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In planta evidences for the involvement of xylanase inhibitors in wheat defence against fungal pathogens

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Abstract

Cereals contain xylanase inhibitor (XI) proteins which inhibit microbial xylanases and are considered part of the defense mechanisms to counteract microbial pathogens. Nevertheless, *in planta* evidence for this role has not been reported yet. Therefore, we produced a number of transgenic plants constitutively overexpressing TAXI-III, a member of the TAXI type XI that is induced by pathogen infection. Results showed that TAXI-III endows the transgenic wheat with new inhibition capacities. We also showed that TAXI-III is correctly secreted into the apoplast and possesses the expected inhibition parameters against microbial xylanases. The new inhibition properties of the transgenic plants correlate with a significant delay of Fusarium head blight (FHB) disease symptoms caused by *Fusarium graminearum* but do not significantly influence leaf spot symptoms caused by *Bipolaris sorokiniana*. We showed that this contrasting result can be due to the different capacity of TAXI-III to inhibit the xylanase activity of these two fungal pathogens.

To elucidate the molecular mechanism underlying the capacity of the TAXI-III transgenic plants to limit FHB symptoms, we treated wheat tissues with the xylanase FGSG_03624 strongly expressed by *F. graminearum* during wheat spike infection, previously demonstrated to induce cell death and hydrogen peroxide accumulation (Sella et al., 2013). Experiments performed on glumes of flowering wheat spikes showed that the presence of TAXI-III significantly decreased cell death. Most interestingly, a reduced cell death was also obtained by treating with the same xylanase glumes of TAXI-III transgenic plants as compared to control non transgenic plants. Moreover, the inhibitor counteract the cell death activity of this xylanase also in wheat cell suspension cultures. Molecular modelling predicted an interaction between TAXI-III and the xylanase FGSG_03624. In particular, TAXI-III interacted with a residue within the peptide that is thought to be responsible for the necrotizing activity.

These results suggest that the reduced FHB symptoms on transgenic TAXI-III plants can be due to the direct inhibition of xylanase activity secreted by the pathogen but also to the capacity of TAXI-III to prevent host cell death, possibly by preventing the recognition of the xylanase FGSG_03624 by a plant receptor involved in cell death elicitation, as previously suggested in tomato.

In conclusion, these results provide, for the first time, direct evidence *in planta* that XI are part of the immune system for plant defense against fungal pathogens and suggest the possible molecular mechanism underlying the protective role of XIs.

Key words: xylanase inhibitors, plant immunity, *Fusarium graminearum*, plant cell death, transgenic wheat.

Riassunto

I cereali contengono inibitori proteici delle xilanasi che inibiscono le xilanasi prodotte dai microrganismi e sono considerati parte dei meccanismi di difesa che la pianta usa per contrastare i microrganismi patogeni. Tuttavia, finora non sono state riportate evidenze dirette *in planta* che supportino tale ipotesi. In questo lavoro di tesi sono state prodotte linee transgeniche di frumento duro sovraesprimenti TAXI-III, un membro della famiglia degli inibitori delle xilanasi di tipo TAXI che viene indotto in seguito ad infezione. Le linee transgeniche hanno mostrato che TAXI-III conferisce alla pianta nuove capacità inibitorie, inoltre l'inibitore viene correttamente secreto nell'apoplasto e possiede i parametri inibitori attesi nei confronti delle xilanasi prodotte dai microrganismi.

Le nuove proprietà inibitorie delle piante transgeniche correlano con una riduzione significativa dei sintomi di fusariosi causati da *Fusarium graminearum*, ma non influenzano significativamente i sintomi di necrosi fogliare causati da *Bipolaris sorokiniana*. Questi risultati contrastanti sembrano essere dovuti alla diversa capacità inibitoria di TAXI-III nei confronti delle attività xilanasiche prodotte da questi due funghi patogeni.

Per chiarire il meccanismo molecolare che sta alla base della capacità delle piante transgeniche sovraesprimenti TAXI-III di limitare i sintomi di fusariosi, sono stati trattati i tessuti di frumento con la xilanasi FGSG_03624 di *F. graminearum* che ha la capacità di indurre la morte cellulare e l'accumulo di perossido di idrogeno (Sella et al., 2013). Gli esperimenti condotti sulle glume di spighe di frumento in fioritura hanno mostrato che la presenza di TAXI-III riduce significativamente la morte cellulare e, cosa ancora più interessante, una analoga riduzione è stata ottenuta anche trattando con la stessa xilanasi glume di piante transgeniche sovraesprimenti TAXI-III. Inoltre è stato dimostrato che l'inibitore TAXI-III riduce la morte cellulare, causata dalla xilanasi necrotizzante, anche in colture di cellule di frumento in sospensione.

La modellizzazione molecolare ci ha permesso di predire una interazione tra TAXI-III e la xilanasi FGSG_03624. In particolare, TAXI-III interagisce con un residuo che compone il peptide ritenuto responsabile della attività necrotizzante.

Questi risultati suggeriscono che la riduzione dei sintomi da fusariosi osservati sulle piante transgeniche sovraesprimenti TAXI-III sia dovuta all'inibizione diretta da parte di questo inibitore delle xilanasi secrete dal patogeno ma anche alla capacità di TAXI-III di prevenire la morte cellulare, probabilmente impedendo il riconoscimento della xilanasi da parte di un ipotetico recettore della pianta coinvolto nell'elicitazione della morte cellulare, come precedentemente proposto in pomodoro.

In conclusione i risultati riportati in questa tesi dimostrano, per la prima volta, che gli inibitori delle xilanasi fanno parte del sistema immunitario di difesa della pianta contro i patogeni fungini e suggeriscono il possibile meccanismo molecolare sottostante il ruolo protettivo dell'inibitore TAXI-III.

Parole chiave: inibitori delle xilanasi, sistema immunitario della pianta, *Fusarium graminearum*, morte cellulare, frumento transgenico.

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1. Introduction

1.1 Wheat

Wheat is one of the major crop in the world and has a relevant economic impact in European Union (EU) countries which are the main producers of both bread wheat and durum wheat: 134.5 of the 674.9 million tons of bread wheat and more than 22% of durum wheat produced world-wide in 2012 are from the EU (FAOSTAT).

Wheat is a major ingredient in such foods as bread, porridge, crackers, biscuits, Muesli, pancakes, pies, pastries, cakes, cookies, muffins, rolls, doughnuts, gravy, boza and breakfast cereals, while durum wheat is widely used to produce pasta, cous cous and some traditional breads.

Nowadays the main concern is to gain enough yield to feed population as it is growing continuously. In the last decade cereal production increased constantly (Fig. 1) to meet the world needs, but it is expected that in the next twenty years, the current production will be not anymore sufficient to cover the need.

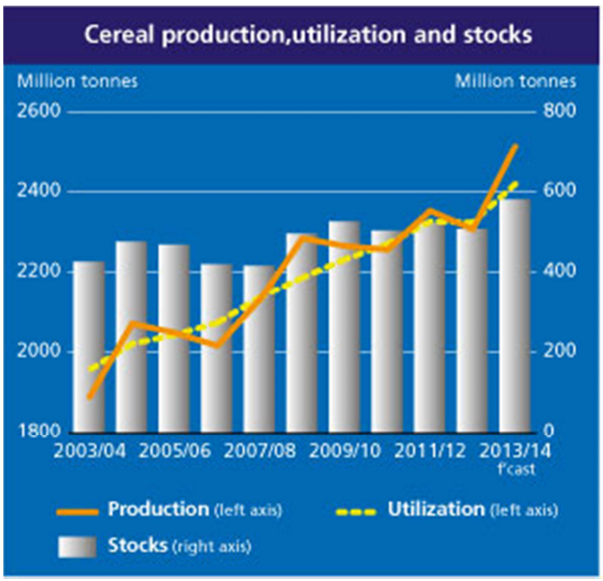

Fig. 1 Cereal production, stocks and utilization in last decade (FAO 06/03/2014).

A concern not less important is the need for high quality products, meaning safe, healthy and nutritional enriched goods and environmental friendly production.

To increase yields there are different approaches, the most successful are those applied during the Green Revolution when the use of better agronomical practices, chemicals, such as fertilizers and pesticides, and new highly productive varieties, produced by traditional breeding, let the productivity increase incredibly.

Nowadays these strategies are still very important and can led to improvements in wheat production but recent knowledge and expertise paved the way for a more effective breeding for crops. The better understanding of plant genetics and physiology and its interaction with the environment, that comprehend even biotic and abiotic stresses, give us new tools to meet the increasing demands for high quality food and feed produced in an environmentally sensitive, sustainable and profitable manner.

On 2012 a draft whole genome sequence of bread wheat (cv Chinese spring) has been published and the International Wheat Genome Sequencing Consortium (IWGSC) is working to complete it. The wheat whole genome sequence data provides direct access to all 96,000 genes and represents an essential step towards a systematic understanding of biology and engineering the cereal crop for valuable traits. Its implications in cereal genetics and breeding includes the examination of genome variation, association mapping using natural populations, performing wide crosses and alien introgression, studying the expression and nucleotide polymorphism in transcriptomes, analyzing population genetics and evolutionary biology and studying the epigenetic modifications. Indeed the availability of large-scale genetic markers generated through NGS (Next Generation Sequencing) technology will facilitate trait mapping and make marker-assisted breeding much feasible.

Moreover an interesting strategy to increase general knowledge of plant physiology and to point breeding for a specific trait is the use of transgenic approach.

1.2 Transgenic wheat for defence against fungal pathogens

Transformation methods are useful tools to study plant physiology, through the overexpression and silencing of genes or gene families, since they allow to study *in planta* the effects of single or multiple modifications.

Genetic engineering is taking a leading role also in breeding programs to ensure increases of food production and quality in a sustainable manner by enhancing crop tolerance to biotic and abiotic stresses.

The first transgenic wheat plant was obtained using the biolistic method by Vasil et al. (1992), indeed, since 1997 the *Agrobacterium*-mediated transformation approach was also used to transform wheat (Cheng et al., 1997). Comparing these two methods, more successful cases with the biolistic mediated wheat transformation have been reported (Li et al., 2012).

To date technological improvements for the production of transgenic wheat plants were proposed including high efficiency regeneration protocols, a temporal and spatial stability of transgene expression, good markers to identify GM lines, strategies to introduce multiple transgenes or to suppress selected genes or even gene families (Dunwell, 2013). Recently genetic engineering offers also New Breeding Techniques (NBTs) such as cisgenesis/intragenesis, site direct mutagenesis, genome editing using zinc finger nucleases or TALENs, RNA dependent DNA methylation and other epigenetic methods (Dunwell, 2013).

So far genetic modification has been mainly regarded as a potential approach to improve input traits, wheat stress tolerance, yield, and quality; however, unlike other crops, transgenic wheat has not been commercialized yet.

Wheat is affected by a number of fungal diseases such as stem rust, powdery mildew, *Septoria*, *Fusarium*, common bunt, and take-all and there has been a great deal of research on wheat defence against fungal pathogens with promising results from both laboratory and field tests.

Several classes of genes have been explored to produce transgenic wheat resistant to fungal pathogens.

An obvious strategy involves the overexpression of R-genes (Brunner et al., 2011) that are effective in controlling diseases and are used in traditional breeding programs also if they do not confer durable resistance in agricultural environments.

Another successful approach propose the expression in transgenic wheat of defence responsive genes that encodes for PR proteins, such as β-1,3- glucanases (PR-2) (Bieri et al., 2003), chitinases

(PR-3) (Bliffeld et al., 1999; Shin et al., 2008; Rana., 2012; Huang et al., 2013), peroxidases (PR-9) (Altpeter et al., 2005), thaumatin-like proteins (TLPs) (PR-5) (Chen et al., 1999; Xing et al., 2008), ribosome-inactivating protein (RIPs) (Bieri et al., 2000; Balconi et al., 2007), plant lipid transfer proteins (LTPs) (PR-14) (Roy-Barman et al., 2006; Zhu et al., 2012) and thionins (PR-13) (Mackintosh et al., 2007).

This approach was extended by combining multiple PR genes or PR gene plus antifungal proteins (Chen et al., 1999; Oldach et al., 2001; Bieri et al., 2003; Rana, 2012).

Examples of pathogens tested with this strategy are reported in Table 1.

Infections tests were mainly performed in greenhouse and while moving to field trials results were confirmed in some cases (Mackintosh et al., 2007; Shin et al., 2008; Huang et al., 2013) or not (Anand et al., 2003).

Indeed it is very important to investigate not only the resistance against pathogens but also the interference with other pathways (Ioset et al., 2007) to avoid unintended side effects and mainly to not affect yields performances.

Another gene that proved its efficacy in fungal disease control is the *Arabidopsis thaliana* non-expressor of PR genes (NPR1) that produced high resistance to Fusarium Head Blight (FHB) in transgenic wheat plants because it regulates the activation of systemic acquired resistance and increases responsiveness to an endogenous activator of plant defense (Makandar et al., 2006). Also if it is very promising this strategy resulted unsuitable for improving overall resistance against different diseases in wheat (Gao et al., 2013).

Also a novel wheat receptor-like protein gene (RLP1.1) overexpressed in wheat plants gave interesting results; in fact, it conferred highly increased resistance to stripe rust caused by *Puccinia striiformis* f. sp. *tritici* (Jiang et al., 2013).

Indeed Xing et al. (2013) showed that the Hv-SGT1, a suppressor of the G2 allele of *skp1*, from *Haynaldia villosa,* that act as a critical signalling component required for mediated host cell death against various plant pathogens, enhanced resistance to both powdery mildew and FHB in transgenic wheat plants.

Another attracting strategy deals with the protection of wheat cell wall against cell wall degrading enzymes (CWDEs) through the overexpression of protein inhibitors to increase resistance against a broad spectrum of pathogens; examples of the effectiveness of this strategy were reported by Janni et al. (2008), Ferrari et al. (2012) and Volpi et al. (2011 and 2013).

Tab. 1 Examples of transgenic plants overexpressing defence responsive genes. For each paper, the gene/s, the protein class and the pathogens against which they confer resistance are indicated.

Genes	Classes	Pathogens	Reference
Wheat peroxidase (TaPERO)	PR-9	*Blumeria graminis* f.sp. *tritici* (powdery mildew)	Altpeter et al., 2005
Maize b-32	RIP	*F. culmorum* (Fusarium Head Blight)	Balconi et al., 2007
Barley seed chitinase, Barley seed β-1,3-glucanase	PR-3, PR-2	*Blumeria graminis* f.sp. *tritici* (powdery mildew)	Bieri et al., 2003
Barley-seed class II chitinase	PR-3	*Erysiphe graminis* (powdery mildew)	Bliffeld et al., 1999
Rice thaumatin-like protein (tlp)	PR-5	*Fusarium graminearum* (Fusarium Head Blight)	Chen et al., 1999
Rice chitinase RC24	PR-3	*Puccinia striiformis* f. sp *tritici* (stripe rust)	Huang et al., 2013
Wheat α-1-purothionin, barley thaumatin-like protein (tlp-1), barley class-II β-1,3-glucanase	PR-13, PR-14, PR-2	*Fusarium graminearum* (Fusarium Head Blight)	Mackintosh et al., 2007
Barley-seed class II chitinase, *Aspergillus giganteus* antifungal protein (Ag-AFP)	PR-3, antifungal protein	*Erysiphe graminis* f. sp. *tritici* (powdery mildew), *Puccinia recondita* f. sp. *tritici* (leaf rust)	Oldach et al., 2001
Chitinase and chitosanase from *Trichoderma harzianum*	PR-3	*Erysiphe graminis (*powdery mildew)	Rana, 2012
Allium cepa antimicrobial protein (Ace-AMP1)	PR-14	*Blumeria graminis* f.sp. *tritici* (powdery mildew), *Neovossia indica* (karnal bunt)	Roy-Barman et al., 2006
Barley-seed class II chitinase	PR-3	*Fusarium graminearum* (Fusarium Head Blight)	Shin et al., 2008
Wheat Thaumatin-Like Protein	PR-5	*Erysiphe graminis (*powdery mildew), *Fusarium graminearum* (Fusarium Head Blight)	Xing et al., 2008
Wheat lipid transfer protein 5 (TaLTP5)	PR-14	*Fusarium graminearum* (Fusarium Head Blight), *Cochliobolus sativus* (common root rot)	Zhu et al., 2012

Additional experiments involved proteins or small peptides with antifungal properties of plant origin or from other kingdoms. For example defensins or puroindolines were successfully used to improve wheat resistance against *Fusarium graminearum* and *Rhizoctonia cerealis* (Li et al., 2011), *F. culmorum* (Gerhardt et al., 2002), leaf rust (Luo et al., 2008) or *Penicillium* seed rot (Kim et al., 2012).

Examples of antifungal proteins from the animal kingdom are lactoferrin, which plays a major role in the immune system of newborns, or a fusion protein containing a *Fusarium*-specific antibody and an antifungal peptide; both enhanced resistance to FHB (Han et al., 2012; Lakshman et al., 2013; Li et al., 2008).

Indeed examples of small peptides includes MsrA2 and 10R, which reduce susceptibility to FHB and powdery mildew (Badea et al., 2013), potato antimicrobial peptide SN1, that increases resistance to take-all pathogen *Gaeumannomyces graminis* var. *tritici* (Rong et al., 2013), $Hpa1_{10-42}$ peptide of the Harpin Protein Hpa1, produced by the rice bacterial blight pathogen, that enhanced

resistance to powdery mildew (Wang et al., 2014) or the 'killer proteins' KP4 from *Ustilago maydis* viruses that increased resistance to *Tilletia caries* both in greenhouse (Clausen et al., 2000) and in field tests (Schlaich et al., 2006).

Another strategy to enhance wheat defence against fungal pathogen is the introduction of novel secondary metabolites for example the expression of the foreign phytoalexin resveratrol, by expressing the stilbene synthase genes from grapevine (*vst1*, *vst2*), improved disease resistance against two wheat-specific pathogens, *Puccinia recondita* f. sp. *tritici* and *Septoria nodorum* Berk (Serazetdinova et al., 2005).

A different approach that showed interesting results in the struggle against *Fusarium* pathogens involves the detoxification of toxins that cause loss of quality of final products because of health concerns (Desjardins et al., 2007) but are also virulence factors for such pathogens (Proctor et al., 2002). Successful wheat modifications using this strategy were reported by Okubara et al. (2002) and Di et al. (2010).

Finally, recent approaches exploit the use of transcription factors (TFs). These includes MYB, ethylene response factor (ERF) and WRKY families, that play important roles in biotic and abiotic stress crosstalks and signalling cascades through regulation of gene expression. The engineering at TF level may provide a transgenic tool for multiple-resistance in wheat breeding programs. For example Zhang et al. (2012) showed that the overexpression of a MYB transcription activator mediates host resistance to *Bipolaris sorokiniana* and drought stresses through regulation of defense- and stress-related genes.

Dong et al. (2010) reported that *TaPIE1*, a pathogen-induced ERF gene of wheat confers host-enhanced resistance to fungal pathogen *Bipolaris sorokiniana* in transgenic lines, while Zhu et al. (2014) showed that ERF1 overexpressing plants are protected against *Rhizoctonia cerealis* and freezing stresses. Interestingly Bahrini et al. (2011) showed that *TaWRKY45* overexpression confers resistance against multiple fungi such as *Fusarium graminearum*, that cause FHB, *Blumeria graminis*, a causal fungus for powdery mildew, and *Puccinia triticina*, a causal fungus for leaf rust.

Wheat wild relatives, naturally resistant to wheat diseases, are an interesting source of TFs for plant defence; Liu et al. (2013) used a *Thinopyrum intermedium* MYB transcription factor *TiMYB2R-1* to enhance resistance to the take-all disease in transgenic wheat. The same wheat wild relative was used to transfer a TF belonging to ERF family, *TiERF1*, whose overexpression enhances resistance to sharp eyespot caused by *Rhizoctonia cerealis* (Chen et al., 2008).

The investigation of the plant immune system and of the molecules involved in defence response will produce more informations that could be effectively used to improve wheat resistance against fungal pathogens.

1.3 The plant immune system

Although lacking an immune system comparable to animals, plants have developed a stunning array of structural, chemical and protein-based defences designed to detect invading organisms and stop them before they are able to cause extensive damage (Freeman et al., 2008).

Immune systems discriminate self from non-self and activate tightly regulated host defense responses to minimize the damage inflicted by harmful agents (Coll et al., 2011).

Typical defence reactions and cellular responses are initiated right after a plant comes into contact with a pathogen; they can be subdivided into very early or early responses (1–30 min post-pathogen contact) and late responses (hours–days post-pathogen contact) (Boller and Felix, 2009). Very characteristic early responses are ion fluxes, the production of reactive oxygen species (ROS), ethylene production, mitogen-activated protein kinase (MAPK) activation and the expression of typical defence-related genes, while late responses include cell wall modifications such as callose deposition or seedling/root growth inhibition (Albert, 2013).

A clearly visible resistance reaction is represented by the hypersensitive response (HR) including programmed cell death (PCD) of the infected tissue. This necrosis is mediated via resistance proteins encoded by resistance genes (R-genes) and restricts the growth of the pathogen to prevent the plant from further damage (Lukasik and Takken, 2009). In addition to the above mentioned locally restricted responses, systemic responses can also be initiated by the plant, termed systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Fu and Dong, 2013).

However, a successful defence is dependent on a highly sensitive and specific recognition system with the ability to sense ‘danger’ and consequently to switch on plant defence. Pathogens, in turn, provide characteristic ‘patterns’ that serve as a ‘molecular identity card’ and allow the plant to identify the external invader (Albert, 2013). Prominent examples for such microbe-associated molecular patterns (MAMPs) derive from typical microbial structures such as fungal chitin (Felix et al., 1993), bacterial peptidoglycan (Gust et al., 2007), or flagellin (Felix et al., 1999). In addition, many pathogens utilize degrading and cleaving enzymes during plant infection that damage plant cells and generate characteristic degradation products that can serve as so-called ‘damage-associated molecular patterns’ (DAMPs), for example oligogalacturonides or cellulose fragments are potent triggers of plant defence and are generated during pathogen or herbivore attacks (Nühse, 2012). DAMPs can also be peptides derived from cytosolic precursor proteins, extracellular secreted precursors or that resulted from degradation of proteins with distinct primary functions (Albert, 2013).

To sense these molecular patterns indicative of a pathogen attack, plants possess an array of different pattern recognition receptors (PRRs). Plant PRRs are transmembrane receptors, the best-studied class of plant PRRs are receptor-like kinases (RLKs), which feature an ectodomain of leucine-rich repeats (LRRs) involved in MAMP perception, and an intracellular kinase domain, involved in signal transduction relay via MAPK cascades, resulting in MAMP triggered immunity (MTI) (Coll et al., 2011).

MAMP-triggered defence responses represent the first layer of immunity; DAMPs might come into play later and might be considered as inducers of a second wave of plant defence, leading to a prolongation or amplification of the cellular defence response (Fig. 2) (Albert, 2013).

Many microbial pathogens evolved mechanisms to suppress this host defence with so-called 'effector proteins' that interact or enter into the plant cell. In turn, many host plants have evolved detection systems for such effectors and mount effective second layers of immunity termed effector-triggered immunity (ETI) (Spoel and Dong, 2012). This interplay between plants and pathogens follows the concept of the so-called 'zig-zag' model defined by Jones and Dangl (2006).

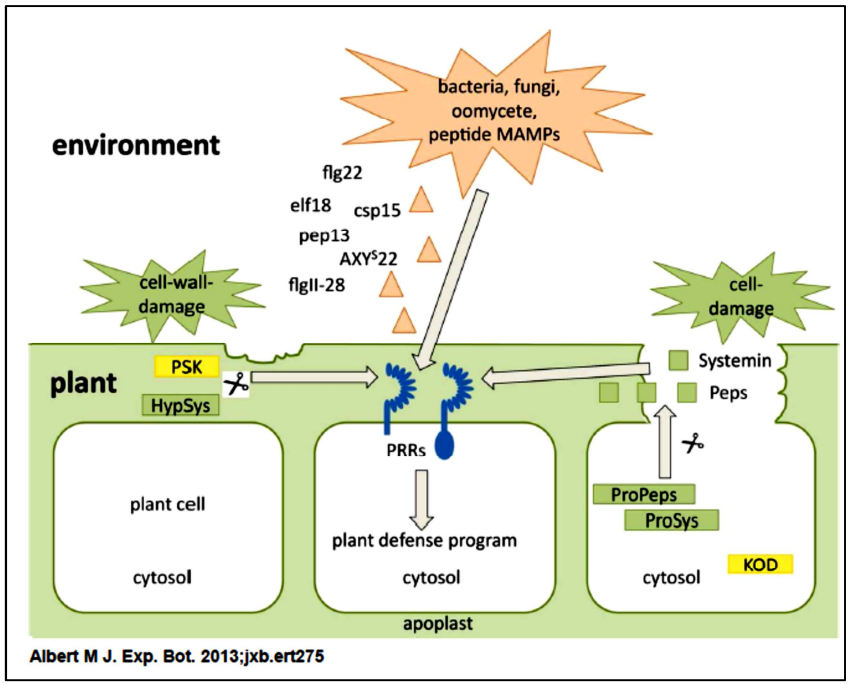

Fig. 2 Attacked plants can sense peptides as microbe-associated molecular patterns (MAMPs; e.g. flg22, elf18, csp15, flgII-28, pep13, axY^{S}22) directly via pattern recognition receptors (PRRs, blue). Furthermore, after cell or cell wall damage, plants sense damage-associated molecular patterns (DAMPs) derived from cytosolic precursor proteins after they are cleaved off (e.g. systemin from prosystemin; Peps from proPeps). Other DAMPs (HypSys, hydroxyproline-rich systemins) or DAMP candidates (PSKs, phytosulphokines) are located to the apoplast where they might become activated after plant damage. Peptides that are not yet defined as DAMPs but might be considered as DAMP candidates are indicated in yellow (PSK, kiss of death (KOD)). (Albert, 2013)

1.4 The plant cell wall: structure and composition

Cell walls are important features of plant cells that perform a number of essential functions, including providing cell shape; moreover, forming the interface between adjacent cells often play important roles in intercellular communication and because of their surface location they are involved in plant-microbe interactions, including defence responses against potential pathogens (Keegrstra et al., 2010).

Plant cell walls are usually divided in primary walls, that surround growing cells or cells capable of growth, and secondary walls that are thickened structures containing lignin and surround specialized cells such as vessel elements or fiber cells.

The most characteristic component found in all plant cell walls is cellulose, a linear polymer of (1–4)-linked β-D-glucose; several cellulose chains are linked to form crystalline aggregates called microfibrils that are deposited around each cell in a random framework and interlocked by hemicelluloses, leading to a strong yet flexible network. The most abundant hemicellulose in dicots is xyloglucan, a linear backbone of (1–4)-linked β-D-glucose substituted at regular sites with D-xylosyl residues that can be further extended by galactosyl, fucosyl or arabinosyl residues. The major hemicellulose in monocots, especially grasses, is xylan, which is a polymer of (1–4)-linked β-D-xylose. This backbone can be decorated with various substituents, including arabinose, glucuronic acid, 4-*O*-methylglucuronic acid and acetyl side groups. The arabinosyl residues can also be esterified with ferulic and *p*-coumaric acid (Lagaert et al., 2009). Xylan can represents up to 40% in the cell wall of grass species, while it represents a minor constituent in dicots (5%) (Vogel, 2008) (Fig. 3).

The cellulose/hemicellulose network is embedded in a jelly-like matrix of pectin, the most complex class of wall polysaccharides. Pectins are a family of complex and highly heterogeneous polysaccharides whose backbones are highly enriched in α-1,4-linked-D-galacturonic acid residues, with various degrees of methyl esterification. Pectins account for about 30% of the primary walls of dicot and non-graminaceous monocot plants and for about 5–10% of the walls of grasses (Vogel, 2008) (Fig. 3). The most abundant and best studied pectic polysaccharides are homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (Harholt et al., 2010).

Plant cell walls also contain many proteins and glycoproteins, including various enzymes and structural proteins (Rose and Lee, 2010).

Approximate composition[a] (% dry weight) of typical dicot and grass primary and secondary cell walls

	Primary wall		Secondary wall	
	Grass	Dicot	Grass	Dicot
Cellulose	20–30[b,c]	15–30[c,d,e]	35–45[c,f]	45–50[c]
Hemicelluloses				
Xylans	20–40[d]	5[c]	40–50[c,g]	20–30[c,g]
MLG	10–30[d]	Absent	Minor	Absent
XyG	1–5[c,d,g]	20–25[g]	Minor	Minor
Mannans and glucomannans	Minor	5–10[d]	Minor	3–5[g]
Pectins	5[c]	20–35[d]	0.1[c]	0.1[c]
Structural proteins	1[d]	10[d,e]	Minor	Minor
Phenolics				
Ferulic acid and ρ-coumaric acid	1–5[c,d]	Minor (except order Caryophyllales)	0.5–1.5[c]	Minor (except order Caryophyllales)
Lignin	Minor	Minor	20[c]	7–10[c]
Silica			5–15[c]	Variable

Fig. 3 Composition of grass and dicot cell walls (Vogel, 2008).

1.5 Cell wall degrading enzymes

To penetrate and use plant cell walls as nutrition source, pathogens secrete a remarkable array of carbohydrate activity enzymes (CAZymes) (Zhao et al., 2014). At present, the CAZymes have been grouped into four functional classes: glycoside hydrolase (GH), glycosyltransferase (GT), polysaccharide lyase (PL), and carbohydrate esterase (CE) on the basis of their structurally-related catalytic modules or functional domain (Cantarel et al., 2009). In particular GHs hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety, such as a protein or a lipid (Cantarel et al., 2009).

Among the CAZymes, classes CE, GH, and PL are often known as cell wall degrading enzymes (CWDEs) due to their important roles in plant biomass decomposition by fungi and bacteria (Ospina-Giraldo et al., 2010).

CWDEs are ubiquitous among pathogenic and saprophytic microorganisms, including bacteria, nematodes, and fungi (Walton et al., 1994; Zhao et al., 2014). They could be required for penetration, for spreading once a pathogen is inside the plant, and/or for obtaining nutrients from wall polymers. They could also be important to a pathogen to survive on dead tissue or in the absence of a suitable host, as a free saprophyte. This is a plausible explanation for the existence of CWDEs that appear not to be important in pathogenesis (Walton et al., 1994).

In general fungal dicot pathogens have more pectinases which agrees with the fact that cell walls of dicots are composed of higher levels of pectin than monocots (Zhao et al., 2014). Although dicot and monocot plants have different amounts of hemicelluloses in their primary cell wall, they share approximately the same level in the secondary cell wall (Fig. 3) and their fungal pathogens have no significant differences in the diversity or number of enzymes related to the hemicellulose degradation (Zhao et al., 2014).

The number of enzymes involved in cellulose depolymerization has no significant differences between dicot and monocot fungal pathogens, which agree with the fact that dicots and monocots are composed of similar levels of cellulose (Fig. 3) (Zhao et al., 2014).

The CWDEs produced by parasites play a central role in host-pathogen interactions; in several plant pathogens pectinases and xylanases were demonstrated to be related to pathogenicity or virulence, these important degrading enzymes will be discussed in next two paragraphs.

1.5.1 Pectin degrading enzymes

Pectin degrading enzymes weaken the plant cell wall and expose other polymers to degradation by hemicellulases and cellulases. They are the first cell wall degrading enzymes that are secreted by pathogens and some of them have been demonstrated to be important virulence factors (Lagaert et al., 2009). Among the best known microbial pectic enzymes are polygalacturonases, pectate lyases, pectin lyases and pectin methylesterases. Polygalacturonases (PGs) (GH28) hydrolyze the linkage between galacturonic acids in unmethylated homogalacturonan. Among pectinolytic enzymes, PGs play a primary role and, in some plant pathogen interactions, they act as virulence factors. Demonstration for this role have been reported for *Botrytis cinerea* on tomato (ten Have et al., 1998), *Alternaria citri* on citrus (Isshiki et al., 2001), *Aspergillus flavus* on cotton (Shieh et al., 1997) and *Claviceps purpurea* on rye (Oeser et al., 2002).

Pectate and pectin lyases (PL and PNL) cleave the same linkage of PGs, but cleavage occurs via a β-elimination reaction resulting in the formation of an unsaturated C4–C5 bond at the non-reducing end of the cleaved polysaccharide; indeed PLs are specific for unmethylated substrates, while PNLs degrade the methylated forms. PL's role during pathogenicity is well documented and they act as virulence factors in many pathogens (Lagaert et al., 2009). Several bacterial and fungal phytopathogens contain genes encoding PNLs, but little information is available on their role during pathogenicity (Lagaert et al., 2009).

Pectin methylesterases (PME) catalyze the demethylesterification of homogalacturonan, thereby making the pectin available for degradation by polygalacturonases and pectate lyases (Lagaert et al., 2009).

PMEs are ubiquitous enzymes and are present not only in bacteria and fungi but also in plants. The importance of pectin de-methylation for pathogen colonization has been also suggested by the induction of host PME following bacterial infection in Arabidopsis (Raiola et al., 2011) and by the reduction of disease symptoms in transgenic plants overexpressing an inhibitor of endogenous PME (Lionetti et al., 2007; Volpi et al., 2011).

1.5.2 Xylanases

Endo-β-1,4-xylanases (EC 3.2.1.8) (hereafter xylanases) catalyze the initial breakdown of xylan by hydrolyzing internal β-1,4 bonds in the polymer backbone and therefore play a key role in the degradation of this complex polysaccharide, although several other hydrolytic enzymes are needed for efficient and complete degradation (Shallom and Shoham, 2003).

Xylanases belong mainly to glycoside hydrolase families 10 (GH10) and 11 (GH11), although some forms are also classified into families 5, 7, 8, and 43 (Collins et al., 2005).

GH10 xylanases typically have a high molecular weight (≥30 kDa) and a low pI (Subramaniyan and Prema, 2002). The overall structure of the catalytic domain of these enzymes is an eight fold β/α barrel resembling a 'salad bowl,' with the active site located in a cleft running across the barrel top (Collins et al., 2005). The functional domain may be accompanied by a carbohydrate-binding domain, which is in most cases specific for cellulose (Kulkarni et al., 1999). Two glutamate moieties act as catalytic residues of the enzymatic reaction (Henrissat and Davies, 1997; Rye and Withers, 2000). GH10 xylanases are generally less selective than GH11; furthermore, GH10 tend to release shorter oligosaccharides (Lagaert et al., 2009).

GH11 xylanases are generally characterized by a low molecular mass (typically around 22 kDa) and a high pI, although xylanases exhibiting acidic pI values are also naturally occurring in this family (Törrönen and Rouvinen, 1997). Their structure adopts a β-jelly roll fold, which has been likened to a right hand with a two–β strand 'thumb' forming a lid over the active site located in the 'palm' (Törrönen et al., 1994). Similarly to GH10 xylanases, two glutamates are implicated in the enzymatic hydrolysis; however, GH11 xylanases are usually more selective and release larger oligosaccharides, since substituents represent a more serious hindrance to their activity (Biely et al., 1997).

Xylanases are produced mainly by microorganisms but can also be found in plants, marine algae, protozoans, crustaceans, insects, and snails (Beliën et al., 2006). They naturally occur in cereals and are believed to be at the basis of a significant part of the variability in biotechnological functional properties of cereals. Variability in xylanase and xylanase inhibition activities in different cereals and the contribution of environment and genotype to this variability has been studied in the HEALTHGRAIN diversity screen (Gebruers et al., 2010).

Because of their ability to break down xylan, xylanases are increasingly used in a number of biotechnological processes, including bread-making, gluten–starch separation, improving nutritional

properties of animal feed, and the bleaching of cellulose pulp in paper manufacturing (Polizeli et al., 2005).

Since xylans represent a large proportion of the hemicellulosic fraction of cereal cell-wall matrices, xylan-degrading enzymes are expected to be important components of the offensive arsenal of cereal pathogens and may have a role similar to that of pectic enzymes in infection of dicotyledons (Beliën et al., 2006).

The role of xylanases in fungal pathogenicity has been suggested by many evidences. Indeed, several xylanase encoding genes have been identified in the genome of most phytopathogenic microorganisms (Zhao et al., 2014) and many of these genes are transcribed and the corresponding proteins secreted in the infected plant tissues (Wu et al., 1997).

Genetic redundancy or the occurrence of multiple xylanases in microorganisms has been put forward as one of the main reasons why the gene disruption mutants of xylanases remain pathogenic (Apel-Birkhold and Walton, 1996; Gómez-Gómez et al., 2002). Indeed, the disruption of none of the individual xylanase genes from *Cochliobolus carbonum* (Apel et al., 1993; Apel-Birkhold and Walton, 1996), *Fusarium oxysporum* (Gómez-Gómez et al., 2001 and 2002), *Erwinia chrysanthemi* (Keen et al., 1996) and *Magnaporthe grisea* (Wu et al., 1995) has been reported to dramatically affect pathogenicity. However, an alternative approach to evaluate the role of multiple xylanases in pathogenesis is the disruption of transcriptional regulators or conserved signal transduction components, as these proteins normally affect whole sets of xylanase genes. In this context, a *Cochliobolus carbonum* mutant disrupted in *SNF1*, a gene encoding a protein kinase required for expression of catabolite-repressed genes, showed reduced virulence and strongly reduced expression of cell wall degrading enzymes, including xylanases (Tonukari et al., 2000).

The contribution of xylanase 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 xylanases can 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 *Trichoderma viride*, respectively, which induce necrosis and defense responses in plants (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 an 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).

Recently Sella et al. (2013) reported that a *Fusarium graminearum* xylanase, expressed during wheat infection and sharing seven of 11 conserved amino acids regarded as essential for eliciting necrosis in plant tissues, is a necrotizing factor but is not essential for virulence in wheat. This was the first finding of a xylanase inducing necrosis in monocot and suggests a wider role of these fungal enzymes, involved not only in cell wall degradation but also in elicitation of cell death. Another defense signaling pathway that plays a role in the response to elicitation with xylanase is the mitogen-activated protein (MAP) kinase cascade. Indeed, the tomato MAP kinase *LeMPK3* is induced at the mRNA level upon treatment with EIX (Mayrose et al., 2004).

The relevance of xylanases in pathogenesis would particularly hold true for necrotrophic pathogens, and differential expression of xylanase genes in hemibiotrophic pathogens maybe associated with switching from the biotrophic relationship to the necrotrophic phase. For example correlation of *in planta* endo-β-1,4-xylanase activity with the necrotrophic phase of the hemibiotrophic fungus *Mycosphaerella graminicola* was shown by Siah et al. (2010).

1.6 Inhibitors of cell wall degrading enzymes

Among the defence mechanisms that plants use to counteract CWDEs there are protein inhibitors which contrast the activity of these degradative enzymes (Juge et al., 2006). These inhibitors include polygalacturonase inhibiting protein (PGIP), xylanase inhibitor (XI), pectin lyase inhibiting protein (PNLIP), xyloglucan-specific endoglucanase inhibitor protein (XEGIP) and pectin methyl esterase inhibitor (PMEI) (Lagaert et al., 2009).

Some inhibitors are organized in gene families and consists of several members with different regulation during development, response under stress conditions and recognition specificity.

The inhibitors of microbial cell wall degrading enzymes are located in the cell wall, as a sort of molecular sentinels, to perceive the potential pathogen and for some of them evidences *in planta* of their involvement in host defence against fungal pathogens have been reported.

Evidences of the protective role of PGIP, PME and XI will be discussed below.

1.6.1 Polygalacturonase inhibiting protein

Polygalacturonase inhibiting proteins (PGIPs) are plant cell wall leucine-rich repeat (LRR) glycoproteins that inhibit fungal and insect endopolygalacturonases (PGs) (De Lorenzo et al., 2001).

The genes encoding PGIPs have been characterized in a number of plants, including both monocot and dicot species. Different *Pgip* genes of a single species can undergo a different regulation following plant growth and stress conditions and PGIPs can also differ in their inhibition properties (De Lorenzo et al., 2001). It has been suggested that this variability reflects the need to adapt both their regulation and recognition features to combat pathogens more efficiently (D'Ovidio et al., 2004).

The hexaploid wheat (*Triticum aestivum*, genome AABBDD) genome contains one *Pgip* gene per genome: *Tapgip1* (B genome) and *Tapgip2* (D genome) are expressed in all tissues (Janni et al., 2006), whereas *Tapgip3* (A genome) is inactive because of a long terminal repeat (LTR), *Copia* retrotransposon insertion within the coding region (Di Giovanni et al., 2008).

Tapgip1 and *Tapgip2* are induced following fungal infection and wounding suggesting an involvement in the wheat defence response at the site of pathogen entry (Janni et al., 2013).

The involvement of PGIP in plant defence has been demonstrated in different plant species, including tomato (Powell et al., 2000), Arabidopsis (Ferrari et al., 2003 and 2006), tobacco

(Manfredini et al., 2005; Joubert et al., 2006), grape (Agüero et al., 2005) and wheat (Janni et al., 2008; Ferrari et al., 2012). In all cases, the overexpression of PGIP was correlated with a reduction of symptoms caused by fungal and bacterial pathogens. In particular, the expression of a bean polygalacturonase-inhibiting protein (PvPGIP2) in wheat transgenic plants limits disease symptom development caused by *B. sorokiniana* and *F. graminearum* (Janni et al., 2008; Ferrari et al., 2012). Recently, PvPGIP2 has been shown to be effective in improving tobacco plant resistance against a broad spectrum of pathogens and in one case the protection was maintained also in field experiments (Borras-Hidalgo et al., 2012).

1.6.2 Pectin methyl esterase inhibitor

Pectin methyl esterase inhibitor (PMEI) does not affect fungal or bacterial PME activity but inhibits the de-methylation activity of the endogenous PME (Giovane et al., 2004; Pelloux et al., 2007). The importance of this inhibitor in plant defence is related to its capacity to maintain a high degree of pectin methyl esterification, while inhibits the endogenous PME, and this should reflect in a reduced ability of PG to hydrolize the methyl esterified cell wall pectin (Bonnin et al., 2002; Limberg et al., 2000).

As reported for model plant species, wheat contains a *Pmei* family whose members are highly variable in sequence and can play tissue specific roles in controlling the endogenous activity of specific PME isoforms (Rocchi et al., 2012).

The effectiveness of PMEIs in controlling PME activity was demonstrated *in vivo* through the overexpression of PMEI from Arabidopsis and kiwi in transgenic plants of Arabidopsis and wheat, respectively. These transgenic plants showed also an increased level of pectin methyl esterification and a reduced disease symptom development caused by fungal and bacterial pathogens (Lionetti et al., 2007; Volpi et al., 2011; Raiola et al., 2011). Arabidopsis transgenic plants overexpressing PMEI showed also a reduced virus spread (Lionetti et al., 2014).

1.6.3 Xylanase inhibitors

Cereals contain xylanase inhibitor (XI) proteins which inhibit the activity of endo-β-1,4-xylanases of microbial origin. These inhibitors were discovered as thermo-labile, water soluble proteins that interfere with the activity of xylanases in food processing (Debyser et al., 1997).

Since now three classes of XI has been discovered: *Triticum aestivum* xylanase inhibitor (TAXI) (Debyser et al., 1999), Xylanase inhibitor protein (XIP) (McLauchlan et al., 1999) and Thaumatin like- xylanase inhibitors (TL-XI) (Fierens et al., 2007) .

XIs have different inhibition abilities: XIP-type inhibitors can counteract xylanases from GH10 and GH11 families, while TAXI type and TL-XI are able to inhibit only GH11 xylanases. These inhibitors have also different kinetic properties that is competitive for TAXI and XIP and non-competitive for TL-XI (Dornez et al., 2010a).

XIP and TAXI include several family members with different inhibition specificities while to date only one TL-XI is known (Dornez et al., 2010a).

Although there is variation in the expression of these genes, most of them are mainly expressed in the endosperm tissue where the corresponding proteins accumulate at a remarkable level (Croes et al., 2009; Dornez et al., 2010a). Immunoblot analyses also showed the lack of accumulation of XI in leaf, stem, and root tissues of wheat plants at the ripening stage (Croes et al., 2009).

The XIs classes and observations suggesting the involvement of these inhibitors in plant defence are discussed below.

1.6.3.1 *Triticum aestivum* xylanase inhibitor (TAXI)

The first TAXI protein was purified from wheat in 1999, it is characterized by two molecular forms, an A form that is a single polypeptide chain of ~40 kDa and a B form that is a product of limited proteolysis of the former one and consists of two polypeptides of approximately 30 and 10 kDa, held together by a disulfide bridge (Debyser et al., 1999). TAXI-I has alkaline pI value (Goesaert et al., 2004) and is a slightly glycosylated protein (Sansen et al., 2004; Croes et al., 2008).

Different isoforms of TAXI have been discovered in wheat (TAXI-I, TAXI-IB, TAXI-II, TAXI-IIB, TAXI-III); they are active against bacterial and fungal xylanases of GH11 family but not against GH10 xylanases from different origin, including all the plant xylanases identified so far (Simpson et al., 2003). TAXI isoforms showed different inhibition profile and because of this they can be classified in two groups based on the ability to inhibit GH11 xylanases from *A. niger* and *B.*

subtilis; group I (TAXI-I, TAXI-IB, TAXI-III) can inhibit xylanases from both microorganisms, while group II (TAXI-II, TAXI-IIB) can inhibit only the *B. subtilis* xylanase (Dornez et al., 2010a).
TAXI-type xylanase inhibitors were found also in other cereals such as durum wheat, rye and barley, while so far no TAXI isoforms has been purified from rice, maize, oats and buckwheat (Goesaert et al., 2004).
The determination of the structure of TAXI-I showed that the protein folds in two β-barrel domains with a few helical segments, divided by an extended cleft and displays structural homology with the pepsin-like family of aspartic proteases but is proteolytically non-functional (Sansen et al., 2004).
The structure of the complex TAXI-I-*Aspergillus niger* xylanase I indicated a competitive inhibition strategy due to the direct interaction of the inhibitor with the active site region of the enzyme, mimicking substrate contact and filling the whole substrate-docking region (Sansen et al., 2004).
Mutational and biochemical analyses of TAXI-I, TAXI-II and xylanase A of *Bacillus subtilis* showed that inhibition strength and specificity depend on the identity of only a few key residues of inhibitor and xylanase (Pollet et al., 2009a).
The first *Taxi* gene (AJ438880) was isolated by Fierens and colleagues in 2003 from *T. aestivum* cv Estica. This gene is composed of an uninterrupted open reading frame of 1,205 bp that encodes a signal peptide of 21 amino acids followed by a mature protein of 381 amino acids with a predicted molecular mass of 38.8 kDa (Fierens et al., 2003). The recombinant TAXI-I produced in *E.coli* maintained the inhibition properties of the protein purified from wheat flour (Fierens et al., 2003).
Moreover, *Taxi-I* gene was localized on chromosome 3B by PCR on nulli-tetrasomic lines of *T. aestivum* cv. Chinese spring (Raedschelders et al., 2004).
Other *Taxi* genes have been identified in wheat: *Taxi-IIA* (AJ697849), *Taxi-IIB* (AJ697850), *Taxi-IB* (AJ697851) (Raedschelders et al., 2005), *Taxi-III* (AB114627.1), *Taxi-IV* (AB114628.1) (Igawa et al., 2004), *Taxi-I 725ACCN* (EU082811.1), *Taxi-I 725ACC* (EU082810.1), *Taxi-801NEW* (EU082815.1), *Taxi-602OS* (direct submission by Raedschelders et al., 2007).
Taxi-IB and *Taxi-III* are considered isoforms of the same gene due to the high identity 99,6% and equal inhibition abilities and the same is for *Taxi-IIB* and *Taxi-IV* (99,8% identity) (Dornez et al., 2010a). Sequences belonging to *Taxi* family were found also in other cereals such as durum wheat, spelt wheat, emmer wheat, einkorn wheat, rye and barley, indicating a conserved distribution of these genes in grass species (Dornez et al., 2010a).

Transcript analysis in bread wheat showed that genes of the *Taxi* family have different expression profiles: *Taxi-I* is expressed in roots till 10 days after germination (DAG) and in shoot only at 3

DAG; this gene is expressed also in immature embryo and ovary (Igawa et al., 2005). In comparison with *Taxi-I*, *Taxi-III* and *Taxi-IV* showed the same level of expression in shoots, a higher expression in roots till 10 DAG and a lower expression in ovary and immature embryos (Igawa et al., 2004).

Taxi-I, Taxi-III and *Taxi-IV* are all induced by wounding but are insensitive to Salicilic acid (SA). However, they undergo a different regulation with other stressors. For example, following *Erisiphe graminis* and *Fusarium graminearum* infection and methyl jasmonate (MeJA) treatment, *Taxi-III* and *Taxi-IV* are more strongly induced than *Taxi-I* (Igawa et al., 2004 and 2005).

These findings suggests a different role of these genes. *Taxi I* that is less inducible than *Taxi III* and *Taxi IV* is supposed to be involved in a basal defense response while the high inducibility of the other two genes suggests a major involvement in the induced stress response (Igawa et al., 2004 and 2005).

In contrast with these expression data, proteomic studies in bread wheat caryopsis after spikelets infection with *Fusarium graminearum ΔTri5,* a less virulent mutant, found a two-three fold increase of TAXI-I and TAXI-II while other TAXI isoforms were not induced and TAXI-III was not detected (Dornez et al., 2010b).

1.6.3.2 Xylanase inhibitor protein (XIP)

XIP-I was the first member of XIP family that was purified from wheat flour; it is a glycosylated, monomeric, basic protein of 29 kDa with a unique N-terminal sequence and can inhibit the activity of *Aspergillus* sp. xylanases (McLauchan et al., 1999).

Similarly to all the xylanase inhibitors, XIP-types inhibitors are mainly present in kernel where XIP-I, XIP-III and XIP-R2 were found (Croes et al., 2009). Moreover, XIP-type inhibitors are widespread in cereals, with a higher level in wheat and rye respect to durum wheat, oats and barley (Gebruers et al, 2010).

Different XIP family members showed different inhibition specificities. For example XIP-R1 does not affect the activity of a GH11 xylanase from *A. niger* that instead is inhibited by XIP-I (Takahashi-Ando et al., 2007). Although the inhibition properties of XIP-III have not been characterized yet, it is expected that it could have different inhibition abilities respect to XIP-I since their limited amino acid differences are located in two regions involved in the inhibition of GH10 xylanases.

Among the XIP-type inhibitors, XIP-I has a broad spectrum of inhibition. It inhibits xylanases from

both GH10 and GH11 families and it is also able to block the activity of two barley amylases (AMY1 and AMY2) belonging to GH13 family (Sancho et al., 2003).

The three dimensional structure of XIP-I, determined by X-ray crystallography, is represented by a $(\beta/\alpha)_8$ barrel fold decorated at the top by loops arranged to form a long depression along one side of the molecule (Payan et al., 2003). The crystal structures of XIP-I in complex with GH10 *Aspergillus nidulans* xylanase on one hand, and with GH11 *Penicillium funiculosum* xylanase on the other hand, revealed that XIP-I possesses an independent enzyme-binding site for each family of xylanases and that it is a competitive inhibitor, as reported for TAXI-type inhibitors (Payan et al., 2004). Substrate mimicry in the active site is the key element of the competitive inhibition and structural properties of xylanases are important for inhibitor recognition. Indeed, extended loops insertion in xylanases neighbouring the active site causes steric clashes with the inhibitor bringing to no recognition (Payan et al., 2004).

The first *Xip-I* gene (AJ422119) was cloned from bread wheat in 2002; the gene is a 822 bp open reading frame that codes 274 amminoacids, with a predicted signal peptide 30 amino acids long, and the protein has a calculated molecular mass of 30.2 kDa (Elliott et al., 2002). The recombinant *Xip-I* expression in *E. coli* confirmed the inhibitory activity against xylanases even if the protein rapidly lost its biological activity because of lack of appropriate glycosylation and disulphide bond formation (Juge et al., 2006).

Other two *Xip-I* sequences were reported from different wheat accessions: KC342667.1 from Zhengmai9023 (direct submission by Chen et al., 2012) and GU318402.1 from Zhengmai366 (direct submission by Chen et al., 2009).

Since now three *Xip* genes sequences, different from *Xip-I*, have been identified in bread wheat: *Xip-III* (AB204556.1) (Igawa et al., 2005), *Xip-R1* (AB302972.1), *Xip-R2* (AB302973.1) (Takahashi-Ando et al., 2007), and only one in durum wheat *Xip-II (*AJ318884 *)* (Elliott et al., 2009), but only XIP-I and XIP-R1 have been characterized for their ability to inhibit xylanases.

Xip genes were also reported from other cereals as spelt wheat, emmer wheat, einkorn wheat, rye, barley, rice, sorghum, pearl millet and oats (Dornez et al., 2010a).

The study of *Xip* expression revealed that *Xip-I* is mainly expressed in caryopsis but it was found also in immature embryos and in young seedlings, shoots and roots (Igawa et al., 2005). *Xip-I* expression is induced by wounding, elicitors such as SA and MeJA, and also by biotrophic pathogens such as *Erisiphe graminis* (Igawa et al., 2005). It was also reported that *Xip-I* is not induced following *Fusarium graminearum* infection (Igawa et al., 2005). Proteomic studies following wheat infection with a less virulent mutant strain of *F. graminearum* confirmed this

results by showing that only XIP-III is induced during infection while other XIP isoforms are not induced and glycosylated isoforms are repressed (Dornez et al., 2010b).

1.6.3.3 Thaumatin like- xylanase inhibitors (TL-XI)

The last XI type identified in wheat is Thaumatin like- xylanase inhibitor (TL-XI). This inhibitor was purified from wheat whole meal where it is less present than the other XIs. In fact, while purification yields for TAXI and XIP were approximately 14-15 mg/Kg (McLauchan et al., 1999; Gebruers et al., 2002), the yields for TL-XI was 2.5 mg/Kg (Fierens et al., 2007).

The name of this class of inhibitors derives from the high (>60%) similarity with thaumatin-like proteins (TLPs) and because it contains the Prosite PS00316 thaumatin family signature.

TL-XI is a basic protein (pI 9.3), with a molecular mass of approx 18 kDa and it occurs in wheat with varying extents of glycosylation (Fierens et al., 2007).

The *Tl-xi* gene was cloned from bread wheat genome and encodes for a mature protein of 151 amino acids preceded by a signal peptide of 26 amino acids (Fierens et al., 2007).

The mature TL-XI protein was expressed successfully in *Pichia pastoris*, resulting in a 21 kDa recombinant protein. Polyclonal antibodies raised against TL-XI purified from wheat react with epitopes of rTL-XI as well as with those of thaumatin, demonstrating high structural similarity between these three proteins (Fierens et al., 2007).

The TL-XI structure model, generated with the crystal structure of thaumatin as template, shows the occurrence of five disulphide bridges and three β-sheets that makes the protein very stable, much more than the other two xylanase inhibitors from wheat (Fierens et al., 2009).

TL-XI belongs to a group of smaller TLPs, which are found mainly in cereals. Only one TLP, zeamatin, showing 43% identity with TL-XI, is known to inhibit enzymes, namely α-amylases and trypsin (Schimoler-O'Rourke et al., 2001), but TL-XI is the first known to have xylanase inhibiting activity.

TL-XI has a unique inhibition specificity. Unlike the previous described XIs that showed a competitive inhibition and binds to active site of xylanases, TL-XI is a non-competitive inhibitor. It inhibits a number of glycoside hydrolase family 11 xylanases from both fungal and bacterial origin, but it is inactive towards high pI GH11 and GH10 xylanases (Fierens et al., 2007).

TLPs belong to the class of PR-5 proteins due to their antifungal ability (Van Loon et al., 2006); although the mechanism is not fully understood, it has been hypothesized that the binding of TLPs to fungal β-D-glucans prevents proper fungal wall assembly during hyphal extension (Osmond et

al., 2001). Because of the high homology with TLPs and the similar strategy for antifungal activity, that is proposed to be due to the binding to fungal β-D-glucans (Fierens et al., 2008), leading to disturbance of proper cell wall synthesis and/or modulation (Fierens et al., 2007), TL-XI can be classified into the PR-5 class (Dornez et al., 2010a).

1.6.3.4 The hypothesis of a XIs role in plant defense

The involvement of xylanase inhibitors in plant defense has been proposed early after their discovery and is supported by many observations.

First of all, XIs inhibits only xylanases from microbial origin. All the plant endogenous xylanases identified to date belongs to GH10 family (Simpson et al., 2003) against which TAXI and TL-XI are ineffective and also XIP-I is unable to inhibit them because their structure contains extended insertions that sterically prevent XIP-I inhibition (Payan et al., 2004).

Another argument that support XIs as defence related proteins is the large polymorphism that has been found in XIs families and also the different specificity against xylanases that resembles a co-evolution between enzymes and inhibitors as it has been suggested for other protein inhibitors involved in plant defense such as PGIPs (D'Ovidio et al., 2004).

Moreover XIs shares structural homology with pathogenesis related (PR) proteins: XIP can be classified in the PR-8 class due to the homology with type III chitinases (GH18) whose role in plant defense was demonstrated (Brogue et al., 1991). TL-XI can be allocated among PR-5 because of the homology with TLPs (Dornez et al., 2010a). TAXI was not annotated in a PR class but shares structural homology with proteins that are supposed to be involved in plant defense such as an extradermal glycoprotein from carrot (EDGP) that is induced by wounding (Satoh et al., 1992; York et al., 2004; Shang et al., 2005) and a xyloglucan specific endoglucanase inhibitor from tomato (XEGIP) that inhibits cell wall degrading glucanases (GH12) (Qin et al., 2003; York et al., 2004). Due to its novelty respect to known PR proteins, TAXI can be assigned into a new PR family (Dornez et al., 2010a).

Further interesting observations include the apoplastic localization of XIs, deduced by signal peptide analysis and demonstrated experimentally for XIP-R2 and rice XIPs (Takahashi Ando et al., 2007; Tokunaga et al. 2008). Moreover, XIs can bind to wheat arabinoxylans (Fierens et al., 2008) so they can localize in the cell wall and this support the hypothesis of a protective role against pathogens attack; this behaviour is similar to PGIPs that bind to plant cell wall and act as defense proteins (D'Ovidio et al., 2004).

XIs are also able to bind to fungal β-D glucans (Fierens et al., 2008), moreover the growth of certain fungi in a medium containing glucose as only carbon source is inhibited by TAXI and XIP, this may be due to the disturbing of proper cell wall synthesis and/or modulation as previously proposed for TLPs (Fierens, 2007).

The accumulation of XIs during grain development (Croes et al., 2009) supports their implication in plant defense infact this pattern was also observed in several defense proteins such as endo-β-1,3-glucanase, chitinases, α-amilase and wheatwin proteins (Dornez et al., 2010a).
Another aspect that support the hypothesis that XIs are involved in plant defence come from the analysis of 5' flanking regions of *Xi* genes. These regions showed *cis* acting elements implicated in stress response. For example in the upstream region of *Taxi-III* are present GCC boxes, W box, core sequence activation sequence-1 and a MYB type-I binding site, which are reported to be involved in stress response (Igawa et al., 2004). Also the 5' flanking regions of *TdXip-II* and *Tl-xi* contains *cis* acting elements that are supposed to activate expression in stress conditions (Elliott et al., 2009; Rombouts et al., 2007).
As reported above, *Xi* genes are induced by different stress conditions, including wounding, pathogens infection and stress elicitors treatment.
In conclusion, many findings support the hypothesis of an involvement of XIs in plant defense but until now there are no more direct evidences for their role *in planta.*

1.7 Aim of the work

Since the reinforcement of the cell wall barrier can be an efficient strategy to protect the host plant against microbial pathogens, the aim of the present work was to investigate the role of xylanase inhibitors in wheat defence against fungal pathogens.

To this aim we produced transgenic durum wheat plants overexpressing the xylanase inhibitor TAXI-III and tested these plants against the fungal pathogens *Fusarium graminearum* and *Bipolaris sorokiniana*. We analyzed also the capacity of TAXI-III to prevent the necrotizing activity of a xylanase secreted by *F. graminearum* and verified by modelling analysis the possible interaction between TAXI-III and this xylanase.

2. Materials and Methods

2.1 Isolation of *Taxi-III* gene

Genomic DNA from *T. aestivum* cv. Chinese Spring, *T. aestivum* cv. Bobwhite, and *T. durum* cv. Svevo was isolated using the protocol described by Tai and Tanksley (1990).

Taxi-IIICS, *Taxi-IIIBW*, and *Taxi-IIISV* were isolated by PCR from genomic DNA using the specific primer pair Taxi-III 101spF/ Taxi-III 101spR (Tab. 2), developed from the *Taxi-IIII* sequence of *T. aestivum* cv. Norin61 (AB114627) (Igawa et al., 2004).

PCR reaction was performed in a final volume of 50 µl using 100 ng of genomic DNA, 2.5 U of FastStart High Fidelity PCR system (Roche Diagnostics), 1× Taq PCR buffer, 50 ng of each of the two primers, and 100 µM of each deoxyribonucleotide. Amplification conditions were 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final step at 72°C for 5 min.

The amplicon size was confirmed by electrophoresis on a 1% agarose gel prepared in 1x TBE buffer (Tris, Boric acid and EDTA) containing 0.5 µg/ml ethidium bromide, using a molecular ruler (GD 1kb DNA Ladder, Genedirex).

DNA bands of expected sizes were purified using the "Wizard® SV Gel and PCR Clean-Up System" kit (Promega) and analysed by nucleic acid sequencing (MWG service, Ebersberg, Germany).

Sequences were aligned using the DNAMAN software (Lynnon Biosoft, Quebec, Canada).

The *Taxi-IIICS* gene was ligated into the pGEM-T Easy vector (Promega) following the manufacturer's protocols to obtain the pTaxi-IIICS and used to transform *E.coli* DH5α competent cells.

2.2 *Taxi-III* localization on wheat genome

Genomic DNA isolated from chromosome 3 nulli-tetrasomic and ditelosomic wheat (cv. Chinese Spring) was used for PCR localization of *Taxi-III* on wheat genome.

PCR reactions were performed in a final volume of 20 µl, using 50-100 ng of genomic DNA, 0.5 µM of the *Taxi-III* specific primer (Taxi-III 170F/ Taxi-III 5R) (Tab. 2) and GoTaq® Green Master Mix 2X (Promega). Each sample was also checked for housekeeping gene amplification using the same conditions and specific primers for Actin (TaAct 77F/ TaAct 312R) (Tab. 2) (AB181991).

Amplification conditions were 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 5 min.

Amplicons were confirmed by electrophoresis and nucleic acid sequencing.

Tab. 2 List of oligonucleotides used for gene localization, transcript analysis, screening and characterization of transgenic wheat lines by PCR.

Primer	Sequence (5'-3')
Taxi-III 101spF	CCATCTCCGTCCACTGGTGCGAGC
Taxi-III 101spR	GCCGTAGTCGACGAAGTACATTGT
Taxi-III 170F	GTCCACGTGCGAGGGTAGT
Taxi-III 5R	CGGGTGTTCTCCACTTTGAT
TaAct 77F	TCCTGTGTTGCTGACTGAGG
TaAct 312R	GGTCCAAACGAAGGATAGC
Taxi-III_BamHI_1F	AATAGGATCCATGGCACGGGTCCTCCTCCTG
Taxi-III_BamHI_2R	AATAGGATCCCTAGCTGCCGCAACCCGTAAAG
Ubi A2	CCTGCCTTCATACGCTATTTATTTGC
Taxi-III 2R	TGGAAGGGGATGGTGTAGAG
Taxi-III 1F	ATGGCACGGGTCCTCCTCCTG
Taxi-III 1R	CTAGCTGCCGCAACCCGTAAAG

2.3 RNA extraction and reverse transcription

Total RNA from different plant tissue (root, coleoptide, stem, leaf, caryopsis) was extracted using the RNeasy Plant Mini kit (QIAGEN) following the instructions of the manufacturer.
Reverse Transcription of 1 µg of total RNA was performed using the QuantiTect Reverse Transcription Kit (QIAGEN) and following the producer's protocol.

2.4 Transcripts analysis

2.4.1 RT-PCR

The analysis of transcript in different plant tissues was performed by RT-PCR in a total reaction volume of 20 µl using 1 µl of cDNA, 0.5 µM of each primer (Taxi-III 101spF/ Taxi-III 101spR), GoTaq® Green Master Mix 2X (Promega) and volume was adjusted with water.
Reaction conditions were: one cycle at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final cycle at 72°C for 5 min.
Each sample was also checked for housekeeping gene amplification (Actin, as above) using the same conditions.
The amplicons was confirmed by electrophoresis on agarose gel and by nucleic acid sequencing.

2.4.2 qRT-PCR

qRT-PCR experiments were performed using the iCycler (Bio-Rad Laboratories) and the Master Mix iQ^{TM}SYBER Green supermix (Bio-Rad Laboratories) containing the flurogenic SYBER Green I DNA binding dye. Oligonucleotide primer pairs used were Taxi-III 170F/ Taxi- III 5R for *Taxi-III* and TaAct 77F/ TaAct 312R for Actin (Tab. 2). The homolog genes *TaPr-1-1*, *TaPr-1-2* and *TaPr-1-3*, located on the short arm of wheat homologous group 5 (Lu et al., 2011, hereafter named *TaPr-1-1/2/3*), were simultaneously amplified by using previously developed primers for *Pr1.1* (Pr1.1 1F/ Pr1.1 290R) (Lu et al., 2006). The specificity of the primers was verified by nucleotide sequence of the specific amplicon. The Actin gene was used as housekeeping gene.
Total reaction volume was 15 µl and included 7.5 µlof (2×) Master Mix, 100 ng of cDNA, 0.5 µl of 10 µM each forward and reverse primers, and volume adjusted with water. Each reaction was made in triplicate. Reaction conditions were as follows: one cycle at 50°C for 2 min and 95°C for 5 min;

then, 40 cycles at 95°C for 40 s, 61°C for 40 s, and 72°C for 40 s; and, finally, 90 cycles at 55°C for 10 s, increasing the set point temperature after cycle 2 by 0.5°C.

The relative expression analysis was determined by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) (User Bulletin Number 2-P/N 4303859; Applied Biosystems, Foster City, CA, U.S.A.). Calculation and statistical analyses were performed by Gene Expression Macro Version 1.1 (Bio-Rad Laboratories).

The qRT-PCR experiments were performed on RNA samples from two different biological replicas.

2.5 Preparation of the pUBI::Taxi-IIICS construct

The pUbi::Taxi-IIICS construct was prepared by inserting the complete coding region of *Taxi-IIICS* into the *Bam*HI site of pAHC17 (Christensen and Quail 1996) under control of the maize *Ubiquitin1* promoter and *NOS* terminator. The *Bam*HI sites flanking the complete coding region of *Taxi-IIICS* were generated by PCR amplification using the forward and reverse primers Taxi-III_BamHI_1F and Taxi-III_BamHI_2R (Tab. 2), respectively. PCR reactions were performed in a reaction volume of 50 µl with 10 ng of plasmid DNA (pTaxi-IIICS), 2.5 U of FastStart High Fidelity PCR system (Roche Diagnostics), 1× Taq PCR buffer, 50 ng of each of the two primers, and 100 µM of each deoxyribonucleotide.

Amplification conditions were 1 cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 5 min.

The amplicon was digested with *Bam*HI (Promega) following the producer's protocol and then ligated using the T4 Ligase (Promega) into the *Bam*HI site of pAHC17, generating the pUbi::Taxi-IIICS construct (5,923 bp) (Fig. 4A). The correct sequence of pUbi::Taxi-IIICS and the insertion sites were confirmed by nucleic acid sequencing. The pUbi::Taxi-IIICS construct was used to transform *E.coli* DH5α competent cells.

The isolation of plasmid DNA was performed using plasmid Mini kit or Maxi kit (QIAGEN) depending on the desired yields, according to the protocols described by the producer.

2.6 Production of wheat transgenic plants expressing TAXI-IIICS

2.6.1 Media

Modified Murashige and Skoog (MS) media for wheat cellular cultures were used (Sears and Deckard, 1982) with MS Salt, Maltose, Thiamine-HCl, L-asparagine at pH 5.85 and Phytagel as gelling agent. After autoclaving at 121°C for 20 min, 2,4-dichlorophenoxyacetic acid (2,4-D) was added and the medium poured in 100 mm x 15 mm Petri dishes (except for the bombardment medium for which the Petri dishes were 60 mm x 15 mm) and let it solidify.
Media compositions are the same as indicated in Weeks et al. 1993 and are reported in Table 3.
Selection marker phosphinotricin (PPT) is present only in regeneration and rooting media.

2.6.2 Embryos isolation

Caryopses at 10 to 18 days post anthesis (Zadoks stage 72) from *Triticum durum* cv. Svevo plants, grown in the field, were collected and surface-sterilized using sodium hypochlorite 1% (10 min), 70% ethanol (15 min) followed by three times washing (5 min) with distilled water.
Immature embryos of 0.5 to 1 mm long were aseptically removed under the stereo microscope, placed with the scutella exposed on the dissecting medium (Fig. 5A) and stored in the dark at 23°C for 5 days for the callus induction.

Tab. 3 Culture media compositions.

Dissecting medium	• Murashige & Skoog Salt mixture 4.3 g/l; • Maltose 40 g/l; • Thiamine-HCl (25 mg/500 ml) 10 ml/l; • L-asparagine 0.15 g/l • 0.25% v/v phitagel • 2,4 D (0.5 mg/ml) 2 ml/500 ml
Recovery medium	• Murashige & Skoog Salt mixture 4.3 g/l • Maltose 40 g/l; • Thiamine-HCl (25 mg/500 ml) 10 ml/l; • L-asparagine 0.15 g/l • 0.25% v/v phitagel • 2,4 D (0.5 mg/ml) 2 ml/500 ml
Bombardment medium (Bomb sucrose)	• Murashige & Skoog Salt mixture 4.3 g/l; • Maltose 40 g/l; • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phitagel • Sucrose 171.5 g/l • 2,4 D (0.5 mg/l) 2 ml/500 ml
Regeneration medium	• Murashige & Skoog Salt mixture 4.3 g/l; • Maltose 40 g/l; • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phitagel • 2,4 D (0.5 mg/ml) 0.2 ml/500 ml • PPT (5 mg/l)
Rooting medium	• Murashige & Skoog Salt mixture 2.15 g/l; • Maltose 20 g/l; • Thiamine-HCl (25 mg/500 ml) 5 ml/l • L-asparagine 0.075 g/l • 0.25% v/v phitagel • PPT (5 mg/l)

2.6.3 Coating gold particles with DNA, bombardment and plant regeneration

Before the bombardment the coating of gold particles with DNA was performed.

30 mg of gold particles (0.6 µm) (Bio-Rad Laboratoires) (Fig. 5B) were resuspended in 500 µl of 100% ethanol. 35 µl of the suspension were aliquoted into 1.5 ml tubes, spun and the ethanol removed. The microprojectiles were then washed with cold sterile water (200 µl), spun and the supernatant discarded.

At this point, the gold particles were coated with plasmid DNA: the plasmid pUbi::bar (5,505 bp) (Fig. 4B), carrying the *bar* gene that confers resistance to phosphinotricin, was co-bombarded with pUbi::Taxi-IIICS (5,923 bp) (Fig. 4A) in a 1:3 molar ratio.

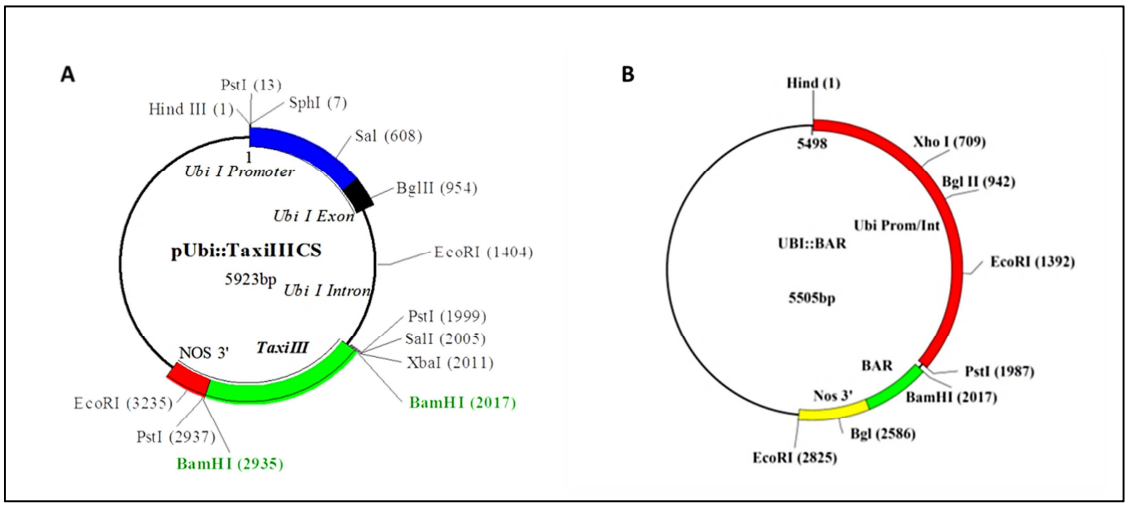

Fig. 4 Plasmids maps: pUbi::TaxiIIICS (A) and pUbi::bar (B) used for the biolistic bombardment.

Constructs were introduced into immature embryos-derived calli as described above.

15 µg of pUbi::bar containing the marker gene was used.

The following formulas were used to calculate the µl of plasmid DNA needed for coating the gold particles: Gene plasmid (pUbi::Taxi-IIICS) (µl): bp gene/bp marker x 15 µg x (1/ plasmid concentration) x 3.

Marker plasmid (µl): 15 µg x (1/plasmid concentration).

The microprojectiles pellet was resuspended in a solution containing 2.5 M $CaCl_2$ (250 µl), spermidine (50 µl), plasmid DNA and water (250 µl - µl of plasmid). The tubes were shaken with a vortex mixer at 4°C for 15-20 min and briefly centrifuged. The supernatant was removed and the pellet was washed with 600 µl of 100% ethanol. The DNA-coated gold pellets were resuspended in 36 µl of 100% ethanol and stored on ice until they were used.

About 100 embryos-derived calli were transferred onto the bombardment medium (characterized by a high osmolarity) 4 hours before the bombardment.

For each bombardment, 10 μl of the DNA-gold suspension was placed in the centre of a macroprojectile and bombarded using a Model PDS-1000/He Biolistic particle delivery system (Bio-Rad Laboratoires) (Fig. 5C) as described in Weeks et al. (1993). The distance from the stopping plate to the target was 13 cm, and the rupture disc strength was 1100 psi .

Immediately after the bombardment, calli were kept in the bombardment medium (Fig. 5D) and stored in the dark at 23°C for 24 hours; then they were maintained in the recovery medium for 4 weeks transferring them to a fresh medium at 2 week intervals (Fig. 5E).

For the regeneration, calli were transferred to the regeneration medium containing the selective marker PPT for 6 weeks at 27°C with 16 h light period (43 $\mu E/m^2$) transferring them to a fresh medium at 2 week intervals. At this stage, new shoots come from the calli resistant to the herbicide, usually starting from the third week (Fig. 5F).

Shoots were then transferred to Pyrex tubes containing the rooting medium, where they were under herbicide selection for additional 2-3 weeks. At this stage, plants capable to form long, highly branched roots in the selective medium were defined as resistant (Fig. 5G left). Sensitive plantlets exhibited yellow necrosis and reduced vigour within 1 week and did not produce roots (Fig. 5G right), whereas resistant plantlets thrived in the rooting media (Fig. 5G left).

Once the plants had sufficient leaves and roots, they were transferred into pots and kept in a growth chamber completely covered with plastic bags to increase the humidity, at 23°C, 16 h day light for 5-10 days to allow them to acclimate to greenhouse conditions (Fig. 5H). After that, plants were transferred to bigger pots; these primary regenerants are called T_0 plants .

Some of the lines showed normal levels of fertility and seed set. However, some of them showed reduced seed set compared with non-transformed Svevo plants and some of them were sterile.

The total process starting from the excision of the embryos up to the anthesis of regenerated T_0 plants, lasts about 168 days. The presence of the transgene in the regenerated plants was checked by PCR and Southern blot analysis.

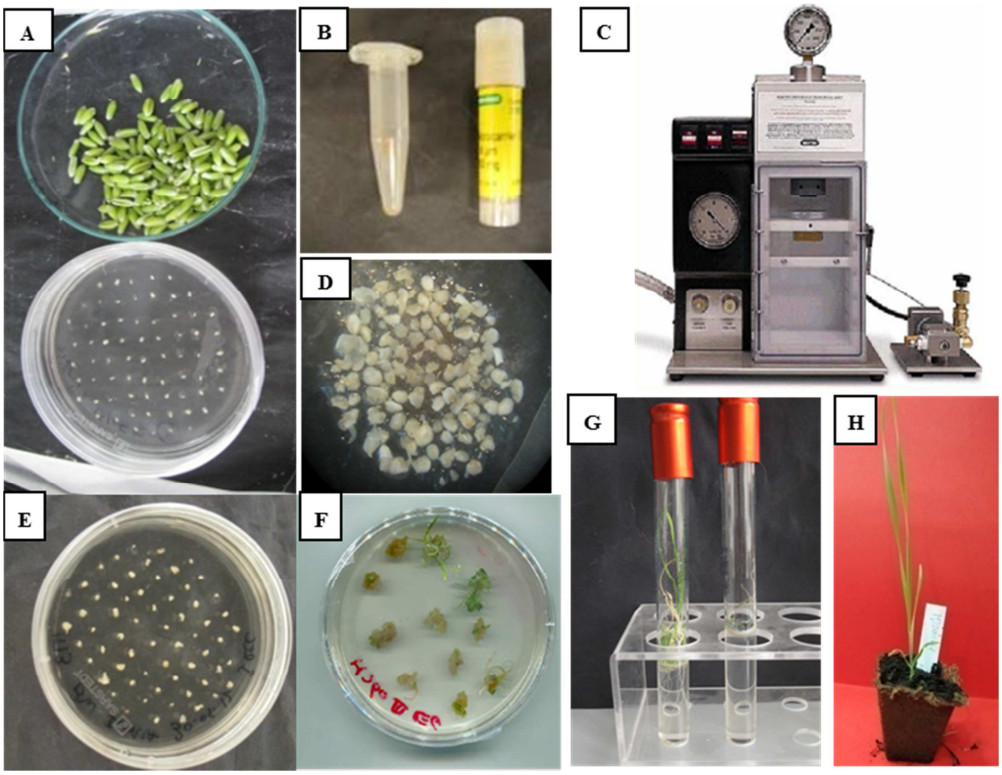

Fig. **5** Production of transgenic wheat plants: **A,** embryos isolated from caryopsis and placed in dissection media for callus induction, **B,** gold particles used to prepare microprojectiles, **C,** the Model PDS-1000/He Biolistic particle delivery system (Bio-Rad Laboratoires) used for bombardment, **D,** bombarded embyos in Bomb sucrose medium, **E,** calli in recovery medium, **F,** calli in regeneration medium, **G,** resistant (left) and susceptible (right) shoots in rooting media tubes, **H,** regenerated plant in soil.

2.7 Screening of transgenic plants

Genomic DNA was extracted from T_0 plants leaves using the method described by D'Ovidio and Porceddu (1996) for PCR and by Tai and Tanksley (1990) for Southern blot analysis.

DNA amplification was carried out according to the procedures specified for GoTaq Green Master Mix (Promega) at an annealing temperature of 60°C by using the specific primers pair Ubi A2 and Taxi-III 2R (Tab. 2) that produces an amplicon of 198 bp. Amplicons were confirmed by electrophoresis.

Southern blot analysis were performed as reported by Janni and associates (2008), using a *Taxi-III* digoxigenin-labelled probe produced following the procedure reported by D'Ovidio and Anderson (1994). 1 µl of pUbi::Taxi-IIICS DNA (10 ng) was added to the reaction mix containing: 73.5 µl of H_2O, 10 µl of 10x buffer, 10 mM dATP, dCTP, dGTP, 1 mM dTTP, 0.1mM DIG-11dUTP-alkali labile (Roche Diagnostics), 100 mM primers Taxi-III 1F/ Taxi-III 1R (Tab. 2), 2.5 U of GoTaq Polymerase (Promega). The DNA amplification conditions were: 94°C for 1 min, 65°C for 1 min

and 72°C for 1 min, repeated for 35 cycles. The amplified DNA was then purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega). The efficiency of the labelling process was verified by DIG Quantification Test strip (Roche Diagnostics) following the manufacturer's procedure.

2.8 Plant total and extracellular protein extraction

Total protein extracts were obtained by homogenizing wheat tissues in the presence of McIlvaine buffer at pH 5.0 (0.2 M disodium hydrogen phosphate and 0.1 M citric acid).

The homogenate was shaken for 1 h at 4°C and centrifuged 20 min at 10,000 × g, and the supernatant recovered.

Extracellular fluids (EF) were extracted from wheat seedlings (Zadoks stage 61) by vacuum-infiltration with McIlvane buffer (discussed above) as described by Salvi and associates (1990). Cytoplasmic contamination was verified by determining the glucose-6-phosphate dehydrogenase activity of the extracts.

Protein concentrations were determined using the Bio-Rad Protein assay kit (Bio-Rad Laboratories) following the manufacturer's protocol.

2.9 TAXI-III purification and protein analysis

TAXI-III was purified from crude protein extract by affinity chromatography on a xylanase-Sepharose conjugate column. Homogeneous *A. niger* endo-1,4-β-xylanase M4 (Megazyme) was covalently bound to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's instructions. The XylM4-Sepharose conjugate was suspended in McIlvaine buffer (pH 5.0) and packed in a column (Poly-Prep Chromatography Column, 9 cm high, 2-ml bed volume, 0.8 by 4 cm) that was used for TAXI-III purification.

N-terminal sequencing and MS analyses of TAXI-III were performed by the Molecular Structure Facility at University of California (Davis). N-terminal amino acid sequencing was performed by means of an Applied Biosystems Procise 494 sequencer.

Purified TAXI-III was fractionated on SDS-PAGE and electroblotted with the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories), by using 10 mM CAPS buffer pH 11, at 4°C for 1 h at 70 V. Staining was performed with 50% Methanol and 0.1% Coomassie blue R-250.

MS analyses were performed on purified TAXI-III previously fractionated by SDS-PAGE, stained with colloidal Coomassie CBBG-250 (Neuhoff et al., 1988), and subjected to tryptic digest procedures according to the In-Gel Digest protocol used by the UC Davis Proteomics Core Facility, where the analyses were performed. Tandem MS (MS/MS) was performed by using Sequest (version SRF v. 3; Thermo Fisher Scientific) that was set up to search against the specific TAXI-IIICS protein sequence, assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 1 Da and a parent ion tolerance of 2 Da. Oxidation of methionine and iodoacetamide derivative of cysteine were specified in Sequest as variable modifications.

Scaffold (version Scaffold_3_00_06; Proteome Software Inc., Portland, OR, U.S.A.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least ΔCn scores of greater than 0.10 and XCorr scores of greater than 1.2, 2.0, 2.5, and 3.0 for singly, doubly, triply, and quadruply charged peptides, respectively. Protein identifications were accepted if they contained at least two identified peptides.

2.10 Determination of kinetic parameters of TAXI-III against the endo-1,4-β-xylanase M4 of *Aspergillus niger*

Kinetic parameters of endo-1,4-β-xylanase M4 of *Aspergillus niger* (AnM4Xyl) (Megazyme) were determined by the DNS assay using 0.041 U of AnM4Xyl and different concentrations (0.0050 to 0.0250 mg/ml) of low-viscosity wheat arabinoxylan (Megazyme), adjusting with McIlvane buffer pH 5.0, in a reaction volume of 250 µl. Reactions (in triplicate) were assayed for xylanase activity at 40°C and terminated after 4 min by adding 250 µl DNS, then samples were boiled for 5 min.

Absorbance of samples was measured at 545 nm using monomeric D-xylose as standard. 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.

K_m and k_{cat} values were calculated using non linear regression Michaelis-Menten equation.

We used DNS assay to test the AnM4Xyl (0.041 U) in presence of different amounts of purified TAXI-IIICS (8.0 nM, 14.0 nM and 20.0 nM), we calculated K_m and Vmax using the Linewear-Burk curve with increasing concentrations of the purified inhibitor. We used secondary plot of slopes from the double-reciprocal plot against inhibitor concentration to calculate K_i.

2.11 Fungal growth and xylanases heterologous expression

F. graminearum Schwabe strains PH1 and 3824 and *Bipolaris sorokiniana* (Sacc.) Shoemaker DSMZ 62608 were cultured at 25°C on potato dextrose agar (PDA) (Difco Laboratories).
Xylanase activity was induced as proposed by Dong and associates (2012), with some modification. Five agar plugs (5 mm in diameter), taken from the edge of actively growing colonies, were used to inoculate 250 ml flasks containing 50 ml of synthetic medium supplemented with glucose at 20 g/l. These cultures were incubated on an orbital shaker at 25°C and 100 rpm. After 3 days of growth, 200 µl of each culture containing the fungal propagules were used to inoculate 4 g of wheat bran hydrated with 4 ml of modified synthetic medium in petri dishes. The plates were grown for 14 days at 25°C. Bran-fungal mat (8 g) was sliced and ground with 40 ml of extraction buffer (50 mM sodium acetate, 10% glycerol, 2.5 M EDTA, 0.5 M NaCl, and 2.5 M dithiothreitol) containing 0.4 g of polyvinylpolypyrrolidone. The mixture was filtered through a cheesecloth and the crude extract was collected in a flask, while the solid fraction was used for a second extraction with 20 ml of extraction buffer. The collected filtrates were centrifuged at 8,000 rpm for 15 min at 4°C and supernatants were used to determine the xylanase activity.

The FGSG_03624 xylanase gene was cloned and expressed in *P. pastoris* as reported by Sella and associates (2013). The entire coding sequences of FGSG_10999, FGSG_11487, and FGSG_11304 xylanase genes were amplified by using the cDNA obtained from total RNA extracted from infected wheat spikelets at 3 dpi. Reverse transcription was performed by using an Oligo(dT) primer and the ImPromII reverse transcriptase (Promega), following the manufacturer's instructions. Amplification from cDNA was carried out by using the REDTaq ReadyMix PCR reaction mix (Sigma- Aldrich) with the specific primers pairs reported in Table 4.
The PCR was performed by repeating for 35 times the following cycle: 1 min at 94°C, 30 s at the proper annealing temperature, and 1 to 1.5 min at 72°C. The amplification products of the expected size were purified using the "Wizard SV Gel and PCR Clean-Up System" kit (Promega) and were then cloned individually into the pGEM-T Easy vector (Promega), following the manufacturer's instructions. The cloned cDNAs were sequenced in order to check the correctness of the nucleotide sequences and then amplified with the Pfu DNA polymerase (Promega) by using specific primers containing adaptors for *Eco*RI and *Xba*I recognition sequences (Tab. 4).
The amplification was performed by repeating for 35 times the following cycle: 1 min at 94°C, 30 s at the proper annealing temperature, and 1 to 1.5 min at 72°C. The PCR amplicons were purified

and individually ligated into the pPICZαA expression vector and each plasmid was used to transform *P. pastoris* by electroporation as reported in Sella and associates (2013).

Some positive colonies were tested by PCR using specific primers (Tab. 4) and the REDTaq ReadyMix PCR reaction mix (Sigma- Aldrich).

Colonies showing the expected insert were grown and induced with methanol according to the manufacturer's instructions (Invitrogen Life Technologies). After 96 h, liquid cultures were centrifuged at 10,000 × g for 10 min and supernatants were assayed for xylanase activity and subjected to SDS-PAGE. Supernatants from recombinant colonies showed xylanase activity, whereas the supernatant from control colonies (not containing the xylanase genes) did not show any xylanase activity.

SDS-PAGE analysis (12%) was performed according to Laemmli and associates (1970) by using 25 µl of the *P. pastoris* culture medium. Gels were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich).

Tab. 4 List of primers used for the cloning of xylanases in *P. Pastoris.*

Specific primer pairs for complete gene sequences amplification from c-DNA			
Primer name	**Sequence (5'-3')**	**Ta**	**Amplicon Size**
10999compFor	ATGGTCTCGTTCAAATCC	52 °C	696 bp
10999compRev	TTAACTAGTCTGGACATAGATAGA		
11487compFor	ATGAAGTTCTCTTCCCTCCT	55 °C	984 bp
11487compRev	TTAACGGAGAGCGTTGAC		
11304compFor	ATGCACTTCCTAGGACTCG	56 °C	1146 bp
11304compRev	TTAGTTGAGGGCGTTGCT		
Specific primers containing adaptors used for cloning and colonies screening			
Primer name	**Sequence (5'-3')**	**Ta**	**Amplicon Size**
10999for WSP	ACAGAATTCCGTCCCTTTGACTTCCT	52 °C	639 bp
10999rev WSP	TGTTCTAGATTAACTAGTCTGGACATAGATAGA		
11487for WSP	ACAGAATTCATGCCTGCCTCCATCGA	57 °C	939 bp
11487rev WSP	TGTTCTAGATTAACGGAGAGCGTTGACAAC		
11304for WSP	ACAGAATTCCAGTCTGCTATCTGGGGAC	55 °C	1098 bp
11304rev WSP	TGTTCTAGATTAGTTGAGGGCGTTGCT		

2.12 Enzymatic assays

Xylanase activity of *P. pastoris* cultures expressing *F. graminearum* xylanases (FGSG_11487, FGSG_10999, FGSG_ 11304, and FGSG_03624) was determined by measuring the reducing sugars released from 0.5% (wt/vol) beech-wood xylan (Carl Roth GmbH) dissolved in 50 mM sodium citrate buffer at pH 5 according to the DNS method described by Miller (1959) and modified by Bailey and associates (1992). The assay was performed by incubating 50 to 200 µl of culture filtrates in a reaction volume of 500 µl.

Absorbance of samples was measured at 545 nm using monomeric D-xylose as standard. 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.

Xylanase inhibition activity of crude plant extract or purified TAXI-III was measured by radial gel diffusion assay or by using the DNS assay. Radial gel diffusion assay was performed using a modification of the procedure reported by Emami and Hack (2000). Total protein extract and 0.005 U of AnM4xyl (Megazyme) were added to 0.5 cm wells on plates containing McIlvane buffer (pH 5.0), 1% (wt/vol) xylan from beech wood (Sigma- Aldrich) and 1% agarose. Plates were incubated for 16 h at 30°C and the halo caused by enzyme activity was visualized after 30 min of treatment with ethanol 95% that revealed clear halo of degradation against an opaque background (Royer and Nakas, 1990).

DNS assay was performed with 1% (wt/vol) low-viscosity wheat arabinoxylan (Megazyme) in a reaction volume of 500 µl. Reactions (in triplicate) containing xylanase (0.015 U of heterologously expressed *F. graminearum* xylanase or 0.050 U of crude filtrate of *F. graminearum* or *B. sorokiniana*), alone or preincubated (5 min at 30°C) with protein extract or purified TAXI-III, were assayed for xylanase activity at 40°C and terminated after 4 min by adding DNS.

2.13 Plant growth

Wheat seeds (*T. durum* Svevo) were surface sterilized with sodium hypochlorite (0.5%, vol/vol) for 20 min and then rinsed thoroughly in sterile water. Plants were vernalized at 4°C for 2 weeks and grown in a climatic chamber at 18 to 23°C with a 14-h photoperiod (300 µE m^{-2} s^{-1}).
The growth stages were counted using the Zadoks method (Zadoks, 1974).

2.14 Plant infection assays

For plant inoculation with *B. sorokiniana* 62608 DSMZ, conidia were recovered from fungal cultures grown on petri dishes containing PDA medium (BD).
The infection experiment was carried out following the procedure reported by Janni and associates (2008). Lesion sizes were determined 72 hpi by scanning the leaf surface and calculating the pixels of the infected area and of the total area by using the Adobe Photoshop program (Microsoft).
The average area per lesion was calculated as weighted arithmetic mean.
T_1 or T_2 progenies were used in the different experiments.
At least 13 plants for each genotype were used.

For plant inoculation with *F. graminearum* strain 3824, conidia were recovered from fungal cultures grown on synthetic nutrient agar (SNA) plates (Urban et al., 2002).
Infection experiments of wheat plants were performed by single-spikelet inoculation.
A conidia suspension (20 µl, 2.5×10^4 conidia/ml) supplemented with 0.1% Tween-20 was pipetted directly through the glumes of two opposite central florets of a wheat head during anthesis. Infected spikes were covered with plastic bags for 2 days in order to maintain high humidity. Disease symptoms were assessed by counting the number of visually diseased spikelets at different days post-inoculation and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets.
For each experiment, at least 13 plants of T_2 progeny for each genotype were used.

Data from both *B. sorokiniana* or *F. graminearum* infection experiments were analyzed statistically applying the Student's *t* test.

2.15 Fungal biomass quantification

Quantification of fungal biomass was performed by qPCR assay for the *Tri-6* gene with the primers Tri6_10F and Tri6_4R (Horevaj et al., 2011). Briefly, at the end of the infection experiments, all dry caryopses of each transgenic and control genotype obtained from each experiment were pooled to make whole wheat flour. Total DNA from whole flour was extracted with DNeasy Plant Mini kit (Qiagen) according to manufacturer's instructions. qPCR experiments were performed using the iCycler (Bio-Rad Laboratories) and the Master Mix iQ SYBR Green Supermix (Bio-Rad Laboratories) containing the flurogenic SYBER Green I DNA binding dye. The Actin gene (discussed above) was used as housekeeping gene. The specificity of the primers was verified by nucleotide sequence of the amplicon.

2.16 Histochemistry of wheat tissues treated with FGSG_03624 xylanase

Lemma tissues excised from durum wheat spikelets at anthesis stage were treated with a 10 µl drop containing the purified FGSG_03624 (200 ng) (Sella et al., 2013), or the same amount of xylanase boiled for 30-45 min to destroy its enzymatic activity, or the above mentioned xylanases co-incubated for 10 min with TAXI-III purified protein (300 ng), or with TAXI-III only. The phosphate buffer saline (PBS) 0.01 M pH 7.4 was used to bring samples to final volume and as negative control.

The xylanase activity, its inactivation in boiled samples and the inhibition of activity after co-incubation with TAXI-III were tested using radial gel diffusion assay.

A 10 µl drop of one of the previously described samples was putted onto the surface of a detached lemma and tissues were incubated for 24 h in a humid chamber. Staining was performed with 1 mg/l Trypan Blue (Sigma-Aldrich) to detect cell death as detailed in Faoro and Iriti (2005).

The same treatment with the FGSG_03624 xylanase in native and boiled form was performed also using lemmas from TAXI-III plants and from wild type plants as controls, using the same staining conditions.

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

2.17 Cytochemistry of wheat cells treated with FGSG_03624 xylanase

Cell line PC998 (*Triticum aestivum* L. emend. Fiori et Paol. cv. Heines Koga II) (DSMZ) was grown in suspension culture in B5 medium modified after Gamborg et al. (1968).

The medium used is the Gamborg's B-5 Basal Medium with Minimal Organics (Sigma-Aldrich) supplemented with 20 g/l sucrose, adjusted at pH 5.5 and sterilized at 121°C for 20 minutes, after autoclaving the 2,4-D (2 mg/l) was added to maintain callus proliferation.

Cell suspensions were grown in dark conditions at 23°C on a gyratory shaker with a shaking speed of 100 rpm. For propagation we added 25 ml fresh medium to the 125 ml Erlenmeyer flask containing 25 ml old suspension every 15 days, mixed and poured 25 ml suspension each in a fresh Erlenmeyer flask, yielding two Erlenmeyer flasks with 25 ml suspension culture each.

For cytochemical experiments we sampled cell suspension avoiding callus aggregates after 7 days from subculture, when cells are in exponential growth phase, and aliquoted 200 µl in each 1.5 ml tube. Each sampling tube was inoculated with the FGSG_03624 xylanase (1 µg) or the same amount of FGSG_03624 xylanase boiled for 40 minutes or the above mentioned xylanases singularly co-incubated with TAXI-III (1.5 µg) for 10 min at room temperature. We used as negative controls the xylanase elution buffer, the TAXI-III elution buffer or not treated cells, while we used boiled cells (10 min at 100°C) as positive control for cell death.

Each sample was incubated at 23°C, 100 rpm for 24 h and then stained with 1 ml of Trypan Blue, 0.07 mg/l in distillated water, for 10 min. We precipitated cells at 1,000 x g for 3 min, removed the staining solution and washed twice with 1 ml of distillate water. We resuspended cells in 20 µl of distillate water for microscopic analysis. We counted 100 cells for each sample distinguishing among the blue stained and the unstained cells. Blue staining, due to membrane permeabilization, reveals cell death (Iriti et al., 2006).

Two biological and six technical replicas for each sample were analysed.

All samples were examined with the Leica DM5000B microscope (Leica Microsystems), using bright field transmitted light.

2.18 Molecular modelling of the interaction between TAXI-III and FGSG_03624

The primary structure of TAXI-IIICS and FGSG_03624 were used to predict the tertiary protein structures using the CPHmodels-3.0 server (http://www.cbs.dtu.dk/services/CPHmodels/) (Nielsen et al., 2010) that is a web-server predicting protein 3D-structure by use of single template homology modeling.

The ZDOCK SERVER (http://zdock.umassmed.edu/) (Pierce et al., 2014) is a tool for interactive docking prediction of protein-protein complexes. It offers as example the docking between the *Bacillus subtilis* xylanase (as receptor) and the TAXI-I inhibitor (as ligand). Without modifying the software settings we loaded the previous obtained 3D structure of FGSG_03624 xylanase as receptor and of TAXI-III as ligand and let the program perform the docking prediction. We visual analysed the top 5 predictions.

3. Results

3.1 Isolation of *Taxi-III* gene, chromosomal localization and expression analysis

In order to select the *Taxi-III* gene to be used in the genetic transformation of durum wheat cv Svevo (SV), we cloned *Taxi-III* from this cultivar and from the bread wheat cultivars Chinese Spring (CS) and Bobwhite (BW) by using the primer pair Taxi-III 101spF/ Taxi-III 101spR (Tab. 2) developed from *Taxi-III* of *Triticum aestivum* cv Norin61 (AB114627) (Igawa et al., 2004).

The new isolated sequences *Taxi-IIICS* (HF969028.1), *Taxi-IIIBW* (HF969030.1) and *Taxi-IIISV* (HF969029.1) have a coding region of 1,206 bp and sequence comparison (Fig. 7) showed that *Taxi-III* from cv Svevo is identical to that of cv Norin61 but differs by two synonymous substitutions from those of Bobwhite and Chinese Spring. Based on this results we chose the *Taxi-IIICS* gene to prepare the construct for particle bombardment of durum wheat cv Svevo because it make possible to distinguish between the endogenous and transgenic *Taxi-III*.

For this aim specific primers (Taxi-III 170F/ Taxi-III 5R) (Tab. 2) were designed to amplify a region containing the SNP T227C (Fig.7).

Moreover these specific primers allow us to localize *Taxi-III* gene on wheat genome; we analysed by PCR the nulli-tetrasomic lines of CS and localized *Taxi-III* on the long arm of chromosome 3A (Fig. 6).

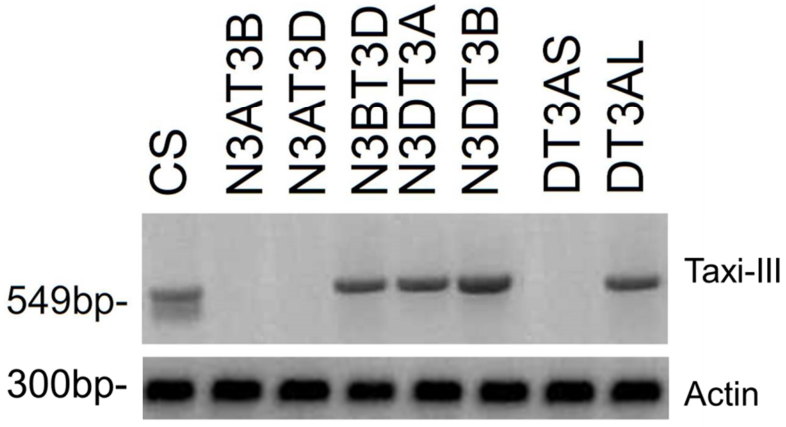

Fig.6 PCR assay for chromosomal localization of *Taxi-III*. The analysis were performed using genomic DNA isolated from nulli-tetrasomic lines of chromosome group 3 and ditelosomic wheat of chromosome 3A (cv. Chinese Spring). PCR was performed using the *Taxi-III* specific primer combination Taxi-III 170F/ Taxi-III 5R and a specific amplification of 549 bp was present in N3BT3D, N3DT3A, N3DT3B, while no amplification was obtained in N3AT3B and N3AT3D. Moreover this specific amplicon was present in DT3AL and absent in DT3AS. Actin was used as housekeeping gene. These results were repeated three times with similar results.

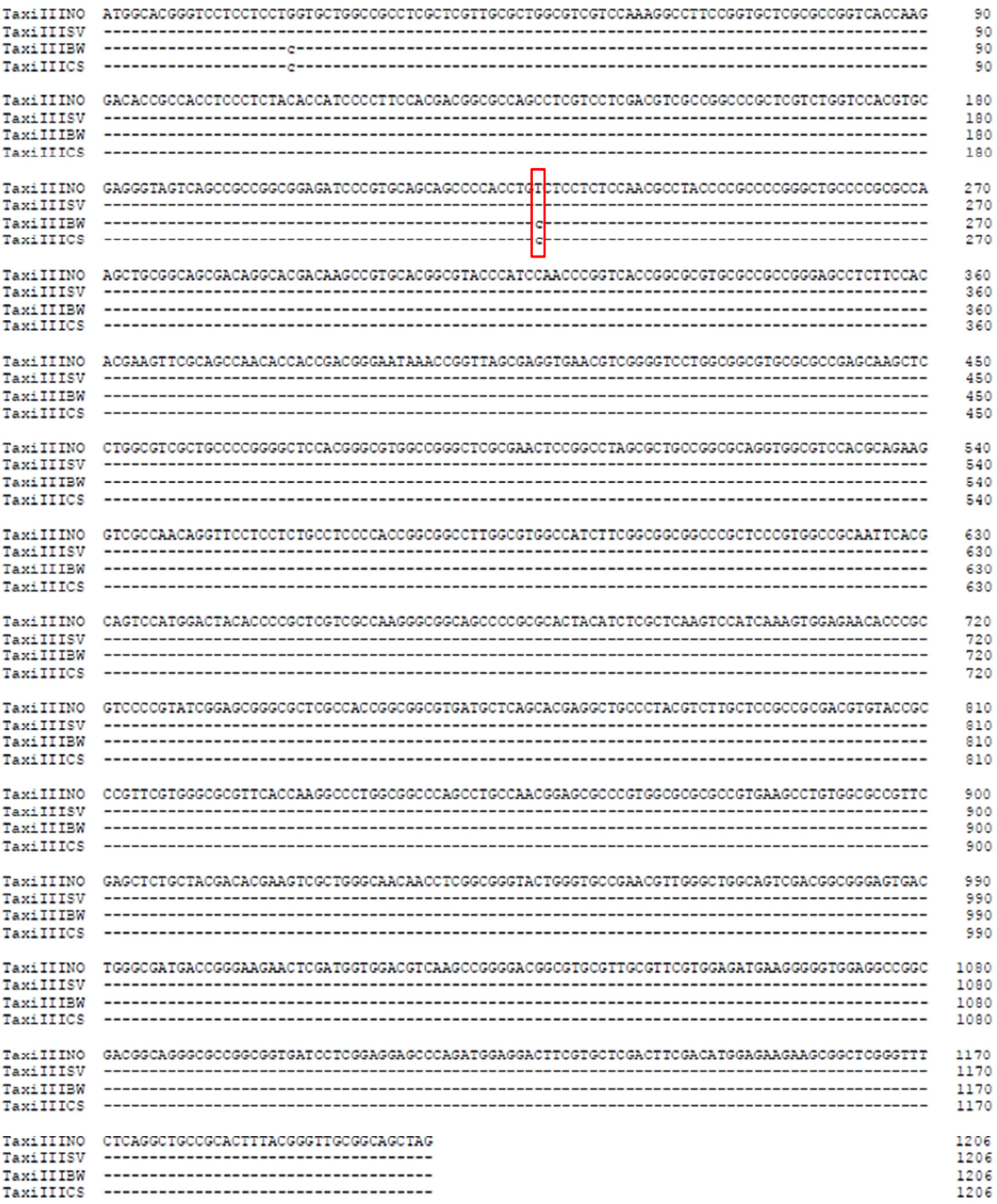

Fig.7 Nucleotide sequence alignment between *Taxi-III* genes of *Triticum aestivum* cv Norin (*Taxi-IIINO*), *T. durum* cv Svevo (*Taxi-IIISV*), *T. aestivum* cv Bobwhite (*Taxi-IIIBW*) and *T. aestivum* cv Chinese Spring (*Taxi-IIICS*). Nucleotides identical to *Taxi-IIINO* are indicated by dash. Single nucleotide polymorphism (SNP) T227C is highlighted in the red box.

Taxi-III transcript expression was evaluated in durum wheat tissues. We found that this gene accumulated at high level in root at early stage of development and it was absent in coleoptide, young leaves, stem and spikelets (deprived of the ovary), while it was present in caryopsis at early stage of development and increases at lates stages (Fig. 8).

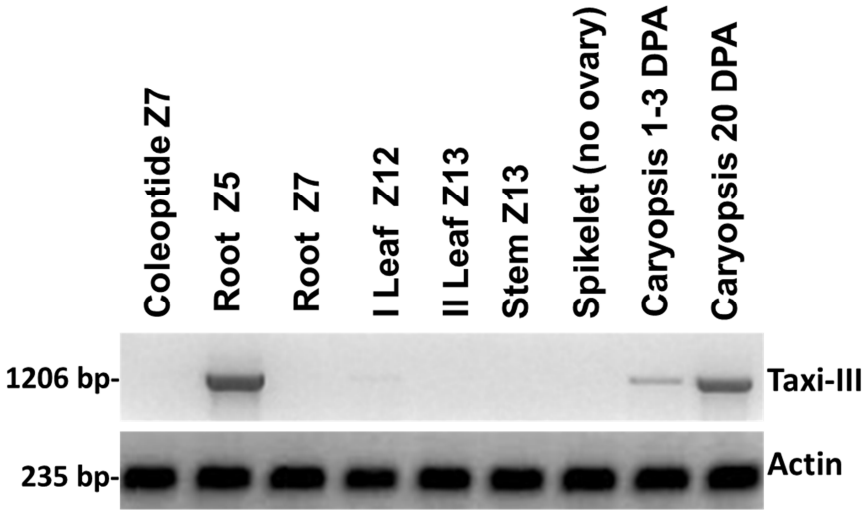

Fig. 8 RT-PCR assay of *Taxi-III* gene on different wheat tissues. The analysis was performed by Taxi-III 101spF/ Taxi-III 101spR primers amplifying the complete coding of *Taxi-III*. We analysed the coleoptide at Zadoks stage 7 (Z7), roots at two stages (Z5 and Z7), first and second leaf respectively at stages Z12 and Z13, stem at Z13, spikelets at anthesis, deprived of the ovary, and caryopsis at early stage (1 to 3 days after anthesis) or at early dough stage (20 days after anthesis). Actin was used as housekeeping gene.

Since *Taxi-III* transcript has been reported to be induced in bread wheat following *F. graminearum* infection (Igawa et al., 2004), we verified if this induction occurs also in durum wheat cv Svevo. qRT-PCR analysis with primers Taxi-III 170F/ Taxi-III 5R (Tab. 2) showed that *Taxi-III* underwent a significant increase at 24 hour post infection (hpi) with about 40 fold increase compared to the corresponding mock inoculated sample. Similarly, qRT-PCR with a primer pair specific for the homeologous genes *TaPr-1-1/2/3* (Lu et al., 2011) used as control of gene induction underwent about 10 fold increase compared to the mock inoculated control sample (Fig. 9).

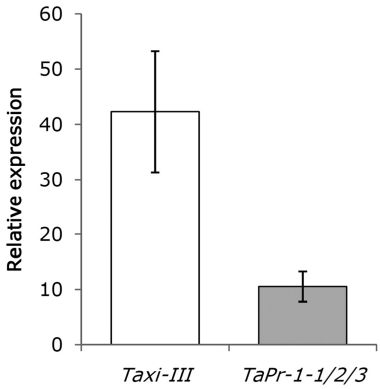

Fig. 9 qRT-PCR of *Taxi III* following infection of durum wheat cv Svevo with *F. graminearum.* Spikelets of *T. durum* cv. Svevo were analyzed 24h after infection. Quantification of gene expression was performed using the comparative $2^{-\Delta\Delta CT}$ method. Relative expression of *Taxi III* and pathogenesis-related protein homeologous genes *TaPr-1-1/2/3* is reported as fold increase of transcript level in infected spikelets relative to values determined in mock inoculated control plants. *TaPr-1-1/2/3* were used as control of gene induction. Actin was used as housekeeping gene. Amplicon sizes were: *Taxi III*, 549 bp and *TaPr-1-1/2/3*, 290 bp. Bars represent average values ± standard errors of two biological replicates and 6 technical replicates.

3.2 Production of wheat transgenic plants expressing *Taxi-IIICS*

In total, 1,932 *T. durum* Svevo immature embryos were co-transformed using pUbi::Taxi-IIICS and pUbi::bar in a single bombardment experiment. *Taxi-IIICS* was prepared under control of the constitutive maize ubiquitin (*Ubi-1*) promoter in order to accumulate the corresponding protein also in tissues that normally do not accumulate TAXI-III or other XIs, as leaves and flowering spikes. This strategy was used to facilitate the identification of TAXI-IIICS activity and the possible correlation between TAXI-IIICS accumulation and disease symptom development.

Seventy one independent regenerated plants were obtained and analyzed for the presence of the transgene by PCR using the specific primers Ubi A2/ Taxi-III 2R (Tab.2).

Twenty two T_0 independent transgenic plants resistant to phosphinotricin and containing *Taxi-IIICS* were obtained, for a transformation efficiency of 1.3% that is within the typical efficiency obtained in stable wheat transformation.

Inhibition activity of transgenic plants containing *Taxi-IIICS*, hereafter called TAXI-III plants, was analysed on radial gel diffusion assay using total leaf protein extract against the *Aspergillus niger* M4 xylanase (AnM4Xyl). The inhibition assays showed that total protein extract (20 µg) of 9 out of 22 T_1 TAXI-III plants inhibited to a varying extent (30-100%) the activity of AnM4Xyl (Tab. 5). In contrast, total protein extract from the control plants did not affect the AnM4Xyl activity (Tab. 5). Moreover, when protein extract of TAXI-III plants was boiled no inhibition activity was observed, excluding the possibility of a non proteinaceous inhibition.

Three TAXI-III lines, MJ30-6, MJ30-10 and MJ30-23 showing a high inhibition activity against AnM4Xyl were selected for subsequent analyses. T_1 plants of these lines did not show any significant difference in morphology and growth compared with untransformed plants (*T. durum* Svevo). T_2 and T_3 progenies from all three primary transformants maintained the inhibition activity against AnM4Xyl, although random transgene silencing occurred. This phenomenon has been observed since the T_1 generation and increased in the subsequent T_2 and T_3 generations. Because transgene silencing occurred randomly in the progenies and also during plant growth and development, all the transgenic plants were monitored during growth and only those showing inhibition activity against AnM4Xyl until flowering were used in subsequent experiments.

Tab. 5. Inhibition activity of total protein extract of T_1 TAXI-III and wild-type (WT) plants against *A. niger* xylanase AnM4Xyl as determined by radial gel diffusion assay*.

Lines	Relative inhibition activity (%)
WT	0
MJ30-1	27± 4.04
MJ30-6	100± 6.15
MJ30-10	100± 7.23
MJ30-10b	100± 8.66
MJ30-12a	33± 6.33
MJ30-23	100± 7.13
MJ30-36	100± 6.21
MJ30-52	40± 3.09
MJ30-68	36 ± 7.89

*Inhibition activity of total protein extract of each transgenic line is expressed as relative percentage considering 0% of inhibition the halo produced by AnM4Xyl in presence of the protein extract of the WT plants and as 100% of inhibition the absence of halo. Data represent the average ± standard errors of three replicates by using 20 μg of total protein extract and 0.005 U of *A. niger* M4 xylanase. Boiled protein extract samples did not exhibit any inhibitory activity against 0.005 U of *A. niger* M4 xylanase.

3.3 Molecular characterization, inhibition activity and apoplastic accumulation of TAXI-IIICS in selected transgenic durum wheat lines

Genomic DNA blots of the selected lines (MJ30-6, MJ30-10 and MJ30-23) were performed using the entire coding region of *Taxi-IIICS* as probe. Digestion with *Bam*HI restriction enzyme, which causes the excision of the *Taxi-IIICS* coding region from the pUbi::Taxi-IIICS (Fig. 10A), revealed the expected hybridization signal of about 1,200 bp in all transgenic lines (Fig. 10B). Digestion with *Sph*I restriction enzyme, which cuts once within the pUbi::Taxi-IIICS, produced in all the transgenic lines a main hybridizing fragment of about 5.9 kb corresponding to the size of the entire construct (Fig. 10C). The strong signal of this fragment and variation in its intensity between lines suggests the integration of multiple copies of the construct in head-to-tail array into the genome of the transgenic lines. Additional hybridizing fragments were also present in all three transgenic lines. Some of these hybridization fragments were also present in the control plants and probably correspond to endogenous *Taxi* genes, whereas others were specific of each transgenic line and most likely represent different insertion sites, or rearranged copies of pUbi::Taxi-IIICS, as well as the terminal fragment of the transgene array (Fig. 10C).

RT-PCR analysis showed that Taxi-IIICS was clearly expressed in the leaves of all three transgenic lines (MJ30-6, MJ30-10 and MJ30-23). Conversely, a weak expression of endogenous Taxi-IIISV was observed in the control plants of cv Svevo. qRT-PCR showed that the level of leaf expression of Taxi-IIICS was similar in all three transgenic lines (Fig. 11A).

In agreement with the results at RNA level, total protein extract from T_1 plants of all three lines inhibited AnM4Xyl at similar extent. In particular, less than 5 μg of total leaf protein extract of each MJ30-6, MJ30-23 and MJ30-10 was sufficient to inhibit by 50% the AnM4Xyl activity (Fig. 11B) and 10 μg inhibited to completion this enzyme (Fig. 11C). Similarly, total protein extract from flowering spikes of transgenic plants inhibited to completion AnM4Xyl (Fig. 11C). On the contrary, no inhibition activity was observed with total protein extract (up to 30 μg) from leaves or flowering spikes of control plants (Fig. 11C). In the experiments involving flowering spikes the ovary was removed before extraction to avoid the interference with possible endogenous XI activity accumulated in the early stage of embryos development.

Since TAXI-IIICS contains a predicted signal peptide for the apoplastic targeting, we extracted and assayed the extracellular leaf fluids (EF) of line MJ30-23 and control plants against AnM4Xyl. Radial gel diffusion assay showed a clear inhibition activity in the EF of MJ30-23 and the lack of this activity in the EF of control plants (Fig. 11C), indicating that TAXI-III is targeted into the

apoplastic compartment. Extracellular fluids from both samples had negligible glucose-6-phosphate dehydrogenase activity, ruling out cytoplasmic contamination (data not shown).

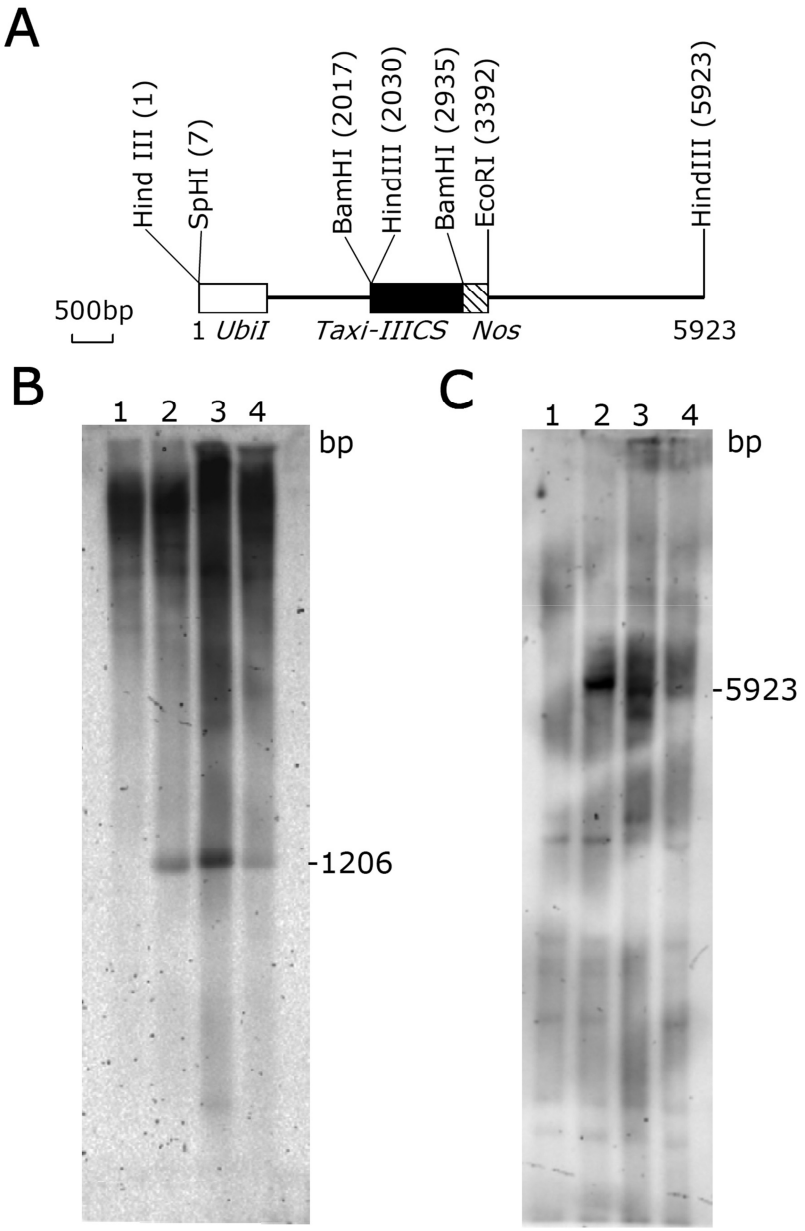

Fig.10 Schematic representation of the pUbi::Taxi-IIICS construct and Southern blots of regenerated transgenic lines. **A**, The pUbi::Taxi IIICS construct was prepared by cloning the *Taxi IIICS* gene into the *Bam*HI site of pAHC17 under control of the constitutive maize *Ubiquitin1* promoter and *NOS* terminator. Genomic DNA (10 µg) of T_2 transgenic lines was digested with **B,** *Bam*HI and **C,** *Sph*I probed with a digoxigenin-labeled coding region of *Taxi IIICS*. Lane 1, *Triticum durum* cv Svevo (untransformed control); lane 2, MJ30-6; lane 3, MJ30-10; lane 4, MJ30-23.

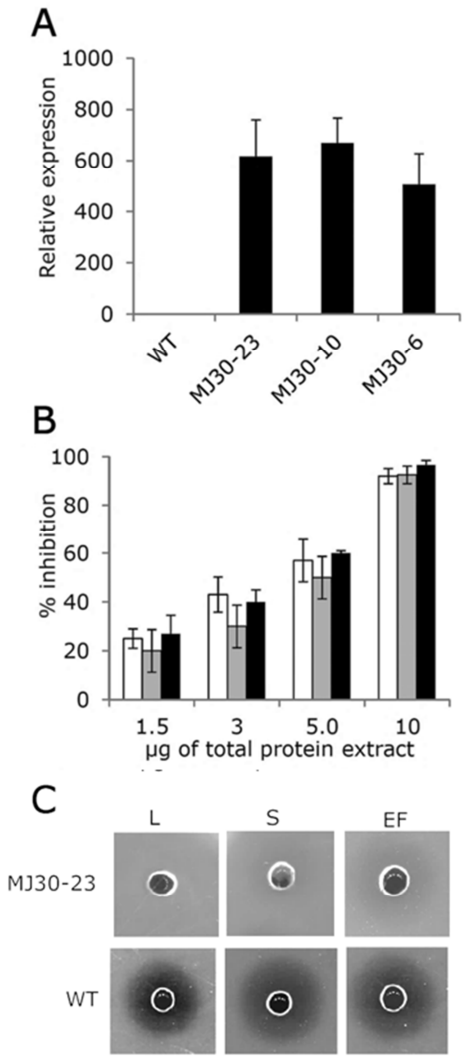

Fig. 11 Expression and XI activity of selected wheat lines overexpressing *Taxi- IIICS*. **A,** qRT-PCR on RNA extracted from mature leaves (Zadoks stage 47) of control plants (WT) and three transgenic lines (MJ30-23, MJ30-10, MJ30-6) using primers specific for *Taxi -IIICS* or *Actin* that was used as housekeeping gene. *Taxi-IIICS* amplicon of the expected size was present in all three transgenic lines. At least three biological replicates were performed and similar results were obtained. **B,** Radial gel diffusion assays for XI activity of total leaf protein extract of transgenic line MJ30-23 (white), MJ30-10 (gray) MJ30-6 (black). Assays were performed using different amounts of total protein extract against AnM4Xyl. Bars represent the average ± standard error of at least three replicates. **C**, Radial gel diffusion assay for XI activity of total protein extract (10 µg) from leaf (Zadoks stage 12) and spikelets (Zadoks stage 68), and of extracellular fluids (3 µg) of transgenic line MJ30-23 and wild type plants (*T. durum* cv Svevo, WT) against AnM4Xyl; the absence of the halo indicates the presence of XI activity. L, leaf; S, spikelets; EF, extracellular fluids.

3.4 TAXI-III purification and identification of the cleavage site of the signal peptide

TAXI-IIICS was purified from total leaf protein extract of line MJ30-23 by affinity chromatography on a Sepharose-AnM4Xyl column. SDS-PAGE showed a single band of the expected size of about 40 kDa (Fig. 12) on those fractions containing inhibition activity against AnM4xyl. Mass spectrometry (Fig. 13) and N-terminal sequence (Fig. 14) confirmed the correspondence of the protein. This latter analysis showed also that the mature TAXI-IIICS starts with Lys at position 19 (KGLPVLAPVT) of the complete deduced protein from the *Taxi-IIICS* gene (Fig. 14B). This result is slightly different from that predicted by SignalP that indicated the putative cleavage sites between residues 21 and 22 (Fig. 14A).

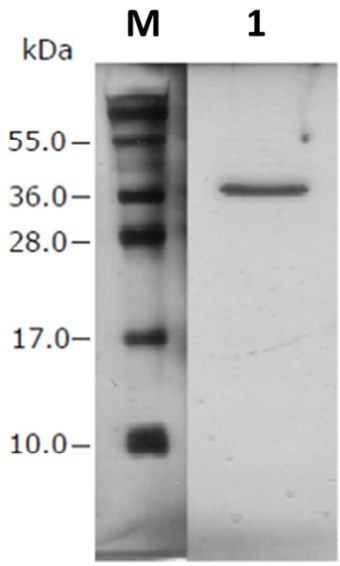

Fig. 12. SDS-PAGE of TAXI-IIICS purified by affinity chromatography on a *Aspergillus niger* M4 xylanase (AnM4Xyl)-Sepharose conjugate column: M, molecular weight standard, lane 1, purified TAXI-IIICS. Proteins were loaded on a 12% polyacrylamide gel then stained with Coomassie Brilliant Blue R250.

Fig. 13 Identification of TAXI-IIICS by mass spectrometry. Amino acid sequence of TAXI-IIICS and of the different peptides (underlined) determined by tandem mass spectrometry (MS/MS) of TAXI-IIICS purified from leaves of the transgenic line MJ30-23 by affinity chromatography on a *Aspergillus niger* M4 xylanase (AnM4Xyl)-Sepharose conjugate column. Sixteen peptides were identified for a 39% coverage of the TAXI-IIICS sequence.

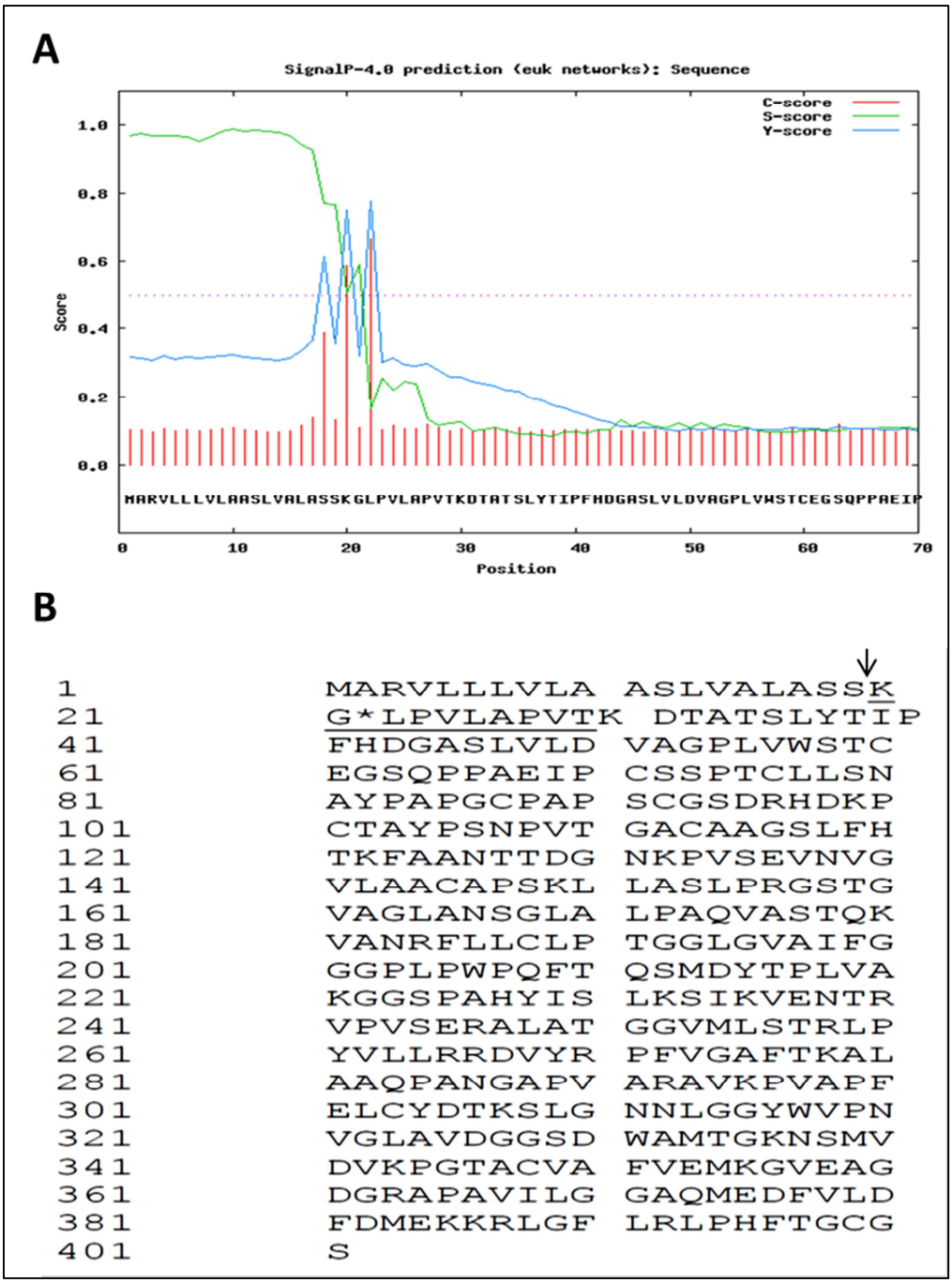

Fig. 14 A, Amino acid sequence of TAXI-IIICS was used to predict the cleavage site using the SignalIP (http://www.cbs.dtu.dk/services/SignalP/). Results showed the most probable cleavage site between pos. 21 and 22: SKG-LP, D=0.848, D-cutoff=0.450. **B,** Amino acid sequence of TAXI-IIICS and of the N-terminus (underlined) identified from TAXI-IIICS purified from leaves of the transgenic line MJ30-23 by affinity chromatography on a *Aspergillus niger* M4 xylanase (AnM4Xyl)-Sepharose conjugate column. Asterisk indicates cleavage site determined by SignalP. Arrow indicates cleaveage as determined by N-terminal sequence.

3.5 Determination of kinetic parameters of TAXI-III against AnM4Xyl

To determine the kinetic parameters of the AnM4Xyl we performed Dinitrosalicylic acid (DNS) assays using different concentrations of low viscosity wheat arabinoxylan; this enzyme exhibits K_m of 0.018 mM and k_{cat} of 5855.60 s^{-1}.

We tested the AnM4Xyl in presence of different amounts of purified TAXI-IIICS and we found that Vmax did not change but K_m increased with increasing concentrations of the purified inhibitor (Fig. 15A). Lines convergence at the Y axis of the double reciprocal plot indicated that the inhibition is competitive (Fig. 15A). A secondary plot of slopes against TAXI-IIICS concentration gave a K_i of 18.8 nM at pH 5 (Fig. 15B).

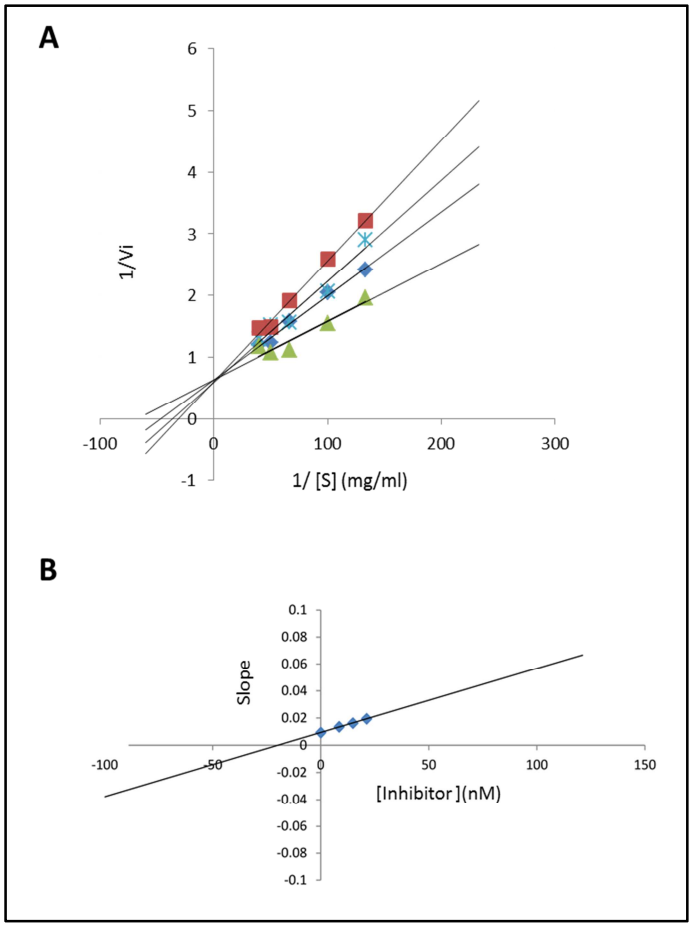

Fig. 15 Kinetic analyses of TAXI-IIICS. **A.** Double-reciprocal plots of the inhibition of AnM4Xyl by TAXI-IIICS using low viscosity wheat arabinoxylan as substrate. Xylanase activity with no inhibitor (▲), in presence of 8.0 nM (♦), 14.0 nM (✳) and 20.0 nM (■) of inhibitor. **B.** Secondary plot of slopes from the double-reciprocal plot against inhibitor concentration to give K_i.

3.6 Inhibition properties of TAXI-III against the xylanase activity of the fungal pathogens *Fusarium graminearum* and *Bipolaris sorokininana*

Purified TAXI-IIICS was also assayed against the xylanase activity of the fungal pathogens *Bipolaris sorokiniana* and *Fusarium graminearum* grown on wheat bran as sole carbon source. TAXI-IIICS inhibited only partially the total xylanase activity of *F. graminearum*. Conversely, no inhibition activity was observed against the total xylanase activity of *B. sorokiniana* (Fig. 16A). Inhibition assays using total protein extracts from leaves or spike of TAXI-III plants showed the same results, that is an incomplete inhibition and no inhibition against total xylanase activity of *F. graminearum* and *B. sorokiniana*, respectively (Fig. 16B).

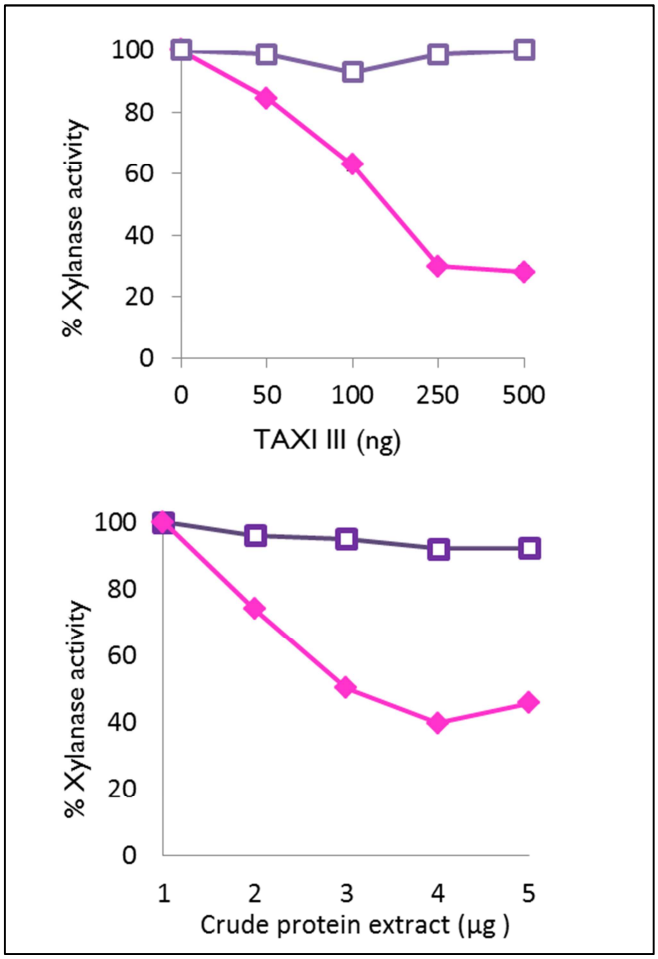

Fig. 16. Inhibition activity of purified TAXI-IIICS or crude protein extract of transgenic line MJ30-23 against *F. graminearum* and *B. sorokiniana* xylanase activities. **A**, Xylanase activities determined in presence of increasing amounts of purified TAXI-IIICS. **B,** Xylanase activities determined in presence of increasing amounts of leaf crude protein extract of transgenic line MJ30-23 overexpressing TAXI IIICS. Inhibition assays performed against the same xylanases in the same conditions with leaf crude protein extract of control plants (*T. durum* cv Svevo) did not show any inhibition activity. Culture filtrates of *F graminearum* (■) and *B. sorokiniana*(□) grown on wheat bran.

In order to verify the origin of the partial inhibition of TAXI-IIICS against the total activity of *F. graminearum*, four xylanases FGSG_11487, FGSG_10999, FGSG_11304 and FGSG_03624, expressed in *Pichia pastoris* (Sella et al., 2013), were used in inhibition assays. Culture filtrates of *P. pastoris* expressing these xylanases (Fig. 17) were used to perform DNS assays with purified TAXI-IIICS or total protein extracts from the transgenic line MJ30-23.

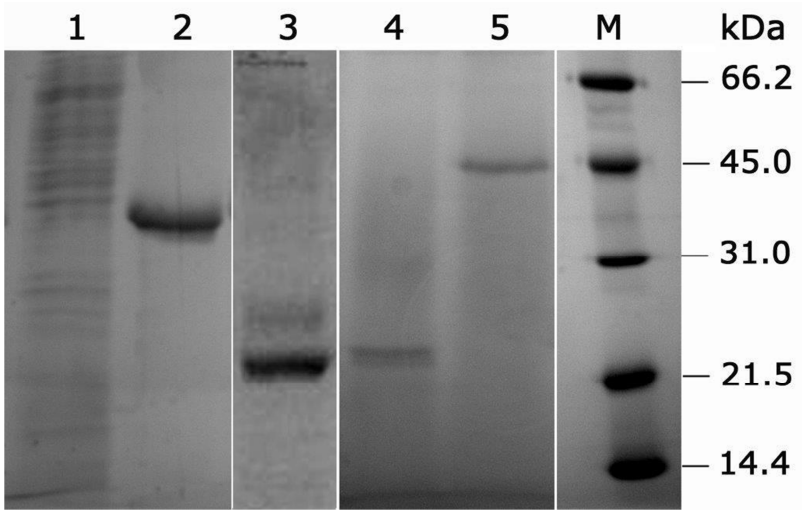

Fig. 17 SDS-PAGE analysis of the secretome of a WT (lane 1) and the *P. pastoris* colonies (lanes 2-5) transformed to express the *F. graminearum* FGSG_11304 (~34 kDa), FGSG_03624 (~23 kDa), FGSG_10999 (~24 kDa) and FGSG_11487 (~40 kDa) xylanases, respectively. Twenty five microliters of *P. pastoris* 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.

The xylanase FGSG_03624 was completely inhibited by 250 ng of TAXI-IIICS, whereas the same amount of inhibitor reduced by 75% the activity of xylanase FGSG_10999. Increased amount of the inhibitor did not modify the inhibition capacity against these two xylanases (Fig. 18A). Conversely, the activity of FGSG_11487 and FGSG_11304 was unaffected by TAXI-IIICS (Fig. 18A). Similar results were obtained by performing the inhibition assays using total protein extract of the transgenic line MJ30-23 (Fig. 18B).

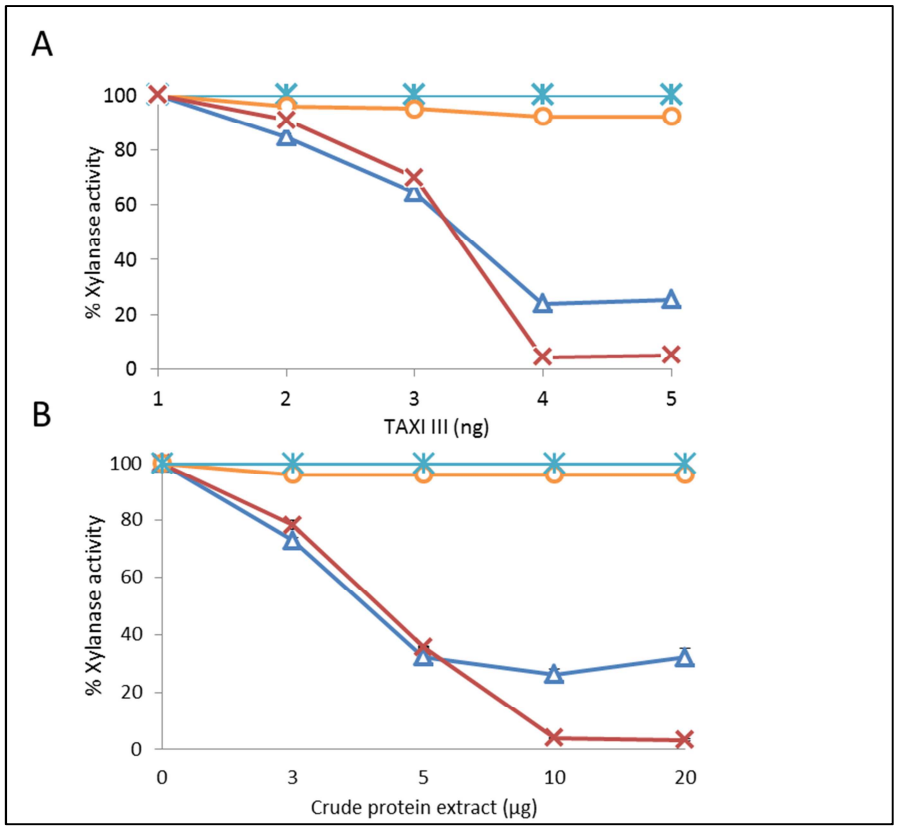

Fig. 18. Inhibition activity of purified TAXI-IIICS or crude protein extract of transgenic line MJ30-23 against *P. pastoris* culture filtrates FGSG_11487 (✳), FGSG_10999 (Δ), FGSG_11304(O) and FGSG_03624 (X). **A**, Xylanase activities determined in presence of increasing amounts of purified TAXI-IIICS. **B**, Xylanase activities determined in presence of increasing amounts of leaf crude protein extract of transgenic line MJ30-23 overexpressing TAXI IIICS. Inhibition assays performed against the same xylanases in the same conditions with leaf crude protein extract of control plants (*T. durum* cv Svevo) did not show any inhibition activity.

3.7 Transgenic wheat TAXI-III lines show increased resistance against *F. graminearum* infection but not against *B. sorokiniana*

Infection experiments with *B. sorokiniana* were performed on the first emerged leaf of TAXI-III lines MJ30-6, MJ30-10, MJ30-23 and wild type plants (*T. durum* cv Svevo). Disease symptoms were visible 48 hours post infection (hpi) and appeared as reddish brown spots of variable size on the leaf surface. In order to facilitate the analysis of single lesions, data were collected 72 hpi. Disease symptoms were evaluated as the ratio between leaf area showing symptoms and the total leaf area, expressed as percentage (Fig. 19). Statistical analysis of data indicated that none of the transgenic lines showed a significant reduction of disease symptoms compared to control plants (Fig. 19).

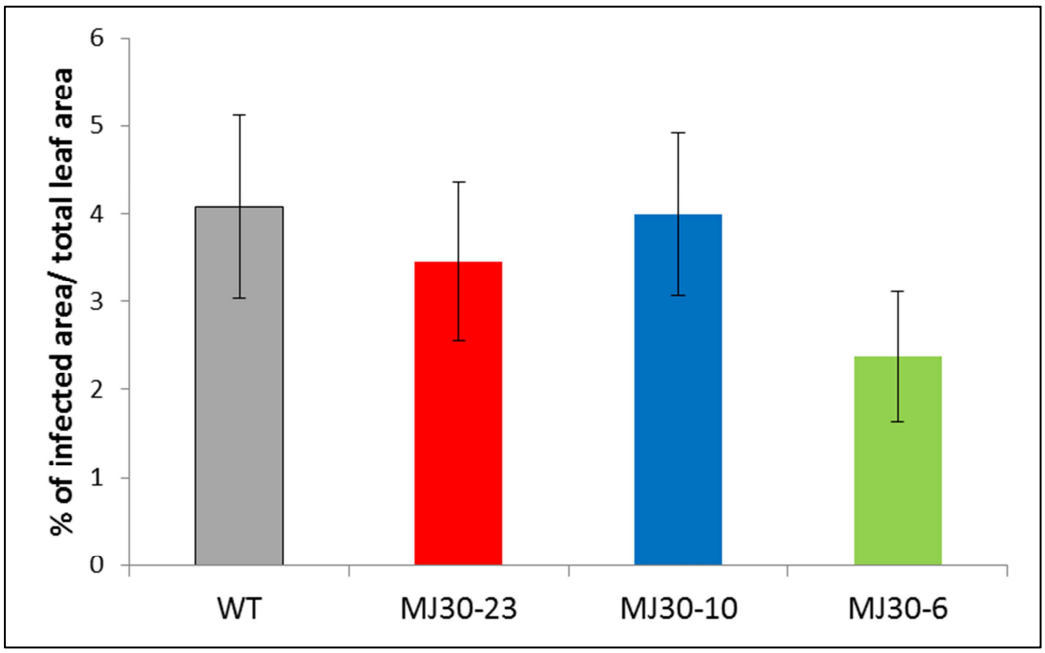

Fig. 19 Quantification of symptomatic leaf area of transgenic lines MJ30-6 (green), MJ30-10 (blue), MJ30-23 (red) and wild type plants (*T. durum* cv Svevo, WT) (grey) inoculated with conidia of *B. sorokiniana*. Symptom severity (lesion expansion area [pixel]/total leaf area [pixel]) × 100 at 72 h post infection (hpi). Data represent the average ± standard errors of four experiments for control plants, three experiments for lines MJ30-10 and MJ30-23 and two experiments for MJ30-6, all performed with at least 13 replicates. The average values were not significantly different according to Student's *t* test. WT, *T. durum* cv Svevo.

Flowering spikes of transgenic lines MJ30-6, MJ30-10, MJ30-23 and wild type plants (*T. durum* cv Svevo) were also subjected to infection with the fungal pathogen *F. graminearum*. Infection experiments were performed by point inoculation of the opposite central spikelets in primary spikes (Zadoks stage 68) of transgenic and control plants. FHB disease symptoms, manifested as spikelet bleaching, usually appeared 3 days post infection (dpi) and the spread of the disease was visually examined for a period of 20 days (Fig. 20). All three transgenic lines showed a significant reduction of symptoms from 3 to 11 dpi compared to control plants. Line MJ30-6 continued to show a significant symptom reduction until 14 dpi, whereas line MJ30-23 until the end of the analysis (20 dpi). However, the maximum symptom reduction was observed between 8 and 10 dpi when the reduction of FHB symptoms was higher than 25% for all three transgenic lines compared to control plants.

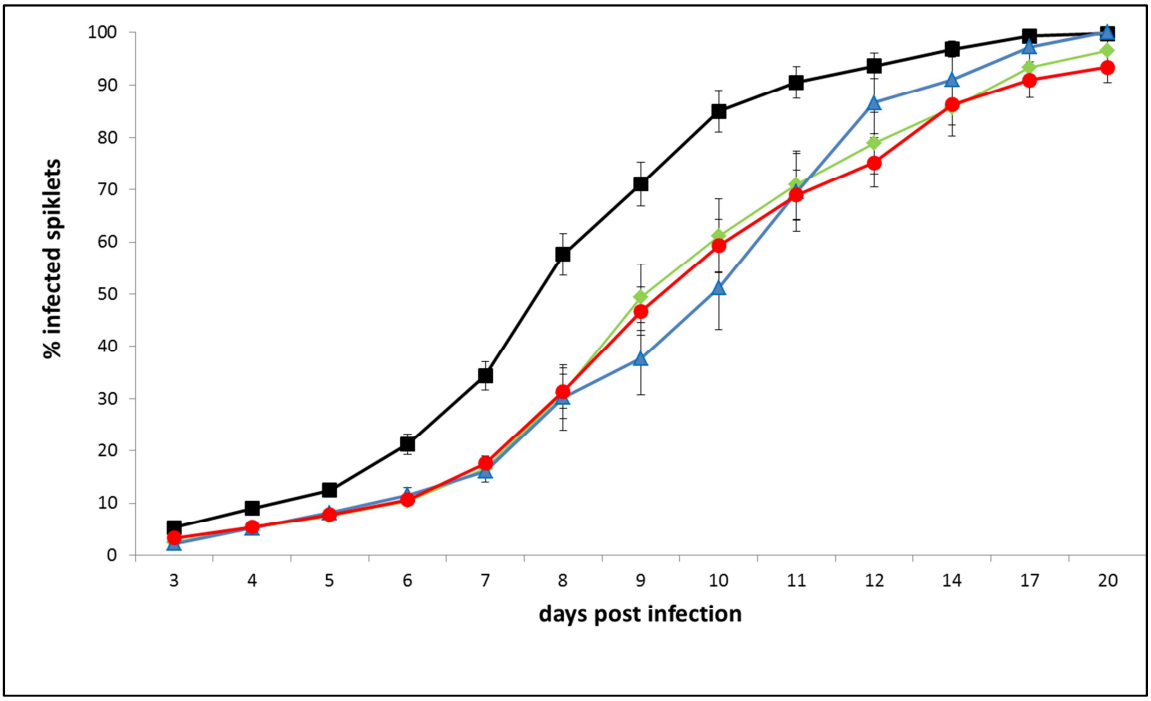

Fig. 20. Time-course analysis of Fusarium head blight symptom development following *Fusarium graminearum* infection of transgenic lines MJ30-6 (♦), MJ30-10 (▲), MJ30-23 (●), and wild type plants (■) (*Triticum durum* cv Svevo, WT). Disease symptoms are expressed as percentage of spikelets showing symptoms on the total number of spikelets per spike. Data represent the average ±standard errors of three experiments for line MJ30-23 and WT plants, two experiments for line MJ30-6 and one experiment for line MJ30-10 performed with at least 12 plants per genotype. The average values of lines MJ30-23, MJ30-10 and MJ30-6 are significantly different from the WT plants according to Student's *t* test ($P \leq 0.01$ or $P \leq 0.05$) until 20 dpi, 14 dpi and 11 dpi respectively .

The reduced FHB symptoms were associated to a reduced accumulation of fungal biomass in the caryopses of transgenic plants. qPCR analyses with primers specific for the fungal *Tri-6* gene, Tri6_10F and Tri6_4R (Horevaj et al., 2011), on total DNA extracted from whole flour of the

transgenic lines and control plants showed about two-fold reduced biomass in the transgenic samples compared to the control sample (*T. durum* cv Svevo) (Fig. 21). Probably, this reflect a slightly reduced growth of the pathogen in the early and advanced phase of the infection of the transgenic plants compared to the control.

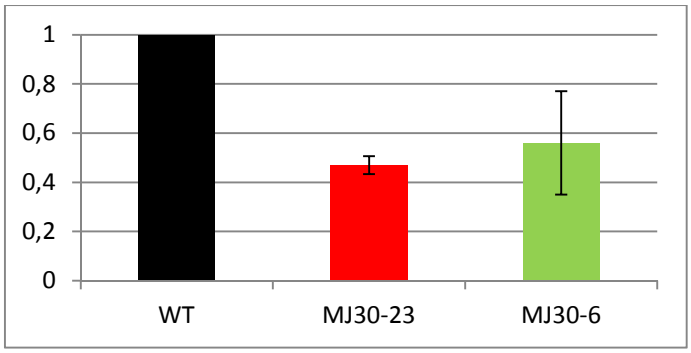

Fig. 21 Relative fungal biomass quantified using qPCR on *Tri-6* gene of flour from *Fusarium* infection experiments. Bars represents average values ± standard error of two biological samples and nine technical replicas for line MJ30-23 (red) and control (WT) (black) and one biological sample with six technical replicas for line MJ30-6 (green).

3.8 TAXI-III limits plant cell death caused by a *Fusarium graminearum* xylanase in lemma tissues

Since the xylanase FGSG_03624 of *F. graminearum* can cause cell death and oxidative stress on bread wheat tissues (Sella et al., 2013), we investigated the effect of TAXI-III on the cell death caused by this xylanase. We performed histochemical analyses on wheat lemmas, since spikelets are the preferred penetration sites of *F. graminearum* (Yang et al., 2012).

Lemmas of durum wheat cv. Svevo were inoculated with 10 µl of purified FGSG_03624 xylanase (20 ng/µl) or with the boiled inactivated enzymes in absence or presence of purified TAXI-III. The amount of TAXI-III used (10 µl, 30 ng/µl) was sufficient to inhibit to completion the activity of FGSG_03624 xylanase.

Microscopic analysis of durum wheat lemmas treated with the xylanase, both in native and in boiled forms, showed the occurrence of cell death, as revealed with the Trypan Blue staining (Fig. 22 b and c). Moreover lemmas treated with FGSG_03624 xylanase previously co-incubated with TAXI-III showed significant reduction of cell death (Fig. 22 d and e). Lemmas treated with the buffer only (Fig. 22 a) or with the same amount of TAXI-III alone (data not shown) appeared unaltered.

These results were confirmed by using lemmas of transgenic TAXI-III plants. In these experiments, the same amount of FGSG_03624 xylanase was used to treat lemmas from TAXI-III plants and control wild-type plants. As expected, the FGSG_03624 xylanase and the boiled sample caused cell death on lemmas of wild-type plants (Fig. 23 c and e). Conversely, this capacity was almost completely abolished on lemmas of TAXI-III plants (Fig. 23 d and f).

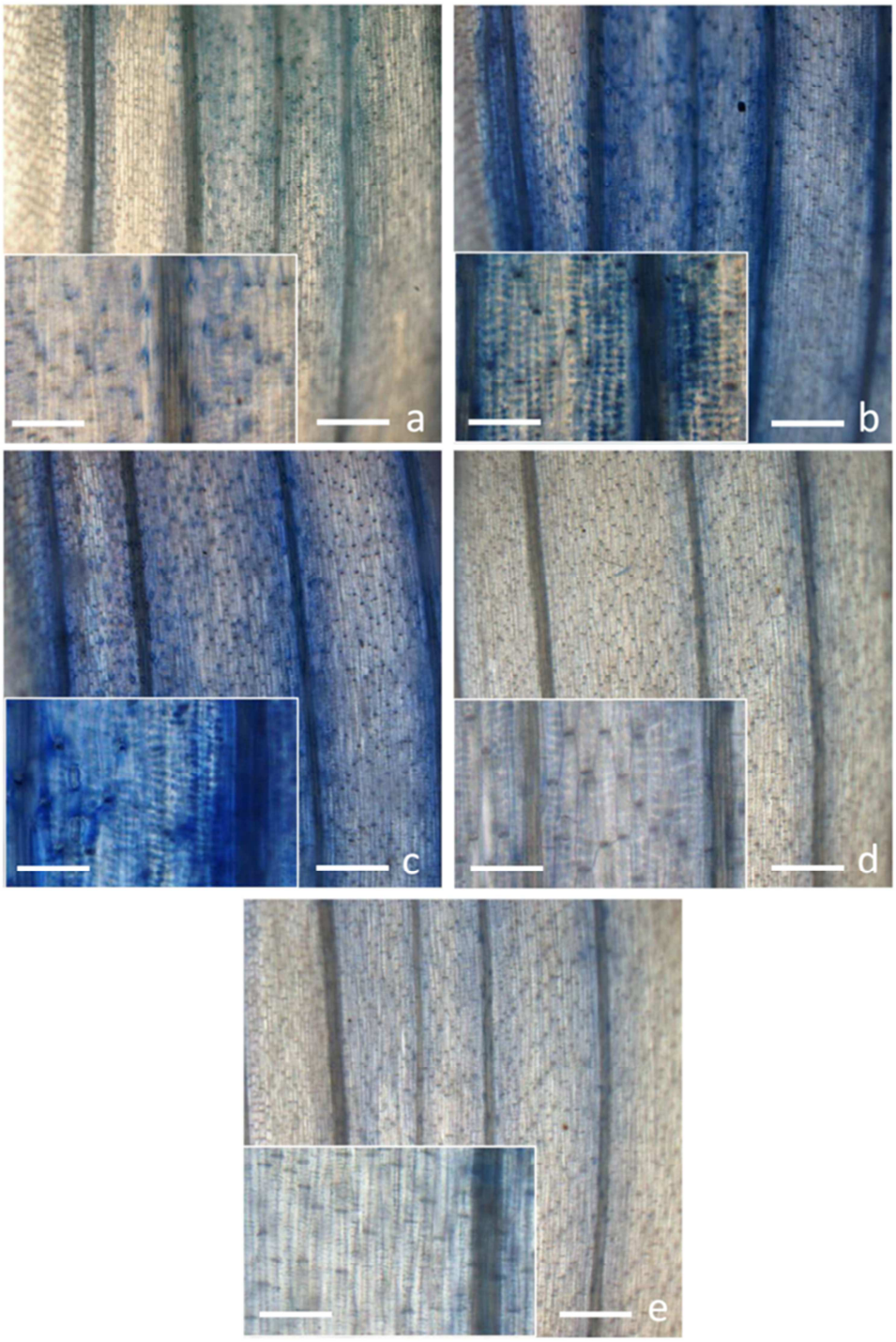

Fig. 22 Lemma tissues of wheat spikelets treated with buffer as control (a) or with FGSG_03624 xylanase (b) or with the same amount of xylanase boiled for 30 min (c) or with the same amount of native and boiled xylanase co-incubated with TAXI-III (d and e, respectively) and stained after 24 h with Trypan Blue. Mesophyll cells under the areas treated with both non-boiled (b) and boiled (c) xylanase are dead (stained in blue), while those treated with both non-boiled and boiled xylanase co-incubated with TAXI-III (d and e, respectively) appear significantly less affected by cell death. Lemmas treated with buffer only (a) appear unaltered ; all bars = 500 µm; bars in the insert = 50 µm.

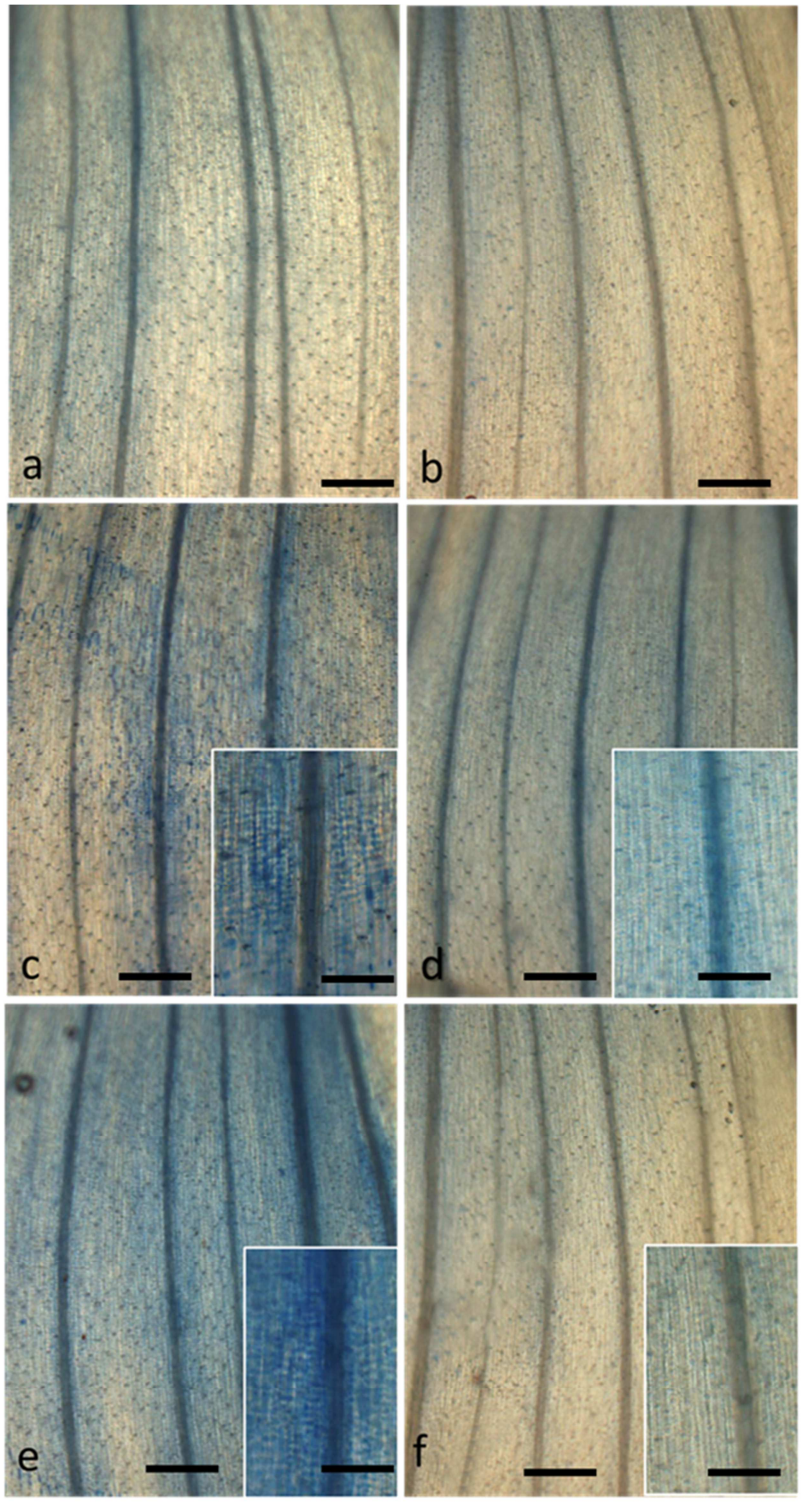

Fig. 23 Lemmas of wheat control plants (left side) and TAXI-III plants (right side) treated with buffer as control (a, b) or with FGSG_03624 xylanase (c, d) or with the same amount of xylanase boiled for 30 min (e, f) and stained after 24 h with Trypan Blue. Mesophyll cells in lemmas of control plants treated with both non-boiled (c) and boiled (e) xylanase are dead (stained in blue), while lemmas of TAXI-III plants treated with xylanase (d) or boiled xylanase (f) appear significantly less affected by cell death. Lemmas treated with buffer only (a, b) appear unaltered. All bars = 500 µm; bars in the insert = 50 µm.

3.9 The effect of TAXI-III in reducing cell death caused by a *Fusarium graminearum* xylanase is also evident in wheat cell suspensions

Wheat cell are a useful tool to study the effects elicited by specific molecules. In our experiments, we treated cell suspensions of the wheat cell line PC998 (DSMZ) with the *F. graminearum* xylanase FGSG_03624 responsible for cell death in wheat tissue. An aliquote of cell suspension (200 µl) in exponential growth phase was treated for 24 h with FGSG_03624 xylanase (1 µg) or with the boiled inactivated xylanase in absence or presence of purified TAXI-III (1.5 µg). Untreated cells or cells treated with only buffers were used as negative controls while a boiled cell suspension was used as positive control for plant cell death. Cell death was visualized by using the vital stain Trypan Blue (Iriti et al., 2006) and assessed by counting blue stained (dead) and unstained (alive) cells in each sample.

The treatment with xylanase or boiled xylanase caused 33.5% and 31.4% of death cells, respectively; whereas a co-incubation of TAXI-III with the xylanase or the boiled xylanase prior the addition to the cell suspensions significantly reduces the number of death cells to 18.7% and 20.6%, respectively (Fig. 24). These results confirmed those obtained on the glumes and indicate that TAXI-III has the capacity to reduce the cell death caused by FGSG_03624 xylanase.

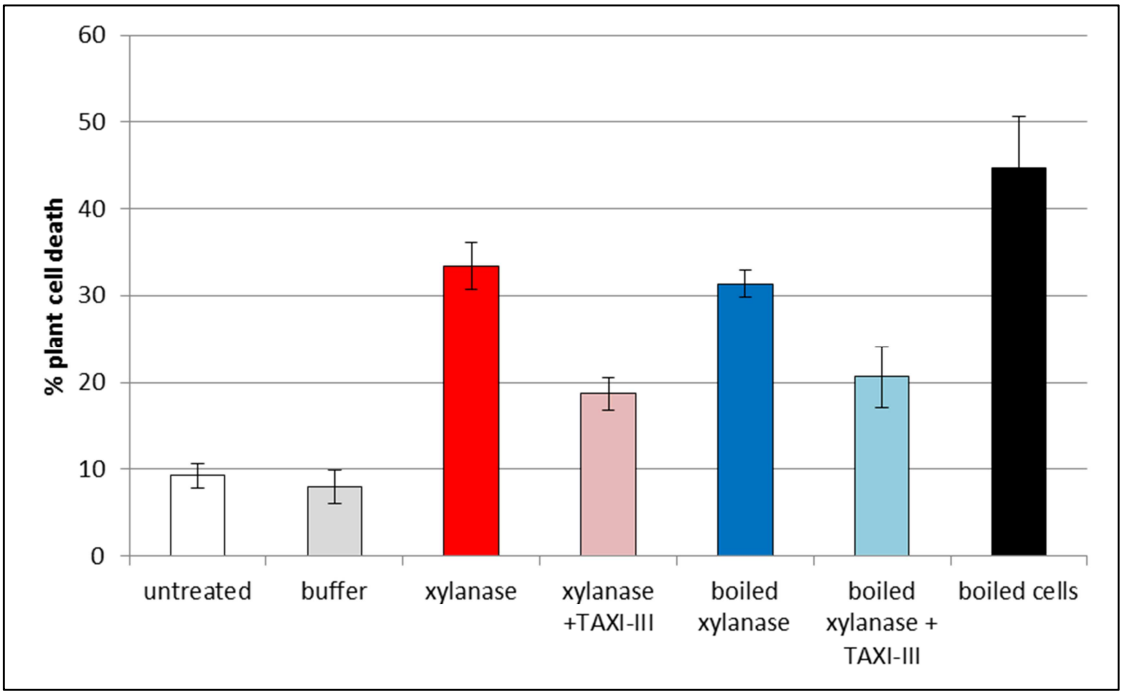

Fig. 24 Analysis of plant cell death in wheat cell suspensions. Cells treated with FGSG_03624 xylanase (red) showed 33.5% of cell death, while the co-incubation of xylanase with TAXI-III (pink) significantly ($P<0.01$) reduces cell death to about 18.7%. Cells treated with boiled xylanase (blue) showed 31.4% of cell death while the co-incubation with TAXI-III (cyan) showed a significant ($P<0.05$) reduction in cell death to 20.6%. Untreated cell (white) and cell treated with buffer only (grey) are used as negative control. Cells boiled (10 minutes) (black) are used as positive control for cell death. Data represent the average ± standard errors of two biological and six technical replicates. Data were analysed using the Student *t* test.

3.10 Molecular docking showed that TAXI-III interacts with the necrotizing peptide of *F. graminearum* xylanase

To investigate the interaction between TAXI-III and the FGSG_03624 xylanase we performed molecular modelling using free available software. Starting from the primary amino acidic sequence of TAXI-III or of FGSG_03624 and using the CHPmodels software (Nielsen et al., 2010) we predicted their 3D structure. To analyse the interaction between the inhibitor and the xylanase we searched for a useful and simple software and we chose the ZDOCK SERVER (Pierce et al., 2014) because it shows as example a docking model with a *Bacillus subtilis* xylanase and TAXI-I.
Without modifying the program settings of the docking model between *B. subtilis* xylanase and TAXI-I, we loaded the 3D structure of FGSG_03624 xylanase and of TAXI-III and we obtained the more probable docking models. Focusing on the xylanase region Q139- S149 that is supposed to be responsible of the necrotizing effect (Sella et al., 2013) and is folded as a sheet in the predicted tertiary structure of the xylanase, the model predicts an interaction between the Q139 and the inhibitor (Fig. 25).

Fig. 25. The ZDOCK model of TAXI-III (cyan) and FGSG_03624 xylanase (grey). The model predicts an interaction between the Q139 of the xylanase (indicated by the red arrow) and the inhibitor.

4. Discussion

Sequence comparison showed high sequence similarity between the *Taxi-III* genes of bread wheat cvs. Chinese Spring, Bobwhite, Norin and the durum wheat cv Svevo, with the latter two cultivars showing an identical sequence. The presence of a few SNPs between the *Taxi-III* genes of cv Chinese Spring and cv Svevo were exploited to distinguish the endogenous gene of cv Svevo (*Taxi-IIISV*) and the transgene from cv Chinese Spring (*Taxi-IIICS*).

Increased sequence divergence was observed between *Taxi-III* and the homolog genes *Taxi-I* and *Taxi-IV*. On the basis of their nucleotide sequence differences, specific primers for PCR assays were developed and used to assign *Taxi-III* on the long arm of chromosome 3A. This result extended our knowledge on the genome localization of the *Taxi* genes that was known so far only for *Taxi-I*, assigned to chromosome 3B (Raedschelders et al., 2004).

Similarly to what reported for bread wheat (Igawa et al., 2004 and 2005), we found *Taxi-III* transcripts mainly in roots and caryopsis of durum wheat cv Svevo and confirmed that this gene is induced following *F. graminearum* infection. Based on this result we proceed with the ectopic expression of *Taxi-IIICS* in the durum wheat cv Svevo.

Transgenic expression of *Taxi-IIICS* under control of the constitutive maize *Ubi-1* promoter endowed the transgenic durum wheat plants with the capacity to accumulate the transgene-encoded TAXI-IIICS in all tissues. This accumulation did not cause any major phenotypic change, although transgene silencing occurred at quite high frequency in the progeny and during plant growth. The accumulation of TAXI-IIICS was easily identified by the presence of inhibition activity against microbial xylanases in total protein extracts of transgenic tissues, including those that, in the untransformed plants, do not show any XIs activity.

TAXI-IIICS purified from transgenic tissue showed a competitive inhibition against AnM4Xyl with a K_i of 18.8 nM at pH 5. This K_i value is similar to that found for TAXI-I (20 nM; Gebruers et al., 2004), one of the best studied and most represented XIs in wheat endosperm. Differently to TAXI-I, TAXI-III has not been detected in wheat endosperm, also following infection with a *F. graminearum* strain exhibiting a reduced pathogenicity (Dornez et al., 2010b). Nevertheless, our results and those previous obtained on bread wheat (Igawa et al., 2005) showed that *Taxi-III* is induced by *F. graminearum* infection.

Although the induction of the endogenous *Taxi-III* following fungal infection can contribute to an increase of xylanase inhibition activity, the level reached is probably not sufficient to contrast effectively the activity of the xylanases secreted by pathogens. Differently, the TAXI-III plants possess a high level of xylanase inhibition activity and this is related to a delay of disease symptom

development. Indeed the TAXI-III plants are characterized by the development of symptoms from the inoculated spikelets but showed reduced spread of symptoms to the surrounding spikelets as compared to control plants. The reduction of FHB symptoms is particularly evident in the early phase of fungal infection (8-10 dpi) when the infection level is reduced by about 25%. Probably, this outcome is due to the reduced ability of *F. graminearum* to degrade the cell wall xylans of transgenic plants. These polysaccharides are major components of the wheat cell wall and for their degradation *F. graminearum* secretes a number of xylanases. Among them, six different xylanase genes are clearly expressed during wheat infection (Sella et al., 2013; Lysøe et al., 2011; Paper et al., 2007). These genes code for xylanases with different catalytic properties. For example, those xylanases belonging to glycoside hydrolase family 11 (GH11) are active on unsubstituted regions of xylan, whereas those of family GH10 can also hydrolyze decorated forms of arabinoxylans (Biely et al., 1997). The contemporary secretion of xylanases with different catalytic properties reflects probably the heterogeneity of cell wall xylans and the complexity of the cell wall.

To contrast this variability of xylanases, plants have evolved different types of XIs with different inhibition properties (Dornez et al., 2010a). In the present work, we demonstrated that TAXI-III inhibits the *F. graminearum* xylanases FGSG_10999 and FGSG_03624 of the GH11 family, whereas it is ineffective against the two xylanases belonging to family GH10, FGSG_11487 and FGSG_11304. These results are in agreement with the notion that TAXI-Type XIs are typically effective against xylanases of GH11 family and ineffective against xylanases of family GH10 (Dornez et al., 2010a). In particular, TAXI-I has been tested against the previous mentioned xylanases of *F. graminearum* and the same results have been obtained (Pollet et al., 2009b). We showed also a variation in the extent of the inhibition capacity of TAXI-III towards the two inhibited xylanases, FGSG_10999 and FGSG_03624, being more effective against the latter one. Worth of noting is that the genes encoding these two xylanases are the most expressed ones during wheat spike infection by *F. graminearum* (Sella et al., 2013). Consequently, the capacity of TAXI-IIICS to inhibit these two xylanases should play a primary role in counteracting the degradative potential of the pathogen on the cell wall xylan during the infection process. Nonetheless, the inability of TAXI-III to inhibit all four xylanases tested is reflected on its capacity to inhibit only partially the total xylanase activity of *F. graminearum*. Probably other XIs could complement the inhibition activity of TAXI-III. Indeed, the two xylanases, FGSG_11487 and FGSG_11304, also expressed during *F. graminearum* infection and not inhibited by TAXI-III are inhibited *in vitro* by XIP-I (Pollet et al., 2009b).

Although TAXI-III is not able to inhibit to completion the total xylanase activity of *F. graminearum*, its overexpression in the transgenic plants correlates with a delayed FHB symptom. In contrast, the inability of TAXI-III to inhibit the xylanase activity secreted by *B. sorokiniana* results in the lack of any reduction of spot blotch symptoms in the TAXI-III plants. These results provide clear evidences of the active contribution of xylanases during the infection of wheat spike tissue by *F. graminearum* and suggest that TAXI-III can control the activity of these enzymes during the infection process. Moreover, although the reduced FHB symptoms in the TAXI-III plants is especially evident in the initial phase of infection, its impact is durable since whole flour from caryopses of TAXI-III plants infected with *F. graminearum* showed a reduced accumulation of fungal biomass.

These results with the TAXI-III plants parallel those previously obtained with transgenic plants expressing a polygalacturonase inhibiting protein (PGIP) or a pectin methyl esterase inhibitor (PMEI). Both inhibitors limit the activity of fungal polygalacturonases (PGs), one of the first CWDEs secreted by pathogens during the infection process, and in both cases their effectiveness in limiting microbial pathogen colonization of host tissue have been demonstrated in different species. In particular, transgenic wheat expressing PGIP or PMEI showed a reduction of FHB disease symptom development similar to that observed for the TAXI-III plants, with the disease symptom mostly reduced in the initial-middle stage of infection (Volpi et al. 2011; Ferrari et al. 2012).

Worth of noting is a recent result obtained in rice in which the overexpression of a rice xylanase inhibitor, OsHI-XIP, enhances resistance to the rice striped stem borer and decreases the feeding and oviposition preferences of the rice brown planthopper (BPH) *Nilaparvata lugens*, demonstrating a protective role of xylanase inhibitors against herbivores (Xin et al., 2014).

To shed light on the molecular mechanism underlying the capacity of the transgenic plants expressing TAXI-III to limit FHB disease symptoms, we performed experiments on glumes of flowering wheat spikes with the xylanase FGSG_03624, causing plant cell death in wheat tissues (Sella et al., 2013), and showed that the presence of TAXI-III prevented necrosis in durum wheat glumes. The protective activity of TAXI-III was also evident on wheat cell suspensions treated with FGSG_03624 xylanase, where cell death was reduced of about 40% in presence of TAXI-III. It is notable that results obtained both by co-incubating TAXI-III with the xylanase prior treatment of wheat glumes or cells, were more directly confirmed by treating glumes of transgenic wheat plants expressing TAXI-III.

These results suggests that the delay of FHB symptoms on transgenic TAXI-III plants can be also due to the capacity of TAXI-III to prevent the necrotizing capacity of xylanase FGSG_03624. On the basis of previous findings showing that the recognition of the necrotizing xylanase EIX of *Trichoderma viride* by an LRR receptor-like protein was essential for plant cell death in tomato (Ron et al., 2004), we hypothesize that TAXI-III interacting with the xylanase FGSG_03624 prevents the recognition of this xylanase by a plant receptor from which could depend cell death elicitation. Indeed, molecular docking analyses between the xylanase FGSG_03624 and TAXI-III showed that TAXI-III interacts with the residue Q139 belonging to the peptide Q139-S149, supposed to be responsible for the necrotizing effect of the xylanase FGSG_03624 (Sella et al., 2013).

The capacity of glycosidase inhibitor to act as inhibitor of enzyme activity and prevent the necrotizing activity of the same enzyme has been reported for the interaction between the PGIP of *Brassica napus* and the PG of *Sclerotinia sclerotium*. These capacities of *B. napus* PGIP correlates with the delay of disease symptoms in transgenic Arabidopsis plants (Bashi et al., 2013).

Knock out experiments showed that the xylanase FGSG_03624 is not essential for virulence of *F. graminearum* (Sella et al., 2013). This aspect can be explained by considering that the pathogen produce at least six xylanases during the infection process and probably the lack of a single xylanase gene can be replaced by the redundant function of the other xylanases. In this view, it would be interesting to verify whether the other *F. graminearum* xylanases have the capacity to elicit cell death or if this activity is limited to the xylanase FGSG_03624.

In conclusion, we provide for the first time a direct evidence *in planta* that XIs are involved in plant defense against fungal pathogens (Moscetti et al., 2013). Specifically, we showed that the transgenic wheat plants expressing TAXI-III have delayed FHB symptoms caused by *F. graminearum*. Moreover, we brought evidences that this capacity of TAXI-III can be due to both its ability to inhibit the hydrolytic activity of the xylanases secreted by the pathogen and to its capability to prevent the necrotizing activity of xylanase FGSG_03624.
These results and those previously reported with different proteinaceous inhibitors of CWDEs, like PGIP and PMEI, strengthen also the notion that the integrity of host cell wall polysaccharides, irrespective of their amount and type, play a key role in keeping the cell wall as functional barrier against pathogens.

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