milligrams (mg). Data represent the average \pm standard deviation (indicated by bars) of three flasks per strain and were not statistically different by applying the completely randomized ANOVA and using the Student-Newman-Keuls test at $p < 0.05$.

Infection experiments on wheat spikes (*T. aestivum* cvs. Nandu and Bobwhite) were performed at anthesis with a spore suspension of the three mutant and WT strains. Spikelets were point-inoculated and symptoms were assessed 21 dpi. All three knock-out mutants maintained the capability to infect wheat spikes and there were no significant reductions in symptoms between WT and mutants strains (Fig. 13A and B).

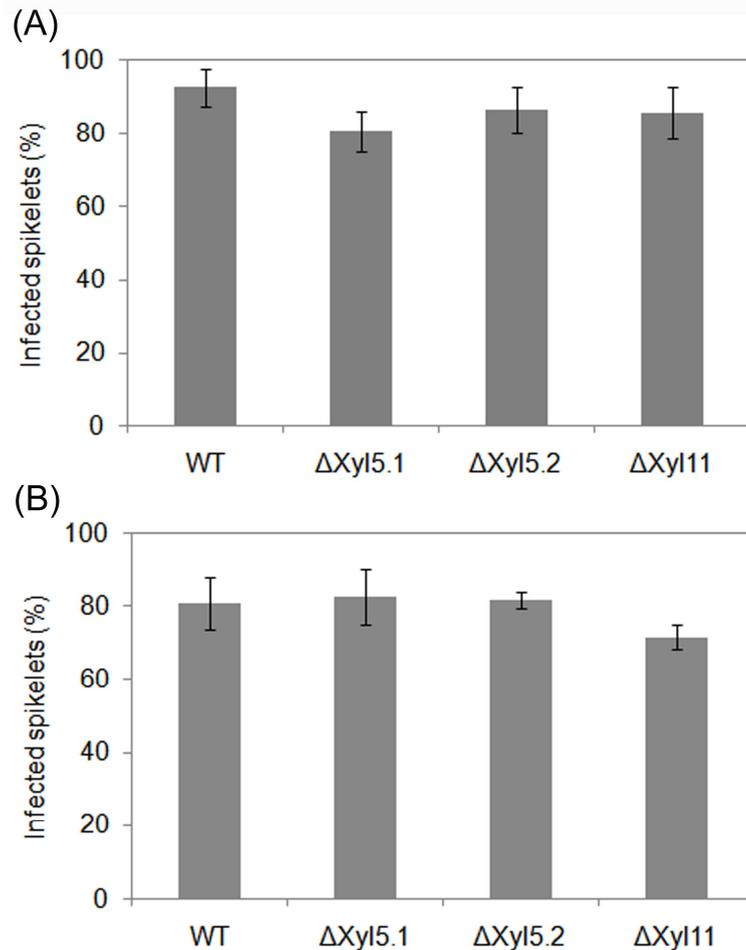


Figure. 13 – Wheat spikelet infection with *F. graminearum* WT and Δ Xyl mutant strains. Disease symptoms were assessed at 21 dpi by counting the number of visually diseased spikelets. Infected spikelets are expressed as percent of symptomatic spikelets on total number of spikelets of the respective head. **(A)** Infection of *T. aestivum* cv. Bobwhite. Data represent the average \pm mean standard error (indicated by bars) of three independent infection experiments performed by inoculating at least 10 plants with 2000 conidia in each independent experiment. Probability (p) of no significant difference between WT and mutant strains, as determined by the two-tailed Student's *t*-test: Δ Xyl5.1 (p>0.08); Δ Xyl5.2 (p>0.39); Δ Xyl11 (p>0.42). **(B)** Infection of *T. aestivum* cv. Nandu. Data represent the average \pm mean standard error (indicated by bars) of two independent infection experiments performed by inoculating with 500 conidia at least 10 plants in each independent experiment. Probability (p) of no significant difference between WT and mutant strains, as determined by the two-tailed Student's *t*-test: Δ Xyl5.1 (p>0.87); Δ Xyl5.2 (p>0.92); Δ Xyl11 (p>0.36).

1.4 Discussion

Xylan is one of the main hemicellulosic components of the plant cell wall, especially abundant in tissues of commensal monocot species such as wheat (Cooper et al., 1988; Vogel et al., 2008). Complete hydrolysis of xylan requires the activity of endoxylanases, which randomly cleave the internal glycosidic β -1,4-bonds of the xylose backbone (Collins et al., 2005; Wong et al., 1988). Among the six endoxylanase genes known to be expressed during infection, the transcript of *FGSG_03624* strongly accumulates within the first 5 dpi, with a maximum accumulation at 3 dpi, when the *FGSG_03624* transcript accumulates about 3–5 times more than the other xylanase genes, except for *FGSG_10999* which shows a similar maximum accumulation and is also the most expressed gene during *in vitro* growth in a medium containing xylan as sole carbon source.

In addition to expression level, the *FGSG_03624* gene also attracted our attention because its encoded protein shares high sequence similarity with *B. cinerea* XYN11A, *T. reesei* XYN2 and *T. viride* EIX endoxylanases shown to induce necrosis in plant tissues (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Noda et al., 2010). In particular, *FGSG_03624* contained seven of 11 amino acids regarded as essential for eliciting necrosis in plant tissues (Noda et al., 2010).

The *FGSG_03624* protein, heterologously expressed in *P. pastoris*, has a molecular mass of 22.7 kDa, possesses xylanase activity *in vitro* and induces necrotic symptoms in wheat tissue. Dead cells were revealed by microscopic observations after Evans blue staining in both infiltrated wheat leaves and treated lemmas. In lemma, a tissue rich in arabinoxylans (Dong et al., 2012), the presence of dead cells in the tissues underneath the epidermis suggests that the penetration of the xylanase occurred through stomatal openings. Cell death was associated with the production of high levels of H_2O_2 , as a consequence of a robust oxidative burst, and these effects were also maintained after heat inactivation of enzyme activity. Thus, it can be assumed that cell death elicitation is independent of the enzyme's catalytic activity, as previously shown for the xylanases of *B. cinerea*, *T. viride* and *T. reesei* (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Noda et al., 2010). Moreover, of this fungus, could depend on specific recognition by a plant receptor. In fact, the relative independence of cell death elicitation from treatments

with different xylanase concentrations suggests that the necrotic process is due to the induction of the hypersensitive-like response rather than to tissue maceration. The ability of this *F. graminearum* xylanase to elicit cell death in wheat tissues, which is consistent with the necrotrophic lifestyle Ron and Avni (Ron et al., 2004) identified a leucine-rich repeat receptor in tobacco and tomato plants which was able to specifically recognize the EIX xylanase of *T. viride* and to induce programmed cell death with H₂O₂ production. Therefore FGSG_03624 would not have a direct toxic effect but could be recognized by a putative plant receptor, not yet identified in wheat, thus activating the hypersensitive response. To our knowledge, this is the first finding of a xylanase inducing necrosis in a monocot species, thus suggesting a wider role of these fungal enzymes, involved not only in cell wall degradation but also in elicitation of cell death.

Forward and reverse genetic approaches have been used to identify pathogenicity or virulence genes in *F. graminearum*. Most of the mutations affecting the infection process have targeted regulatory genes with pleiotropic effects on toxin biosynthesis and fungal development (Kazan et al., 2012). So far, genes predicted to encode CWDE have not been analyzed by a reverse genetic approach, probably because the large number of genes encoding CWDE in the genome of *F. graminearum* (Cuomo et al., 2007) discouraged using this approach to investigate the role of these enzymes during the infection process. In fact, the knock-out of the *F. graminearum* FGSG_03624 gene demonstrated that the mutant strains were not significantly reduced in virulence compared to the WT strain, although when grown in a liquid medium containing xylan as the sole carbon source they showed about 40% reduction of the total xylanase activity compared to WT.

Although FGSG_03624 contributes significantly to total xylanase activity and induces necrosis in the infected tissue, its role in pathogenicity appears dispensable. These data are in contrast with those obtained with *B. cinerea* infecting tomato and grape berries, where the XYN11A xylanase contributes only 30% of total activity but is an important virulence factor (Brito et al., 2006; Noda et al., 2010). Therefore the role of FGSG_03624 during wheat infection remains elusive, as observed with endo-xylanases of *Cochliobolus carbonum* (Apel-Birkhold et al., 1996), *F. oxysporum* (Gómez-Gómez et al., 2002) and *Magnaporthe grisea* (Wu et al., 2006) and with other fungal necrotizing

factors like NLP (Nep1-like proteins), detected in *B. cinerea*, *F. oxysporum* f.sp. *erythroxyli* and *Mycosphaerella graminicola* (Arenas et al., 2010; Bailey et al., 2002; Motteram et al., 2009). In conclusion, we demonstrated that *F. graminearum* endo-xylanase genes were differently expressed during wheat spike infection, with the *FGSG_03624* transcript accumulating strongly. Additionally, the xylanase encoded by this gene caused necrosis of wheat tissue, probably through the induction of hypersensitive response (HR), but appears dispensable in pathogenicity.

Chapter II

**Gene disruption approach to investigate the role of
Fusarium graminearum Xyr1 transcriptional regulator
during host infection**

2.1 Introduction

As shown in the previous chapter, the deletion of the endo- β -1,4-xylanase gene *FGSG_03624* did not affect the virulence of *F. graminearum*. This finding is similar to those obtained by gene disruption of a single xylanase encoding gene in the cereal pathogens *Cochliobolus carbonum*, *Magnaporthe grisea* and *Fusarium oxysporum* (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gomez-Gomez et al., 2002; Wu et al., 2006). Therefore, in these fungi either the endo- β -1,4-xylanase activity has a dispensable role during plant infection or the contribution to total activity by other fungal xylanases is sufficient to support the fungal infection. In order to answer these questions and because of the redundancy of xylanase genes in the *F. graminearum* genome, two main strategies can be used: the simultaneous silencing of all the xylanase genes (Nguyen et al., 2011), or the knocking-out of components of the signal transduction pathway (Tonukari et al., 2000), including the elimination of specific transcriptional activators.

In some Ascomycetes a single transcriptional activator with a zinc binuclear cluster domain regulates the expression of several xylanolytic, cellulolytic and glucanolytic genes, suggesting a general role as regulator of the cellulose and hemicelluloses degradation and metabolism (Calero-Nieto et al., 2007; de Vries and Visser, 1999; Gielkens et al., 1999; Raucher et al., 2006; Striecker et al., 2006; van Pije et al., 1998a).

The XlnR factor was identified as the major transcriptional activator of xylanase genes in the saprophytic fungus *Aspergillus niger* (van Peij et al., 1998a and b). Its deletion blocked the expression of ten hydrolytic enzymes normally transcribed in the WT strain grown on xylan or xylose containing media. The sequence GGCTAA is recognized as the putative binding motif for XlnR, and is located at the 5' regulatory sequence of the affected genes. The role of XlnR orthologues have been investigated in other fungi. AoXlnR of *Aspergillus oryzae*, the homologue of XlnR, seems to be responsible for the expression of four xylanolytic and four cellulolytic enzymes when the fungus is grown on xylan, xylose, microcrystalline cellulose and cellobiose. These genes contains one to three putative binding site for AoXlnR (Marui et al., 2002a and b). The regulatory gene *xyl1* of *Trichoderma reesei* is essential for the production of xylanase, cellobiohydrolase and glucanase activity and this homologous of *xlnR* affects also the D-xylose catabolism by regulating the transcription of *xyrR* gene encoding for the intracellular enzyme D-

xilose reductase, as observed also in *A. niger* and *Hypocrea jecorina* (Hasper et al., 2000; Stricker et al., 2006). The role of the transcriptional activator XlnR of *Fusarium oxysporum* f. sp. *lycopersici* in regulating xylanase genes and virulence has been investigated by Calero-Nieto et al. (2007). They generated $\Delta xlnR$ disrupted mutants as well as strains carrying a *xlnR* allele under the control of a strong constitutive promoter. In this vascular wilt pathogen, *xlnR* transcription is induced by oat spelt xylan and repressed by glucose. The knock-out of *xlnR* resulted in lack of expression of xylanase genes, both in culture and during infection of tomato plants, as well as in dramatically reduced extracellular xylanase activity. When *xlnR* was over-expressed under the control of the *gpdA* promoter, the xylanase activity did not significantly increase, suggesting that XlnR should be regulated not only at transcriptional but also at post-translational level. Interestingly, the $\Delta xlnR$ mutants were still fully virulent on tomato plants, defining the major transcriptional activator of xylanase genes as not essential for virulence in *F. oxysporum* under tested conditions.

A putative transcriptional regulator of endo-xylanase genes, named Xyr1, has been identified in the *F. graminearum* genome (Brunner et al., 2007). While in the MIPS database (<http://mips.helmholtz-muenchen.de/genre/proj/FGDB/>) *xyr1* is identified as a single gene/protein (MIPS entry FGSG_17662), in the Broad Institute database (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/) *xyr1* is splitted in two hypothetical proteins (entries FGSG_12713 and FGSG_12714), with FGSG_12714 being the N-terminus of the putative regulator and FGSG_12713 forming the C-terminal end of the protein (Brunner et al., 2007).

The *xyr1* gene encodes a 873 amino acids protein that show 59% identity and 71% similarity with the *Trichoderma reesei* Xyr1 sequence. The characteristic Zn(2)-C6 fungal DNA binding domain of the *F. graminearum* Xyr1 has been identified between amino acids 95 and 121, matching perfectly with the position of the motif found in *T. reesei*. The two motives share 96% identity, with only one Leu substituted by a Gln in *F. graminearum*. Compared to *A. niger* XlnR, the binding domains of *T. reesei* and *F. graminearum* show 96% and 54% identity at nucleotide and protein level, respectively (Brunner et al., 2007).

F. graminearum xyr1 deletion mutants have been produced and characterized (Brunner et al., 2007). These mutants showed a significant reduction of growth on agar plates containing xylose or xylitol as sole carbon source but the effect of the mutation has not been ascertained.

In the present work new *xyr1* disrupted mutants were produced and tested for xylanase activity and growth on xylan as sole carbon source. These mutants are now available to perform virulence test on *Triticum aestivum*.

2.2 Materials and methods

2.2.1 Fungal cultures, media and growth condition

The *Fusarium graminearum* strain PH1 was cultured at 25 °C on complete medium (CM) (Leach et al., 1982) supplemented with 1.5% (w/v) granulated agar (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) or on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA).

To obtain mycelium for DNA extraction, WT and mutant strains were grown in 30 ml of CM liquid culture inoculated with 3 mycelium plugs (5 mm diameter) and grown at 24 °C for 4 days at 150 rpm on a orbital shaker.

Conidia were obtained by culturing *F. graminearum* WT and mutant strains in 50 ml of carboxymethyl cellulose (CMC) liquid medium (Cappellini et al., 1965) at 25 °C and 100 rpm.

For growth experiments, biomass of WT and transformants was determined on a liquid culture (Szécsi et al., 1990) supplemented with 0.5% (w/v) beechwood xylan (Sigma-Aldrich, Milano, Italy) as the sole carbon source by inoculating 20 ml of medium with 1×10^4 conidia ml⁻¹. After 7 days at 25 °C on an orbital shaker at 100 rpm the flasks were transferred into 50 ml pre-weighed tubes and centrifuged at 8500 x g for 20 min. The mycelium was collected, washed twice with deionized water, oven dried at 80 °C for 3 d and weighed.

Xylanase activity was determined at 4 and 7 dpi on 20 ml cultures inoculated with 1×10^4 conidia ml⁻¹ and grown at 25 °C in the xylan medium. Alternatively, 2.5 ml of the medium were inoculated with one agar plug (5 mm diameter) taken from the edge of actively growing colonies.

2.2.2 DNA manipulation and construction of the gene replacement vector

Genomic DNA from *F. graminearum* WT and mutant strains was extracted as reported by Henrion et al. in 1994 from 200-400 mg of mycelium. All primers used to generate the construct for disrupting the *F. graminearum xyrl* gene (MIPS database entry

FGSG_17662; Broad Institute Fusarium Comparative Genome entries *FGSG_12714* and *FGSG_12713*) were designed by using DNAMAN (Lynnon Biosoft) and PerlPrimer v.1.1.17 programs (Table 2). The *xyr1* flanking homologous regions, necessary for targeted homologous recombination, were amplified by PCR using *F. graminearum* genomic DNA as template. Specific oligonucleotides were chosen to amplify the upstream (primers Fg17662upF and Fg17662upR) of about 900 bp and downstream (primers Fg17662downF and Fg17662downR) flanking regions (Figure 14 and Table 2) of about 1000 bp. The amplification was performed by “REDTaq ReadyMix PCR Reaction Mix” (Sigma, Milano, Italy) in a 50 µl volume. PCR conditions were as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 sec and 72 °C for 1 min. The amplicons obtained were purified and used in a second PCR to fuse the homologous flanking regions with the hygromycin resistance gene (hygromycin B phosphotransferase, *hyg*), used as selection marker (Punt et al., 1987). The fusion PCR reaction was performed with the “REDTaq ReadyMix PCR Reaction Mix” (Sigma, Milano, Italy) in a 50 µl volume using 150 ng of the purified flanking regions containing tails homologous to the 5' and 3' region of the *hyg* gene (Figure 14) and 450 ng of the *hyg* gene cut with *Sma* I (Fermentas, Milano, Italy) from pAN7-1 vector (Voigt et al., 2005). The fusion PCR conditions were as follows: 94 °C for 4 min, followed by 20 cycles of 94 °C for 1 min, 60 °C and 72 °C for 4 min. The fusion PCR product was then used as template in a nested PCR reaction with the primers Fg17662nstF and Fg17662nstR (Table 2 and Figure 14). Nested PCR conditions were as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 sec and 72 °C for 4 min. The amplicon obtained was cut from agarose gel, purified and then cloned into the pGEM-T easy vector (Promega, Milano, Italy) following the manufacturer's instruction. An *E. coli* positively transformed colony was grown in LB-broth high salt culture (Fluka, BioChemika) and the plasmid DNA was extracted with the “MIDI Nucleobond® Ax” (Macherey-Nagel GmbH & Co.) kit following manufacturer's instructions. The construct of 3438 bp was then cleaved from the pGEM-T EASY vector by double digestion using *Bst*XI and *Ap*aI (Fermentas, Milano, Italy) and used to transform protoplasts of *F. graminearum* WT strain.

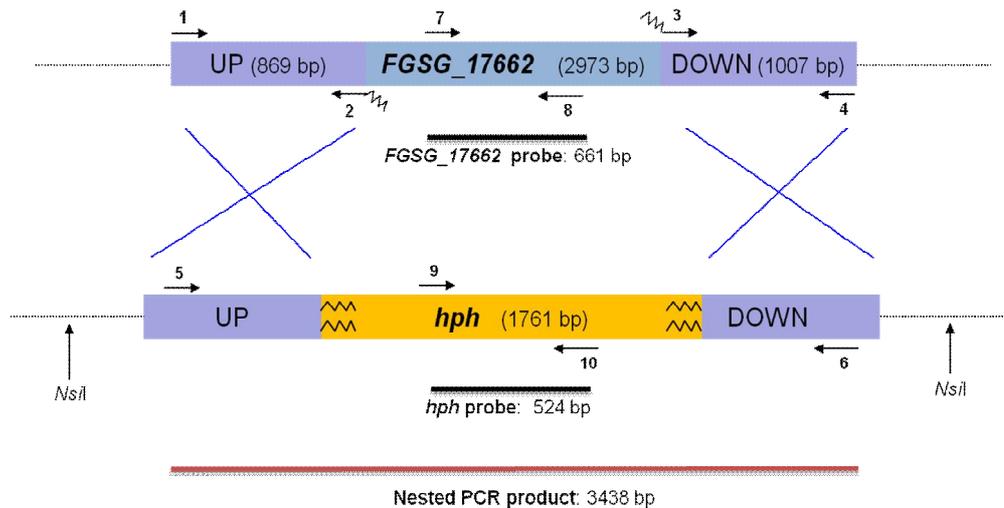


Figure 14 – Schematic illustration of the PCR-based construction of the gene replacement vector.

Flanking homology regions of the *F. graminearum* *FGSG_17662* gene were amplified by PCR using specific primers for each gene: primers 1 (Fg17662upF) and 2 (Fg17662upR) were used for the amplification of the upstream region (UP), and primers 3 (Fg17662downF) and 4 (Fg17662downR) for the downstream region (DOWN). UP and DOWN amplicons were fused with the hygromycin resistance *hph* gene by the “Fusion PCR” technique, using as primers the tails (^^) of primers 2 and 3, complementary to the 5’ and 3’ *hph* regions, respectively. The fusion PCR product was used as template in a subsequent nested PCR reaction, where primers 5 (Fg17662nstF) and 6 (Fg17662nstR) were used to obtain the full construct of 3372 bp. The disruption of the target gene was obtained by homologous recombination via two crossing-over events. Primers pairs 7-8 (Fg17662intF and Fg17662intR) and 9-10 (Hyg-For and Hyg-Rev) were used to obtain the *FGSG_17662* and *hph* probes for Southern blot analysis, respectively. Sites recognized by *Nsi*I, used for DNA digestion, are also indicated.

2.2.3 Fungal transformation-mediated gene disruption and preliminary screening of mutant lines

Protoplast formation and fungal transformation were performed according to Van Nguyen et al. (2012). Hygromycin-resistant colonies were collected and transferred to 30-mm CM plates supplemented with 200 µg/ml of hygromycin B (Duchefa Biochemie, Haarlem, The Netherlands). Resistant mutants were single-conidiated and preliminarily screened by PCR using the primer pair Fg17662int-F and Fg17662-int-R, designed by using DNAMAN (Lynnon Biosoft) and PerlPrimer v.1.1.17 programs (Table 2 and

Figure 14). Transformants without the *FGSG_17662* gene were then tested by Southern blot hybridization for single insertion of the disruption construct.

2.2.4 Southern blot analysis

Genomic DNA samples of 1,5 µg were digested with *Nsi*I (Fermentas, Milano, Italy), separated on a 1% (w/v) agarose/TBE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Italy). Digoxigenin (DIG)-labeled (Roche, Mannheim, Germany) specific probes were generated with primers specific for the *FGSG_17662* or *hyg* genes (Table 2 and Figure 14) by using genomic or plasmid DNA as template, and were used for overnight hybridization at 65 °C. The PCR reaction, performed in a 50 µl volume using DIG-11-dUTP (Roche, Mannheim, Germany), consisted of 1 min at 94 °C, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min.

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

2.2.5 Total xylanase activity assay

Total xylanase activity of *F. graminearum* liquid cultures was determined by measuring the reducing sugars released from 0.5% (w/v) beechwood xylan (Sigma-Aldrich, Milano, Italy) dissolved in 50 mM sodium citrate buffer at pH 5 according to the dinitrosalicylic acid (DNSA) method described by Miller et al. in 1959 and modified by Bailey et al. in 1992. D-xylose (Merck Chemicals) was used as a standard. Xylanase activity of WT and Δ xyr mutant strains was assayed by incubating 100 µl of fungal culture in a 200 µl reaction mixture. 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.

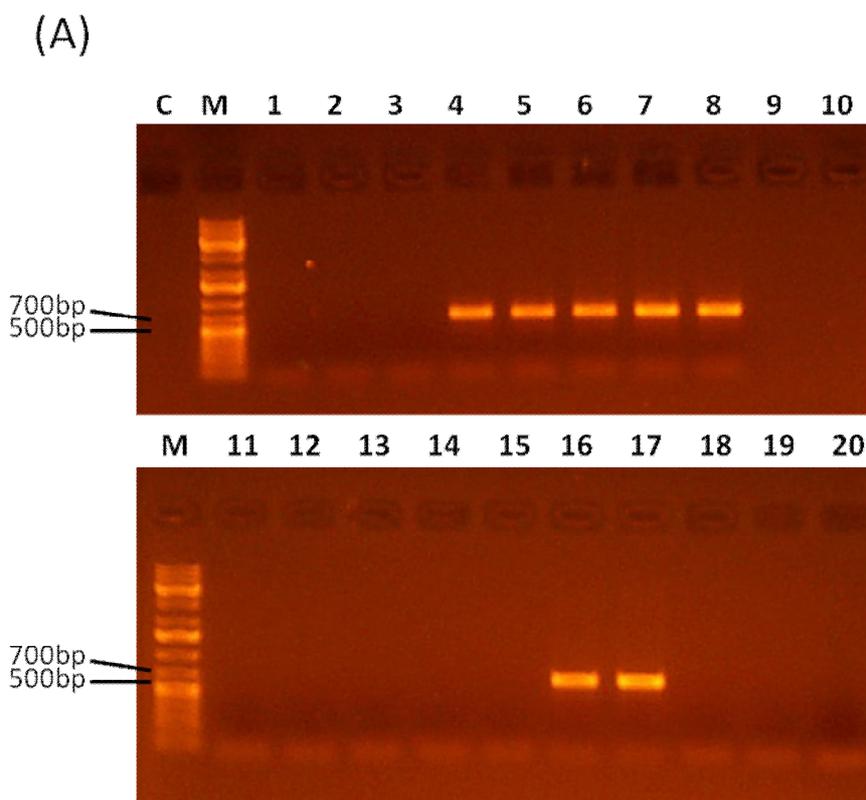
Table2 – List of used primers.

Primer name	Sequence (5'-3')
Primers to produce the disruption construct	
Fg17662upF	ACTTCCCTCCAGGATCCACT
Fg17662upR	AGATGCCGACCGAACAAGAGCTGTCCCCCACATCTTGCCGGTACTCAGG
Fg17662downF	TCAATGCTACATCACCCACCTCGCTCCCCCAACATACTTGGGGCGTCTT
Fg17662downR	GCAACAACACAAGCGAGAAA
Fg17662nstF	TGCCAAACCCATTACAACAA
Fg17662nstR	TACTTCCCCTTGCCCTCTTT
Primers for preliminary screening of Δxyr mutants and for DIG-labeled probes	
Fg17662intF	CTGTTGCTGATGGTGTGCT
Fg17662intR	TTCTTTGCGTGATGCAAGTC
Hyg-For	CTCGATGAGCTGATGCTTTG
Hyg-Rev	GGACAGCTCTTGTTCCGGTCGG

2.3 Results

2.3.1 Targeted disruption of *F. graminearum* Xyr1 encoding gene

The *F. graminearum* (WT) protoplasts were transformed with a disruption vector containing the hygromycin resistance gene in order to replace the *FGSG_17662* gene by two homologous recombination events. Thirty-two hygromycin resistant colonies were selected from regenerated protoplasts. After single conidiation, the transformants were screened by PCR for the absence of an internal 661 bp fragment of the *FGSG_17662* gene. The genomic DNA of eleven mutants did not produce any *xyr1* amplicons (Figure 15A), but resulted positives for *hyg* gene (Figure 15B).



(B)

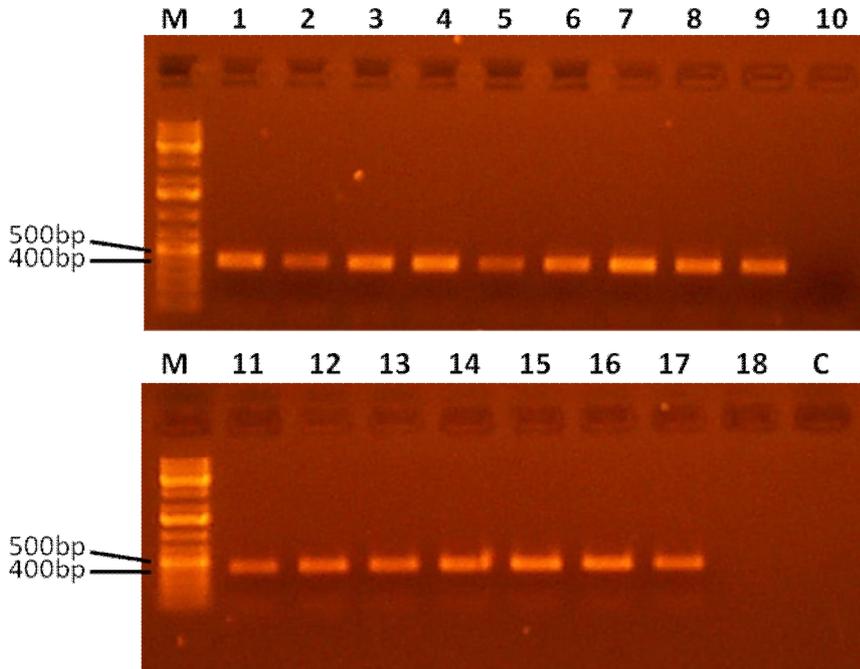


Figure 15 – PCR selection of *F. graminearum* *FGSG_17662* gene disruption mutants. Transformants colonies resistant to hygromycin were screened by PCR using the primer pair Fg17662intF and Fg17662intR or Hyg-For and Hyg-Rev. **(A)** The 661 bp internal fragment of the *FGSG_17662* gene was amplified in WT (lane 17), but not in the mutant strains Δ xyr1.3 (lane 11), Δ xyr2.1 (lane 1), Δ xyr2.2 (lane 2), Δ xyr2.3 (lane 3), Δ xyr2.11 (lane 12), Δ xyr2.14 (lane 13) and Δ xyr2.16 (lane 14). Δ xyr2.17 (lane 15) Δ xyr2.23 (lane 18) and Δ xyr2.24 (lane 19), which exhibited the amplification of *hyg* gene. A negative control was loaded in lane C. M: molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy) are shown on the left. **(B)** The 525 bp internal fragment of the *hyg* gene was absent in WT (lane 18), and amplified in the mutant strains Δ xyr1.3 (lane 11), Δ xyr2.1 (lane 1), Δ xyr2.2 (lane 2), Δ xyr2.3 (lane 3), Δ xyr2.11 (lane 12), Δ xyr2.14 (lane 13), Δ xyr2.16 (lane 14), Δ xyr2.17 (lane 15), Δ xyr2.23 (lane 8) and Δ xyr2.24 (lane 9). A negative control was loaded in lane C. M: molecular size markers (GeneRuler DNA Ladder Mix, Fermentas, Milano, Italy) are shown on the left.

The genome of five interesting mutants were submitted to high-stringency Southern blot using the *FGSG_17662* and the *hyg* specific probes. The knock-out of the gene was confirmed in the all examined mutant strains, but only for the Δ xyr1.3 and Δ xyr2.11 strain the hybridization with the *hyg* probe revealed a unique signal of the expected 7.4

Kb size, indicating a single homologous integration of the deletion construct (Figure 16). The single signal of about 10 Kb showed by $\Delta xyr2.3$, $\Delta xyr2.14$ and $\Delta xyr2.16$ strains was compatible with a targeted double integration of the disruption construct in the correct locus.

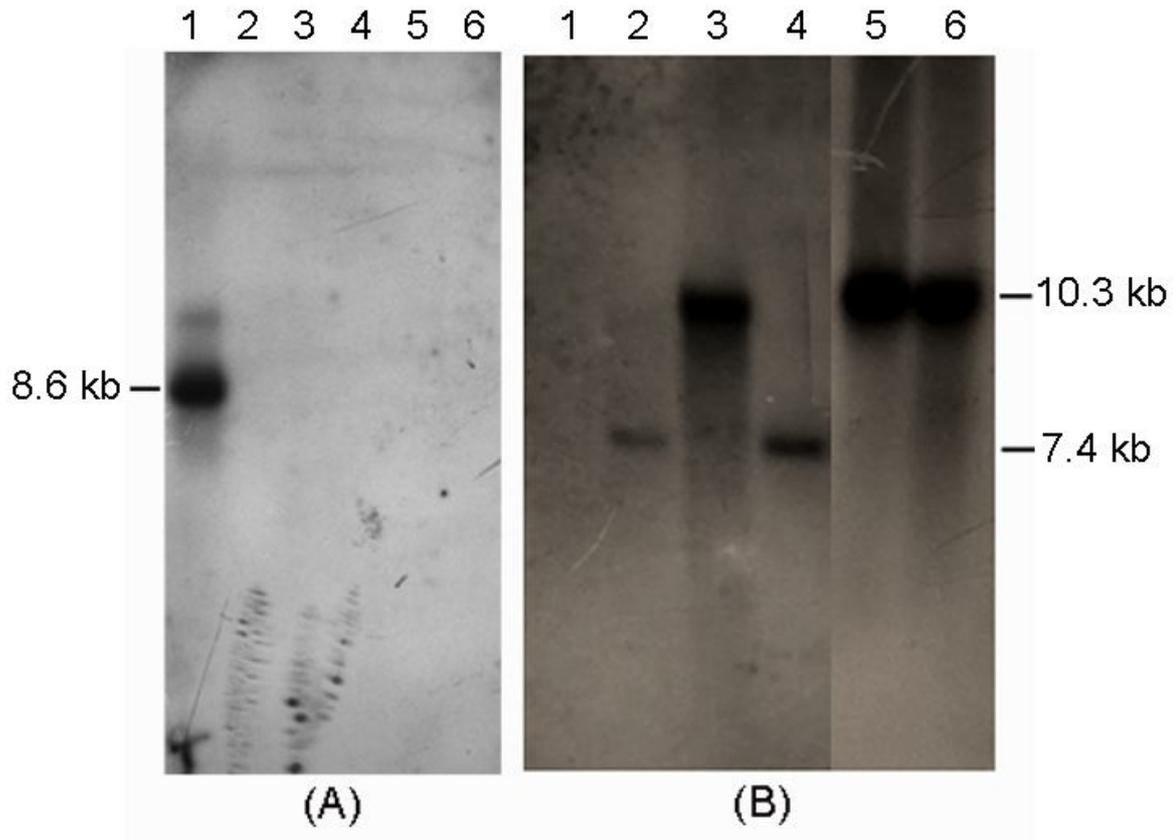


Figure 16 – High-stringency Southern blot analysis of genomic DNA from *F. graminearum* WT and mutant strains digested with *NsiI* (Promega, Milano, Italy). **(A)** A fragment of the *FGSG_17662* gene was used as specific probe. The WT strain (lane 1) showed an hybridization signal of 8.6 Kb corresponding to the *FGSG_17662* gene, while the $\Delta xyr1.3$ (lane 2), $\Delta xyr2.3$ (lane 3), $\Delta xyr2.11$ (lane 4), $\Delta xyr2.14$ (lane 5), $\Delta xyr2.16$ (lane 6), mutant strains did not show this hybridization signal. **(B)** A fragment of the *hyg* resistance gene was used as probe. The WT (lane 1) strain gave no hybridization signal. $\Delta xyr1.3$ (lane 2), $\Delta xyr2.11$ (lane 4) mutant strains showed a single hybridization signal at 7.4 Kb; while in $\Delta xyr2.3$ (lane 3), $\Delta xyr2.14$ (lane 5), $\Delta xyr2.16$ (lane 6) mutants the single signal at 10.3 Kb was higher than the expected size. Dig Marker VII, (Fermentas, Milano, Italy) was used molecular size marker.

2.3.2 *In vitro* characterization of the Fg Δ xyr mutants

To verify if the disruption of the transcriptional regulator gene was effective, the WT and the Δ xyr mutant strains were grown in liquid culture containing xylan as the sole carbon source. The total xylanase activity in the cultural supernatant was determined by the DNSA method at 4 and 7 dpi. At 4 dpi, the total xylanase activity produced by the mutants was 90% lower than the WT strain and no significant differences among mutant strains were observed (Figure 17). The difference observed between mutants and WT strains was also confirmed at 7 dpi (data not shown).

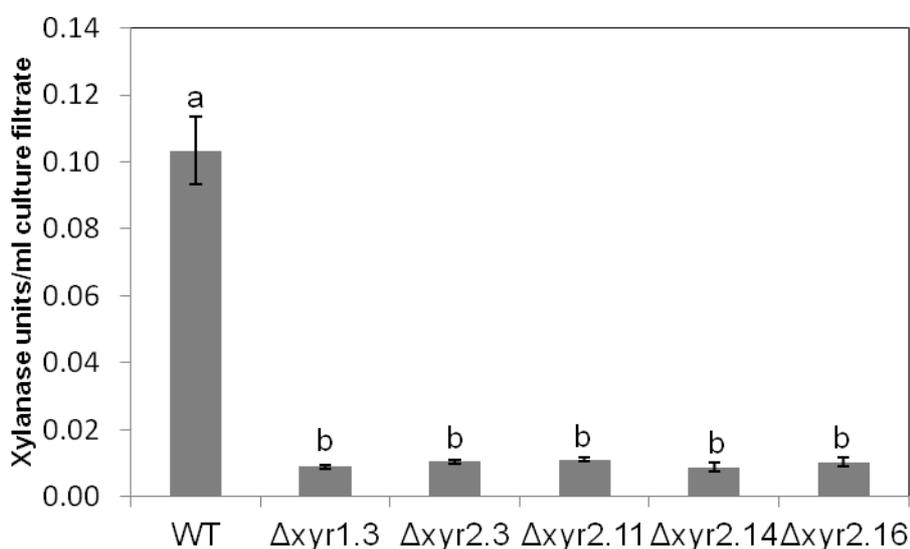


Figure 17 – Total xylanase activity secreted by WT and Δ xyr mutant strains. One hundred μ l of culture filtrates, collected after 4 d of liquid culture in Szécsi medium with xylan as carbon source, were incubated in 1 ml of reaction mixture containing 0.5% (w/v) beechwood xylan. Xylanase activity, measured with the DNSA method, was expressed as xylanase units/ml of culture filtrate. One xylanase unit was defined as the amount of enzyme required to release 1 μ mol of xylose in 1 min under the assay conditions. Data represent the average \pm mean standard error (indicated by bars) of three independent flasks per strain. Treatments were statistically different ($p < 0.01$, $F = 243.89$) by applying randomized complete blocks ANOVA. Different letters (a, b) indicate significant differences at $p < 0.01$ (Student–Newman–Keuls test).

The biomass formation produced by of WT and mutant strains in a liquid culture containing xylan as the sole carbon source was compared after 7 days of growth. Dramatic

differences in dry weight were observed between mutants and the WT strain. (Figure 18). The dry weight of mutant strains resulted from 40% to 70% lower than the WT, even no significant differences among mutant strains have been revealed by performed statistical analysis.

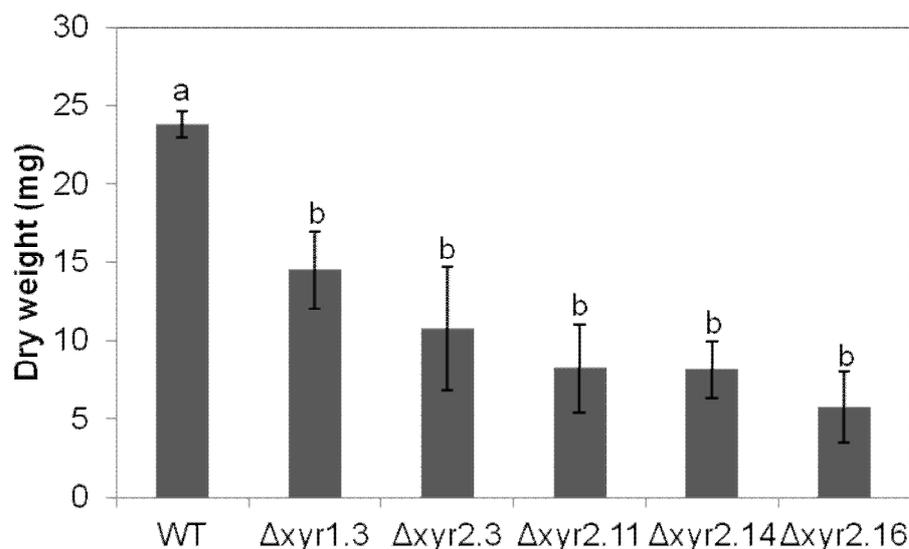


Figure 18 – Dry weight of WT and Δxyr mutant strains grown for 7 days in a liquid culture containing xylan as the sole carbon source. 1×10^4 conidia ml^{-1} of *F. graminearum* WT and mutant strains were grown in 20 ml of Szècsi liquid medium supplemented with 0.5% (w/v) beechwood xylan at 100 rpm and 25 °C. After 7 days mycelia were collected, filtered, washed twice with sterile water, oven dried at 80 °C for 3 days and then weighed. Dry weights are expressed in milligrams (mg). Data represent the average \pm standard deviation (indicated by bars) of three flasks per strain. Treatments were statistically different ($p < 0.01$, $F = 12.3215$) by applying randomized complete blocks ANOVA. Different letters (a, b) indicate significant differences at $p < 0.01$ (Student–Newman–Keuls test).

2.4 Discussion

In the present work, the targeted gene replacement of the *Fusarium graminearum* *xyr1* gene, encoding for the transcriptional regulator of the endo-xylanase genes expression, has been successfully performed. From thirty-two hygromycin resistant transformants screened by PCR, eleven mutants were selected for the lacking of *FGSG_17662* gene. Among them, five were analyzed by high-stringency Southern blot, which revealed that the a single homologous integration of the deletion construct in *FGSG_17662* locus occurred in the $\Delta xyr1.3$ and $\Delta xyr2.11$ mutant strains, while a double homologous integration of the disruption cassette occurred in the $\Delta xyr2.3$, $\Delta xyr2.14$ and $\Delta xyr2.16$ mutant strains.

These Δxyr mutants exhibited a strong impairing in the capability to grow on xylan as sole carbon source, and after seven days of liquid culture the dry weight of mutant resulted very reduced, from 40% to 70% lower than the WT. This result is coherent with the 40% reduction of biomass formation observed by Brunner et al. (2007) for *xyr1* deletion mutants grown on xylan, compared to WT.

The decrease of total xylanase activity secreted in liquid culture by all our Δxyr strains appeared really dramatic, since mutants preserved only 10% of the enzymatic activity produced to WT. Interestingly, Brunner et al. (2007) observed a less dramatic reduction of the total xylanase activity produced by the *xyr1* *F. graminearum* deletion mutants, measured as 30% of the total activity produced by the WT strain when grown on xylan medium.

Taken together, these data seem to confirm the involvement of the *F. graminearum* Xyr1 transcription factor in xylan utilization, as suggested by Brunner et al. (2007) and earlier proposed for other plant fungal pathogens (Hasper et al., 2000; Stricker et al., 2006).

In order to forecast a possible *F. graminearum* Xyr1 binding site in the 5' regulatory sequence of the xylanase genes selected for the expression analysis *in vivo* (Chapter I, paragraphs 1.1 and 1.2.8), an *in silico* analysis was carried out on their 5' regulatory sequences to search for the hypothetical conserved motif GGCTAA: this short sequence is indicated as a putative binding motif for the Xyr1 orthologue XlnR found in *Aspergillus oryzae*, and was reported as located at the 5' regulatory sequence of the affected genes (Marui et al. 2002a and b). All the six *F. graminearum* xylanase encoding

genes analyzed showed only the presence of the central motif GCTA, in at least two copies at the 5' of the ORFs.

Brunner et al. (2007) analyzed the expression of endo xylanase genes only *in vitro* after 48 h of growth on wheat cell wall medium, and demonstrated that all the genes showed a two to five magnitude order reduction in mutants compared to WT; however they did not include in this analysis the expression of the *FGSG_10999* gene, one of the two most transcribed endoxylanase encoding genes during wheat spike infection (Chapter I, paragraph 1.2.8).

The expression of the *FGSG_11258*, *FGSG_11304*, *FGSG_06445*, *FGSG_11487*, *FGSG_03624* and *FGSG_10999* genes will be studied *in vitro* by qPCR analysis. Gene expression will be compared between WT and Δ xyr mutant strains in order to get deeper insight on the transcriptional regulation mediated by Xyr1.

The expression analysis *in vivo* is therefore necessary to verify if this regulation functions also during plant infection. The *xyr1* disrupted mutants now available will be characterized for their virulence by *in-vivo* inoculation experiments of *Triticum aestivum* spikes and for expression of the xylanase genes to verify their regulation during plant infection.

Conclusions and perspectives

Fusarium graminearum is a necrotrophic plant pathogen which has a brief initial short biotrophic stage (Kazan et al., 2012) and is primarily responsible of the FHB disease that affects cereals such as wheat. Some observations suggest that CWDEs secreted by this pathogen can play an important role during pathogenesis. In particular, cell wall degradation of *F. graminearum* infected spike tissue was observed by immunohistological methods (Brown et al 2010., Kikot et al., 2009; Wanyoike et al., 2002). Besides, the observation that wheat transgenic plants expressing inhibitors of fungal pectinases (Ferrari et al., 2012; Volpi et al., 2011) show a reduction of FHB symptoms indicates the importance of these CWDEs during host colonization. The xylanases may also play a major contribution to break the plant cell wall and especially the cell wall of the cereal plants. To better define the role of xylanases during the FHB disease, a two step approach was followed. By first one of the major expressed enzymes belonging to the glycoside hydrolase family 11, the FGSG_03624 endoxylanase, was recognized as the major responsible of the activity detected *in vitro*. However, no clear contribution to virulence of this xylanase was observed when *FGSG_03624* knock-out mutants were used to infect wheat spikes, although a recombinant FGSG_03624, purified after *Pichia pastoris* heterologous expression, displayed a hypersensitive-like effect on glume tissues that seems independent from the enzyme activity. The purified enzyme is now available for future molecular plant-pathogen interaction studies directed to isolate some specific receptor for FGSG_03624 in wheat cells as well as to identify the amino acidic motif possibly recognized by the putative wheat receptor. To this aim, peptides could be synthesized and used also for examining the elicitation of other plant responses and possibly to induce resistance or prime plants against successive pathogen attacks.

The second aim of the research was to establish the importance of the entire xylanase enzymes class produced by *F. graminearum* during infection of host plants. To this purpose the *FGSG_17662* gene, encoding for the *F. graminearum* Xyr1 transcriptional regulator factor, has been deleted by targeted homologous recombination. Disruptant strains are heavily impaired both in secreted total xylanase activity and in biomass formation when grown in liquid culture with xylan as sole carbon source, and are now

available to be assayed in infection experiments with wheat plants. If these mutants will be verified as impaired in virulence, new control measures of the FHB can be investigated. For example, transgenic plants may be produced to counteract the fungal attack by inhibiting the xylanase activity. To this aim three types of xylanase inhibitors (XIs) have been identified in wheat: *Triticum aestivum* XI (TAXI) (Debyser et al., 1997; Debyser et al., 1998), xylanase inhibitor protein (XIP) (McLauchlan et al., 1999) and thaumatin-like XI (TLXI) (Fierens et al., 2007). The most effective of these XI could be expressed to obtain wheat plants more resistant to FHB.

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Errata corrige

Page 17, line 10 and 11: Bailey et al., 1997 **must be changed with** Biely et al., 1997.

Table 1, the primers reported on page 29 must be changed with the followings:

fg03624int-For	CCTACAACCTGGTGTCAACAAT
fg03624int-Rev	CAGAGACAGTCATGGTAGCC
Hyg-For	CTCGATGAGCTGATGCTTTG
Hyg-Rev	GGACAGCTCTTGTTCCGGTCGG

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