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Fusarium graminearum produces different xylanases causing host cell death that is prevented by the xylanase inhibitors XIP-I and TAXI-III in wheat

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ABSTRACT

To shed light on the role of Xylanase Inhibitors (XIs) during *Fusarium graminearum* infection, we first demonstrated that three out of four *F. graminearum* xylanases, in addition to their xylan degrading activity, have also the capacity to cause host cell death both in cell suspensions and wheat spike tissue.

Subsequently, we demonstrated that TAXI-III and XIP-I prevented both the enzyme and host cell death activities of *F. graminearum* xylanases. In particular, we showed that the enzymatic inhibition by TAXI-III and XIP-I was competitive and only FGSG_11487 escaped inhibition.

The finding that TAXI-III and XIP-I prevented cell death activity of heat inactivated xylanases and that XIP-I precluded the cell death activity of FGSG_11487 – even if XIP-I does not inhibit its enzyme activity – suggests that the catalytic and the cell death activities are separated features of these xylanases.

Finally, the efficacy of TAXI-III or XIP-I to prevent host cell death caused by xylanases was confirmed in transgenic plants expressing separately these inhibitors, suggesting that the XIs could limit *F. graminearum* infection *via* direct inhibition of xylanase activity and/or by preventing host cell death.

1. Introduction

Cell-wall-degrading enzymes (CWDEs) are secreted during the infection process by several fungal pathogens to overcome the first line of defense represented by plant cell wall and colonize the host tissue [1]. Endo-β-1,4-xylanases (xylanases; EC 3.2.1.8) are key enzymes in the degradation of xylan, the main cell wall component of commelinoid monocot plants. According to their protein sequence homology, most xylanases are classified into glycoside hydrolase (GH) family 10 and 11 from the CAZy classification, while others belong to the GH5, GH8, and GH43 families (http://www.cazy.org [2]). GH10 family includes plant, fungal, and bacterial enzymes whereas the structurally unrelated GH11 family only includes fungal and bacterial enzymes [3].

The contribution of these enzymes in pathogenesis has been demonstrated for the necrotrophic pathogen *Botrytis cinerea* as the Xyn11A is required to determine full virulence during the infection of tomato leaves and grape berries [4]. Recently, a number of effector candidates have been identified using bioinformatics approaches in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* and one is a putative xylanase related to *B. cinerea* Xyn11A [5].

The activity of microbial xylanases is controlled *in vitro* by specific protein inhibitors (xylanase inhibitors, XI) localized in the plant cell wall. In wheat, three structurally different classes of XIs have been identified: *Triticum aestivum* XI (TAXI) [6], XI protein (XIP) [7], and thaumatin-like XI (TLXI) [8]. Basically, TAXI-type and TLXI-type inhibitors inhibit xylanases from the GH11 family, whereas XIP-type inhibitors inhibit some xylanases from the GH10 and GH11 families [9]. XIs are considered to be part of the defense mechanisms used by plants to counteract microbial pathogens mainly because of their ability to inhibit specifically

Abbreviations: AnM4Xyl, *Aspergillus niger* endo-β-1,4-xylanase M4; CWDE, cell wall degrading enzymes; FHB, Fusarium head blight; GH, glycoside hydrolase; PG, polygalacturonase; PGIP, polygalacturonase inhibiting protein; PvPGIP2, *Phaesolus vulgaris* polygalacturonase inhibiting protein-2; TAXI, *Triticum aestivum* xylanase inhibitor; TlM3Xyl, *Trichoderma longibrachiatum* endo-β-1,4-xylanase M3; TLXI, thaumatin-like xylanase inhibitor; XIs, xylanase inhibitors; XIP, xylanase inhibitor protein.

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microbial xylanases and for their inducibility following pathogen infection [10].

A more direct evidence of their involvement in plant defense has been reported by using transgenic plants. Wheat plants expressing TAXI-III, a TAXI-type XI, showed reduced disease symptoms against *Fusarium graminearum* [11], the major causal agent of *Fusarium* head blight (FHB). Similarly, transgenic plants expressing the XIP-type from rice, RIXI or OsHI-XIP, showed improved resistance against fungal pathogens or herbivores, respectively [12,13]. The capacity of these transgenic plants to limit host tissue colonization by the pathogens has been related to their ability to inhibit the xylanases secreted by the pathogens. However, fungal xylanases could also induce necrosis of the host tissue independently from their enzymatic activity, as shown for the GH11 xylanases EIX, Xyn11A and FGSG_03624 of *Trichoderma viride*, *B. cinerea* and *F. graminearum*, respectively [14–16]. This ability to elicit necrosis has been suggested to depend on the presence of a short stretch of residues in the amino acidic sequence of these xylanases [15,17]. Recently, we showed that TAXI-III is able to prevent the necrotic activity of the xylanase FGSG_03624 secreted by *F. graminearum* [18], suggesting that this property may contribute to delay *Fusarium* head blight (FHB) symptoms caused by *F. graminearum* in transgenic durum wheat plants expressing TAXI-III [11]. However, the observation that *F. graminearum* secretes at least six endo-xylanases during the infection process [16,19,20] raised the question whether these *F. graminearum* xylanases are able to cause necrosis and to which extent XIs can prevent both enzymatic and cell death activities.

In the present work, we investigated the enzymatic and cell death activities of four *F. graminearum* xylanases expressed during wheat spike infection and assessed the capacity of TAXI-III and XIP-I to inhibit these properties. We performed the analyses on wheat cell suspensions and on transgenic durum wheat plants expressing TAXI-III or XIP-I.

2. Materials and methods

2.1. Plant material and nucleic acid analyses

Durum wheat transgenic Taxi-III plants were those reported by Moscetti et al. [11]. Transgenic durum wheat plants constitutively expressing XIP-I were produced by biolistic method co-transforming the constructs pAHC17_Ubi_Xip-I and pUbi::bar [21], carrying the *bar* gene that confers resistance to the bialaphos herbicide, into immature embryos of *Triticum durum* cv Svevo as described by Volpi et al. [22]. The pAHC17_Ubi_XipI construct was prepared by inserting the complete coding region of *Xip-I* (gently provided by Dr. Nathalie Juge, Institute of Food Research, UK), into the *Bam*HI site of pAHC17 [21] under control of the maize *Ubiquitin1* promoter and *NOS* terminator. The *Bam*HI sites flanking the complete coding region of *Xip-I* were generated by PCR amplification using the forward and reverse primers XipI_BamHI_1F (5'-ATAAGGATCCATGGCGCCGCTCGCAGC) and XipI_BamHI_1R (5'-GAAGGATCCTCAGGCGTAGTACTTGATC), respectively. The correct sequence of pAHC17_Ubi_XipI construct (5639 bp) and the insertion sites were confirmed by nucleic acid sequencing.

The presence of pAHC17_Ubi_Xip-I in bialaphos-resistant T_0 plants and their progenies was verified by PCR using total DNA obtained from leaf sections of mature plants [23]. DNA amplification was carried out according to the procedures specified for GoTaqGreen MasterMix (Promega) at an annealing temperature of 60 °C by using the specific primer pair UBI-49F (5′-TCGATGCTCACCCTGTTGTTT) and Xip-I-784R (5′- TGTACCCACTGGTGGGACTTG) which produces an amplicon of 833 bp.

Plants containing the pAHC17_Ubi_Xip-I were also characterized by RT-PCR using the primer pair Xip-I-381F (5′-ACCTCTGGAACTCCTACTTC) and Xip-I-784 reported above. Total RNA extraction was performed from wheat leaf by using the Spectrum plant total RNA kit (Sigma–Aldrich, Milan, Italy). RT-PCR was performed using the Quantitec Reverse Transcription Kit (Qiagen, Milan, Italy).

Growth of wild type (control) and transgenic plants (T_4 generation) was performed by using seeds surface sterilized with sodium hypochlorite (0.5%, v/v) for 10 min and then rinsed thoroughly in sterile water. Plants were vernalized at 4 °C for 2 weeks and grown in a climatic chamber at 18–23 °C with a 14-h photoperiod (300 $\mu E\, m^{-2}\, s^{-1}$). The wheat growth stages were based on the method of Zadoks [24].

2.2. Heterologous expression and purification of xylanases FGSG_03624, FGSG_10999 FGSG_11304 and FGSG_11487

The FGSG_03624, FGSG_10999, FGSG_11487 and FGSG_11304 xylanase genes were cloned and expressed in *Pichia pastoris* as previously reported [11,16]. The purification of the recombinant FGSG_03624 xylanase was performed as reported by Sella and associates [16]. For the recombinant FGSG_10999, FGSG_11487 and FGSG_11304 xylanases, *P. pastoris* cultures were centrifuged at 10,000 × *g* for 5 min and the supernatants were filtered through GFA cellulose acetate membranes (Sartorius Italy, Monza Brianza, Italy) of decreasing pore sizes (0.8, 0.45 and 0.2 μm). The protein was precipitated with ammonium sulphate 75% (*w*/*v*) and recovered by centrifugation at 8000 × *g* for 20 min at 4 °C. The pellets were resuspended in 20 mL of deionized water and centrifuged at 10,000 × *g* for 10 min at 4 °C. The recovered supernatants were then dialyzed in water overnight at 4 °C using Nominal MWCO 3500 membranes (Orange Scientific).

The supernatant containing the recombinant FGSG_10999 xylanase was subjected to preparative isoelectrofocusing (IEF; LKB 110 column) in the pH range 3–10 (Pharmalyte anpholines; GE Healthcare Europe GmbH, Milano, Italy). After the run, the 60 fractions (2 mL each) obtained were assayed for xylanase activity by the dinitrosalicylic acid (DNS) assay as previously reported [16], and the 3 most active fractions were mixed, supplemented with 1 M NaCl to remove ampholytes and then desalted with 50 mM sodium citrate buffer pH 5.0 using a PD-10 desalting column (GE Healthcare Europe GmbH, Milano, Italy).

The supernatant containing the recombinant FGSG_11487 xylanase was purified by an AKTA system (GE Healthcare, Sweden) equipped with a cation exchange S-Sepharose column. Bound protein was eluted with a linear gradient of NaCl 0–0.5 M. The activity of the fractions was assayed for xylanase activity as above reported.

The supernatant containing the recombinant FGSG_11304 xylanase was applied to a Concanavalin A Sepharose 4B column equilibrated with 100 mM sodium acetate buffer (pH 6.0), 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Before loading, the sample was diluted with 4 volumes of the same equilibrating buffer. The column was eluted with a linear gradient of the same buffer supplemented with 250 mM of α-methyl-D-mannopyranoside. The 5 mL fractions eluted were assayed for xylanase activity and the most active fraction was desalted as above reported.

Fractions were analyzed by SDS-PAGE according to Laemmli [25] by a Mini Protean II unit (Bio-Rad). The gel was stained with the colloidal Coomassie Brilliant G-250 blue silver method [26].

2.3. TAXI-III, XIP-I purification and protein analysis

TAXI-III extraction and purification were performed as reported by Moscetti et al. [11]. XIP-I was purified from crude protein extract by affinity chromatography on a xylanase-Sepharose conjugate col-