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Research article

The *Fusarium graminearum* cerato-platanins loosen cellulose substrates enhancing fungal cellulase activity as expansin-like proteins

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ABSTRACT

Cerato-platanin proteins (CPPs) are small non-catalytic, cysteine-rich hydrophobic proteins produced by filamentous fungi. The genome of *Fusarium graminearum*, the causal agent of *Fusarium* head blight disease of wheat and other cereal grains, contains two genes putatively encoding for CPPs. To better characterize their features, the two FgCPPs were heterologously expressed in *Pichia pastoris*. The recombinant FgCPPs reduced the viscosity of a cellulose soluble derivate (carboxymethyl cellulose, CMC). The same effect was not observed on other polysaccharide substrates such as chitin, 1,3-β-glucan, xylan and pectin. Indeed, differently from other fungal CPPs and similarly to expansins, FgCPPs are trapped by cellulose and not by chitin, thus suggesting that these proteins interact with cellulose. A double knock-out mutant deleted of both FgCPPs encoding genes produces much more cellulase activity than the corresponding wild type strain when grown on CMC, likely compensating the absence of FgCPPs. This result prompted us to investigate a possible synergistic effect of these proteins with fungal cellulases. The incubation of FgCPPs in the presence of a fungal cellulase (EC 3.2.1.4) determines an increased enzymatic activity on CMC, filter paper and wheat cell walls. The observation that FgCPPs act with a non-hydrolytic mechanism indicates that these proteins favor fungal cellulase activity in an expansin-like manner. Though the disruption of the FgCPP genes had no demonstrable impact on fungal virulence, our experimental data suggest their probable involvement in virulence, thus we refer to them as accessory virulence genes. Our results suggest also that the FgCPPs could be exploited for future biotechnological application in second-generation biofuels production on lignocellulosic biomasses rich in cellulose. Finally, we demonstrate that FgCPPs act as elicitors of defense responses on *Arabidopsis* leaves, increasing resistance to *Botrytis cinerea* infections.

1. Introduction

Cerato-platanin proteins (CPPs) are small non-catalytic, cysteine-rich and moderately hydrophobic proteins produced by filamentous fungi (Chen et al., 2013). Genes encoding for CPPs have been found in several fungal genomes, with Ascomycete genomes usually containing one or two encoding genes (Chen et al., 2013; Gaderer et al., 2014). These proteins contain a signal peptide and are thus secreted by fungi, and were reported to bind N-acetylglucosamine oligomers and chitin (de Oliveira et al., 2011), being possibly localized at the fungal cell walls (Gaderer et al., 2014; Pazzagli et al., 2014).

Fusarium graminearum Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is a necrotrophic fungal plant pathogen responsible of the Fusarium head blight (FHB) disease of monocot cereal species such as wheat and causes yield reduction and contamination of seeds with mycotoxins such as the trichothecene deoxynivalenol (DON; Brown et al., 2010) and the polyketide zearalenone (ZEA; Voigt et al., 2007). The genome of this fungus contains two genes encoding for proteins with the cerato-platanin domain (Pfam07249). A role played by these two proteins, named FgCPP1 and FgCPP2, was recently demonstrated by producing and characterizing single and double *F. graminearum* knock-out mutants. The mutants were not measurably impaired in virulence after point inoculation of wheat head spikes, thus the two FgCPPs are not essential for fungal infection but they were shown to protect fungal cell wall polysaccharides from enzymatic degradation by β-1,3-glucanase and chitinase, suggesting their possible localization at the fungal cell wall (Quarantin et al., 2016). However, FgCPPs are also predicted to be secreted and phytotoxic (Lu and Edwards, 2016) and could therefore play additional roles during fungal growth and plant infection.

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Fungal CPPs exhibit structural features resembling plant expansins, small proteins of about 26 kDa secreted by plant cells during growth that loosen and disrupt the non-covalent bonding networks of cell wall polysaccharides and are involved in "acidic-induced growth" and other various processes (Sampedro and Cosgrove, 2005; Baccelli et al., 2014b; Gaderer et al., 2014; Cosgrove, 2015). Structurally, expansins consist of two domains: an N-terminal domain (D1), featuring the double ψ-β-barrel fold homologous to the catalytic domain of the glycoside hydrolase family 45 (GH45) (Cosgrove, 2015), and a C terminal domain (D2), having a β-sandwich fold classified as a family-63 carbohydrate binding module (Sampedro and Cosgrove, 2005). An open surface suitable for polysaccharide binding spans the two domains and recent data demonstrate that binding is largely determined by the D2 domain (Cosgrove, 2015). Despite the structural similarity, expansins lack the β-1,4-hydrolytic activity of GH45 enzymes, which in turn lack the wall-extension activity of expansins. Experimental evidence suggests that expansins loosen cell walls via a non-enzymatic mechanism that induces slippage of cellulose microfibrils in the plant cell wall (Cosgrove, 2000).

In fungi, CPPs have a single domain (named "cerato-platanin domain"), which forms a double-ψ-β-barrel fold, quite similar to the D1 domain found in expansin proteins (de Oliveira et al., 2011; de O Barsottini et al., 2013). Therefore, CPPs have been classified as "one domain expansins-like" because of a distantly related phylogenetic origin and the absence of the D2 domain typical of plant and bacterial expansins (Baccelli et al., 2014b; Baccelli, 2015).

In bacteria, the solved crystal structure of the EXLX1 protein secreted by *Bacillus subtilis*, a Gram-positive soil bacterium capable of colonizing the surface of plant roots, shows a canonical plant expansin structure, despite the low sequence similarity (Kerff et al., 2008). Similarly to plant expansins, the EXLX1 protein shows non-hydrolytic cell wall extension activity against major cell wall polysaccharides (Kerff et al., 2008; Georgelis et al., 2015).

Interestingly, the ability of plant expansin and of expansin-like proteins found in microorganisms to weaken cellulose in a non-enzymatic manner is related to the presence of a conserved Asp-77 residue (D77) whose carboxyl group is crucial for loosening (Luti et al., 2017). The D77 residue in the *Ceratocystis platani* CP mature sequence has been also shown to be involved in the oligosaccharide-binding capability together with other residues conserved among the members of the CPP family (de Oliveira et al., 2011). Indeed, it is known that the *C. platani* CP binds to *N*-acetylglucosamine oligomers and chitin (de Oliveira et al., 2011; Baccelli et al., 2014b). It is worth noting that the amino acid sequence of the *C. platani* CP contains also an Asn-84 (N84) residue responsible for the lack of glycoside hydrolase activity, which is conserved in other fungal CPPs (de Oliveira et al., 2011).

The ability of the *C. platani* CP and its orthologous *C. populicola* Pop1 to loosen cellulose in an expansin-like manner (Baccelli et al., 2014b) suggests that CPPs could loosen plant cell walls facilitating hyphae advancement in the plant tissue during infection. Besides, the association to cell wall chitin, demonstrated for the *C. platani* CP, *Trichoderma atroviride* Epl1, *Botrytis cinerea* Bcspl1 and *Verticillium dahliae* VdCP1 (de Oliveira et al., 2011; Frischmann et al., 2013; Frías et al., 2014; Zhang et al., 2017), seems to indicate that CPPs could concurrently favor hyphae growth (Baccelli, 2015).

The observation that treatments of plant tissues with the CPPs of some pathogenic and beneficial biocontrol fungi seem to elicit defense responses and/or necrosis in host and non-host plants (Gaderer et al., 2014; Baccelli et al., 2014a; Pazzagli et al., 2014; Salas-Marina et al., 2015; Wang et al., 2016; Ashwin et al., 2017) indicate that fungal CPPs could act also as microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) or assist tissue colonization of necrotrophic pathogens with their necrosis-inducing activity (Frías et al., 2011, 2013; Gaderer et al., 2014, 2015; Pazzagli et al., 2014; Luti et al., 2016; Zhang et al., 2017). Protein regions recognized as PAMPs by the plant immune system are usually very well conserved in the corresponding protein families, such as in the case of bacterial flagellin (Felix et al., 1999). Accordingly, the alignment of 146 putative CPP sequences showed the presence of two regions with high degree of conservation corresponding to two loops interacting with each other, forming a small protrusion in the protein surface (de Oliveira et al., 2011). In the *B. cinerea* cerato-platanin BcSpl1, these two regions of 10 residues each are included in a 40-amino acid (aa) conserved region and the necrosis-inducing activity seems to reside on these two-peptide motives (Frías et al., 2014). Furthermore, the D77 residue crucial for cellulose loosening, conserved in CPP and expansin proteins, was also demonstrated essential for PAMP activity (Luti et al., 2017).

In order to better define the features of the *F. graminearum* CPPs, we heterologously expressed these two proteins in the yeast *Pichia pastoris*. Firstly, the recombinant proteins were characterized for their ability to loosen cellulose soluble derivate substrates (carboxymethyl cellulose, CMC) with different viscosities and other polysaccharides, and for their ability to bind CMC, filter paper, chitin or 1,3-β-glucan. Since the double mutant $\Delta\Delta fgcpp_{1,2}$ was more sensitive to chitinase treatment than the WT (Quarantin et al., 2016), we secondly, investigated whether the recombinant FgCPPs can protect chitin from chitinase. We demonstrate that the FgCPPs interact with CMC affecting its viscosity and that a double mutant deleted of both FgCPP encoding genes ($\Delta\Delta fgcpp_{1,2}$) produced much more cellulase activity compared to WT strain when grown in CMC containing medium. These results prompted us to investigate a possible synergistic effect of FgCPPs and cellulases. To this aim we tested the FgCPPs in presence of a fungal cellulase on CMC, wheat cell walls and filter paper.

Finally, we also characterized the ability of the recombinant FgCPPs to act as elicitors of defense responses by *in vivo* treatments on the model plant *Arabidopsis thaliana*.

2. Materials and methods

2.1. Sequences analysis and primers design

All the primers used were designed by using PRIMER3 (http://primer3.ut.ee/) and PerlPrimer v.1.1.17 softwares (Supplementary Table 1). Post-translational modifications of heterologously expressed proteins were predicted using proteomic tools (NetNGlyc 1.0 and NetOGlyc 4.0; http://expasy.org/tools/) and signal peptides (SP) were identified with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/).

2.2. Cloning and expression of the FgCPPs in P. pastoris

The cDNAs of the entire coding sequences of the *fgcpp1* and *fgcpp2* genes were obtained from total RNA extracted from wheat spikelets 5 days post inoculation (dpi) with *F. graminearum*. RNA extraction, DNase treatment and reverse transcription were performed as reported in Quarantin et al. (2016). The genes were amplified using the cDNAs as template, the ORF primers pair ORF10212for/rev and ORF11205for/rev (Supplementary Table 1) and the "REDTaq ReadyMix PCR Reaction" (Sigma-Aldrich). The PCR was performed by repeating for 35 times the following cycle: 30 s at 94 °C; 30 s at 50 or 52 °C; 1 min at 72 °C. The amplification products of the expected size (*fgcpp1* ORF: 420 bp; *fgcpp2* ORF: 423 bp) were purified using the "Wizard SV Gel and PCR Clean-Up System kit" (Promega) and then cloned into the pGEM-T Easy vector (Promega) following manufacturer's instructions. The cloned cDNAs were sequenced in order to check the correctness of the nucleotide sequence and then amplified with Pfu DNA polymerase (Promega) by using specific primers (*pPICZαA*for/rev, Supplementary Table 1) containing adaptors for *Eco*RI and *Xba*I recognition sequences for amplification of the coding sequence without signal peptide. The amplifications were performed by repeating for 35 times the following cycle: 30 s at 94 °C; 30 s at 50 °C; 1 min at 72 °C. The PCR amplicons, purified as above reported, were ligated into the *Eco*RI and *Xba*I sites of

the pPICZαA expression vector (Invitrogen Life Technologies) containing an α-factor signal for secretion of the recombinant proteins. The ligation mixtures were then used to transform *Escherichia coli* competent cells, selected in low salt lysogeny broth medium (Fluka) supplemented with 25 μg mL^{-1} Zeocin (InvivoGen). The two recombinant plasmids (named *pPICZαA-FgCPP1* and *pPICZαA-FgCPP2*) were extracted and purified from a corresponding PCR positive colony using the "GenElute HP Plasmid Midiprep" kit (Sigma-Aldrich), linearized with *Sac*I (Supplementary Fig. 1), precipitated with 2 vol of absolute ethanol and 1/10 volume of sodium acetate 3 M (pH 5.2) and resuspended in 20 μL of water. *P. pastoris* transformation was performed as reported in Sella et al. (2013) using the linearized recombinant plasmids *pPICZαA-FgCPP1* and *pPICZαA-FgCPP2* or a *pPICZαA* empty vector as negative control. Some positive colonies were tested by PCR using the specific primers *pPICZαA*for/rev (Supplementary Table 1) and the "REDTaq ReadyMix PCR Reaction", repeating for 35 times the following cycle: 30 s at 94 °C; 30 s at 50 °C; 1 min at 72 °C. The colonies containing the corresponding inserts were grown and induced to produce the recombinant FgCPPs with the addition of methanol according to the Invitrogen Life Technologies manual. After 72 h, liquid cultures were centrifuged at 10000 × *g* for 10 min and the supernatants were checked for the presence of the recombinant proteins by SDS-PAGE.

2.3. Purification of the F. graminearum recombinant CPPs and SDS-PAGE analysis

The culture filtrates of *P. pastoris* transformed with the *FgCPPs* vectors or with the empty vector were adjusted to 20% of saturation with ammonium sulfate (w/v) and stirred for 1 h at 4 °C. After centrifugation at 8000 × *g* for 20 min at 4 °C, the proteins in the supernatants were salted out by ammonium sulfate at 80% of saturation. After centrifugation, the proteins were recovered in 20 mL of deionized water and dialyzed overnight at 4 °C against deionized water (1:100 dilution) using a Nominal MWCO 3500 membrane (Orange Scientific). The two preparations containing the recombinant proteins (FgCPP1 and FgCPP2) and the negative control sample (CK) were subjected to SDS-PAGE on a 16% polyacrylamide gel. Electrophoresis was run by a Mini Protean II unit (Bio-Rad) at constant 200 V for about 45 min. Proteins were stained with Coomassie Blue G250 (Sigma-Aldrich) and the molecular mass of the bands was deduced by comparison with 10 μL of Low Range markers (Bio-Rad). Protein concentration was evaluated by comparison with known amounts of bovine serum albumin (BSA) protein (not shown).

2.4. Effect of FgCPPs on viscosity of polysaccharides

The capacity of the FgCPPs to affect the viscosity of some polysaccharide molecules was determined by Micro-Ostwald capillary viscosimeters (i.d. = 0·70 mm) connected to AVS 310 system units (Schott-Geräte). Viscosimetric assays were performed at 30 °C on 2 mL of 50 mM phosphate buffer (pH 6.0) containing 0.25% (w/v) of the following types of polysaccharide: carboxymethyl cellulose (CMC) with high, medium or low viscosity (Sigma-Aldrich, codes C-8758, C-4888 and C-5013, respectively), chitin (Sigma-Aldrich, code C-7170), 1,3-β-D-glucan (Megazyme, Pachyman, code P-PACHY), larchwood xylan (Sigma-Aldrich, code X-3875) and apple pectin (Sigma-Aldrich, code 76282). Variations of relative viscosity were determined after addition of the recombinant FgCPPs samples, either native or boiled for 30 min, at a final concentration of 0.1 μM. BSA at 0.1 μM or equal volumes of the CK preparation were used as negative controls. The decrease of relative viscosity was determined after 30 min from the addition of the samples to the polysaccharide solutions and was expressed as percentage reduction of the initial relative viscosity. Alternatively, the time employed by the samples to decrease the initial relative viscosity by 10% (T_{10}) and 15% (T_{15}) was taken as a measure of activity.

2.5. Binding assay of FgCPPs to polysaccharides

The polysaccharide binding assay was performed as reported in Baccelli et al. (2014b) with some modifications. In details, about 4 μg of the FgCPP1 and FgCPP2 proteins were incubated in 1 mL of 50 mM sodium acetate buffered at pH 5.0 containing 0.25% (w/v) chitin or medium viscosity CMC or 1,3-β-D-glucan or 1-cm diameter Whatman 3 MM filter paper (Whatman-GE Healthcare). As a control, the proteins were incubated only with the buffer solution. After 48 h of incubation at 38 °C in an orbital shaker at 250 rpm, the samples were centrifuged at 12000 × *g* for 2 min and the unbound proteins present in the supernatants were precipitated by using four volumes of absolute ethanol. Proteins were then suspended in 100 μL sample buffer and 50 μL were analyzed by 16% SDS-PAGE as above reported.

2.6. Chitinase activity assay

To investigate the possible ability of FgCPPs to protect chitin from chitinase activity, an enzymatic assay was performed according to Dinesh et al. (2010). Briefly, 0.3 μM and 0.6 μM FgCPPs or an equal volume of the CK preparation were incubated in a 0.5% (w/v) colloidal chitin solution in the presence or absence of 0.2 U of *Streptomyces griseus* chitinase (Sigma-Aldrich). After 2 h of incubation at 37 °C, the supernatants obtained after centrifugation were supplemented with 1 M borate buffer pH 9.8 and the mixtures were boiled for 3 min and then cooled on ice. After addition of *p*-dimethylaminobenzaldehyde (DMAB) reagent and incubation for 3 min at 37 °C, the μg equivalents of N-acetylglucosamine (Sigma-Aldrich) released from the 500 μL reaction mixture were measured by reading the absorbance at 585 nm in a spectrophotometer. N-acetylglucosamine was used as standard.

2.7. Cellulase activity produced by F. graminearum WT and ΔΔ$fgcpp_{1,2}$ mutant

The *F. graminearum* wild type (WT:8/1) and the Δ*Δfgcpp*$_{1,2}$ mutant strain, produced and characterized as reported in Quarantin et al. (2016), were cultured at 25 °C on potato dextrose agar (PDA, Difco). Conidia were obtained by culturing *F. graminearum* strains in CMC as reported in Sella et al. (2016).

Conidia of the *F. graminearum* WT and the Δ*Δfgcpp*$_{1,2}$ mutant were inoculated at 10^4 mL^{-1}in flasks containing 20 mL of Szécsi medium (Szécsi, 1990) supplemented with 1% (w/v) low, medium and high viscosity CMC. Flasks were incubated at 25 °C on an orbital shaker at 150 rpm in the dark for 4 days. At the end of the cultures (4 days) mycelia of the *F. graminearum* WT and double mutant were collected and the dry weight was measured as reported in Quarantin et al. (2016).

The cellulase activity produced in the cultural medium was determined viscosimetrically as above reported by using 500 μL of culture filtrates and 0.25% (w/v) medium viscosity CMC. One unit of activity was defined as the amount of enzyme that decreases the initial relative viscosity of the substrate by 30% ($Unit_{30}$) or 50% ($Unit_{50}$) in 1000 s.

2.8. Effect of FgCPPs on cellulase activity on CMC and wheat cell walls

To test whether the two FgCPPs affect the fungal cellulase activity, a viscosimetric assay was performed as above reported using 0.25% (w/v) medium viscosity CMC as substrate, 0.04 U of a β-1,4-glucanase from *Trichoderma longibrachiatum* (Sigma-Aldrich; EC 3.2.1.4) and 0.1 or 0.15 μM of the recombinant FgCPPs. As negative controls, assays were carried out replacing the FgCPPs samples with an equal volume of the CK preparation. The activity was expressed in $Unit_{50}$ as defined in the previous paragraph. In a separate experiment, the reducing sugars released in a 500 μL enzymatic mixtures after 1 h of incubation at 30 °C were also determined according to Nelson (1944) using D-(+)-glucose as a standard.

The possible effect of FgCPPs on enhancing the cellulase activity has been tested also using wheat cell walls as substrate. The assay was performed as reported by Paccanaro et al. (2017). Five hundred µL aliquots of a 1% (w/v) *Triticum aestivum* leaf cell wall preparation, suspended in 50 mM sodium acetate buffer at pH 6.0 and containing 0.2 mg mL^{-1} streptomycin sulfate, were treated with 0.04 U of ß-1,4-glucanase in presence of 0.3 µM of FgCPPs or an equal volume of the CK preparation. The mixtures were incubated at 30 °C in an orbital shaker at 250 rpm and after 20 h the reducing sugars were measured by the Nelson method (Nelson, 1944) with D-(+)-glucose as a standard. The experiment was repeated three times.

2.9. Effect of FgCPPs and β-1,4-glucanase on filter paper

The effect of FgCPPs as enhancers of cellulase cleavage activity was tested on Whatman 3 MM filter paper as reported in Baccelli et al. (2014b). Briefly, filter papers were cut into 1-cm-diameter discs (6 mg each), and single discs were placed in 5 mL tubes and incubated with 1 mL of 50 mM sodium acetate buffer (pH 5.0) and 3 µM of FgCPPs. Buffer only or buffer containing an equal volume of the CK preparation or 3 µM BSA were used as negative controls. After 72 h of incubation at 38 °C in an orbital shaker at 320 rpm, 0.2 U of β-1,4-glucanase were added to each tube and the incubation was prolonged for further 24 h at 38 °C and 320 rpm. At the end of the incubation period, the paper discs were removed and the turbidity due to the release of paper fragments in the buffer was measured spectrophotometrically at 500 nm. Absorbance values of the control samples without the fungal β-1,4-glucanase were subtracted to values of the enzyme treated samples. Three different experiments were performed.

2.10. Hydrogen peroxide and necrotic symptoms induced in planta by FgCPPs

The experiments were carried out on 4-week-old *A. thaliana* plants (cv. Col-0) grown in 9 cm plastic pots into a climatic chamber with a 14 h photoperiod and 22/20 °C day/night temperature. Abaxial leaf surfaces were infiltrated with 1.5, 5 or 20 µM FgCPP1 and FgCPP2 protein solutions using a 1 mL syringe without needle; as negative control, leaves were infiltrated with an equal volume of the CK preparation. After infiltration the plants were maintained into the climatic chamber for further 4 h. To reveal the production of hydrogen peroxide (H_2O_2), the infiltrated leaves were detached and dipped into 1 mg mL^{-1} 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) at pH 3.8. After overnight incubation at room temperature in the dark, the leaves were removed from DAB solution and placed into an acetic acid/glycerol/ethanol (1/1/1, v/v/v) solution and boiled for 5 min in a water bath. Subsequently, leaves were maintained in 60% glycerol and photographed. To monitor the development of macroscopic necrotic symptoms, leaves of some *A. thaliana* plants were infiltrated with the FgCPPs or with an equal volume of the CK preparation and incubated into the climatic chamber up to 5 days.

2.11. Resistance induction assay

To assay the ability of the FgCPPs to induce resistance against the necrotrophic fungal pathogen *B. cinerea* (strain B05.10), 30 µL of 20 µM FgCPPs or, as negative control, an equal volume of the CK preparation supplemented with 0.4% (v/v) Tween 20 were spread on the abaxial surface of leaves of 4-week-old *A. thaliana* (cv. Col-0) plants. Untreated leaves were also used as negative control. For each treatment, ten leaves from at least 4 plants were detached 72 h after the treatment and inoculated with 3-mm diameter plugs of PDA with actively growing mycelium. Inoculated leaves were incubated in Petri dishes containing sterile filter paper moistened with deionized sterile water. Petri dishes were kept into a climatic chamber with a 14 h photoperiod and 22/20 °C day/night temperature. Three days after inoculation, leaves were photographed and lesion areas were measured using ImageJ/Fiji 1.46 program. Three independent experiments were performed.

2.12. Relative expression analysis of A. thaliana defence genes

The expression of genes involved in plant defence-signalling pathways such as PR1 (*pathogenesis related 1*), marker of salicylic acid (SA) signalling, PDF1.2 (*plant defensin*), key regulator of ethylene (Et) and jasmonic acid (JA) signalling, COI1 (*coronatine insensitive 1*), key regulator of JA signalling, and ERF1b (*Ethylene-responsive element binding factors*), a transcriptional regulator of some Et-responsive genes, was analyzed by Real-time PCR (qPCR) in *A. thaliana* leaves infiltrated with FgCPPs. In particular, the abaxial surface of 4-week-old *A. thaliana* leaves were infiltrated with 20 µM of the recombinant FgCPPs or an equal volume of the CK preparation by a 1 mL syringe without needle. Plants were incubated in a climatic chamber with a 14 h photoperiod and 22/20 °C day/night temperature.

RNA was extracted as reported in Quarantin et al. (2016) from leaves collected at 0, 16, 24 and 48 h after infiltration. PCR reaction mixtures contained 10 µL of 2× SYBR Green PCR Master Mix (Qiagen), 0.4 µM of each specific primer (Supplementary Tables 1) and 3 µL of cDNA as template. The qPCR was performed with the Rotor-Gene Q 2plex (Qiagen) by repeating 40 times the following cycle: 20 s at 95 °C; 20 s at 56 °C; 30 s at 72 °C. Reactions were performed in triplicates. Four independent qPCR experiments were performed with different RNA preparations.

2.13. Data analysis

Three independent experiments were performed for all the *in vitro* and *in vivo* assays. Data of cellulase activity produced by *F. graminearum* WT and Δ$\Delta fgcpp_{1,2}$ mutant were statistically analyzed by applying the Student's t-test at $P < 0.01$ and $P < 0.05$. To test significance of the data obtained in the other assays, ANOVA Tukey-Kramer test at $P < 0.05$ was performed.

Real-Time PCR expression analysis was performed by using the Rotor-Gene Q version 2.0.3.2 software (Qiagen), calculating the relative expression with the $2^{-\Delta\Delta Ct}$ method by setting to 1 the relative expression of the *A. thaliana* ubiquitin gene (UBQ5, AY139810.1), used as reference gene, and comparing the expression level in the infiltrated sample and that in the negative control sample infiltrated with the CK preparation.

3. Results

3.1. Structural features of the FgCPPs

The alignment of the deduced amino acid (aa) sequences of the *F. graminearum* FgCPP1 and FgCPP2 reveals 61.4% of identity and 76% of similarity.

Most aa residues putatively related to the main functional characteristics of the CPP family are conserved in the two FgCPPs (Supplementary Fig. 2). Namely the SYD motif and the aa Tyr-9, Gly-51, Ser-54 and Asp-77 that are potentially involved in the oligosaccharides binding (de Oliveira et al., 2011). Notably, Asp-77 is known to be co-responsible for the oligosaccharides binding but is also crucial for cellulose weakening and PAMP activity (Luti et al., 2017). In the CP of *C. platani* the Asn-84 residue likely causes its lack of glycoside hydrolase activity in comparison to the endoglucanase enzymes that are structurally related to the CP proteins (de Oliveira et al., 2011; Supplementary Fig. 2). This residue is also conserved in the *F. graminearum* FgCPPs as well in the other CPPs so far characterized.

In the *B. cinerea* BcSpl1, two regions designated as PepA and PepB are recognized as responsible for the necrotizing and elicitor activity in plants (Frías et al., 2014). These regions – that comprise two conserved cysteines forming a disulphide bond connecting the two regions – are

also conserved in the aa sequence of the FgCPPs as well as in that of other seven CPPs from different fungal species (Supplementary Fig. 2). In particular, the PepA regions of FgCPP1 and FgCPP2 have 100% and 90% aa identity, respectively, with the PepA of *B. cinerea* BcSpl1. This peptide is also highly conserved in *T. virens* Sm1, *T. atroviride* Epl1, *M. oryzae* MoSM1, *V. dahliae* VdCP1 and *L. maculans* Sp1. Differently, the PepB regions of FgCPP1 and FgCPP2 have only a 60% and 50% aa identity, respectively, with the PepB of BcSpl1 (Supplementary Fig. 2) and a maximum identity of 80% and 90% aa with that of VdCP1 and Epl1, respectively (Supplementary Fig. 2).

3.2. Heterologous expression of the FgCPPs

The nucleotide sequences encoding the mature FgCPP1 and FgCPP2 were obtained by PCR using specific primer pairs with adaptors for cloning in the *pPICZαA* vector. The *pPICZαA-FgCPP1* and *pPICZαA-FgCPP2* constructs were used to transform *P. pastoris* cells. Five transformed colonies for each construct were tested by PCR and the expected amplicons were obtained from all of them. The culture filtrates of two *P. pastoris* colonies transformed with the FgCPP1 or FgCPP2 constructs were analyzed by SDS-PAGE. Both culture filtrates contained a predominant band of approximately 20 kDa while no significant band was detected in the culture filtrate of a *P. pastoris* colony transformed with an empty *pPICZαA* vector (Fig. 1). Since the expected molecular mass is about 15 kDa, probably the proteins are glycosylated. In fact, both protein sequences possess a putative O-glycosylation site.

3.3. The FgCPPs reduce the viscosity of CMC and are sequestered by this polysaccharide

The effect of the two recombinant FgCPPs on the viscosity of CMC was analyzed on high, medium, and low viscosity CMC preparations. The addition of FgCPPs decreased significantly the viscosity of the substrates. Interestingly, the high and medium viscosity CMC preparations were more affected. FgCPP2 was about 2-folds more effective than FgCPP1 in decreasing the initial substrate viscosity of both medium and high viscosity CMC preparations, and about 3-folds more effective with the low viscosity CMC (Table 1). FgCPP1 and FgCPP2 denatured by heat treatment retained about 18% and 15% activity, respectively (Table 1). As expected, the CK and BSA samples, used as controls, did not show any viscosity reduction during the incubation period (Table 1).

Experiments performed by incubating the recombinant FgCPPs with chitin, 1,3-β-glucan, xylan and pectin did not evidence any reduction of the initial viscosity on these polysaccharides.

A carbohydrate binding assay was performed by incubating the FgCPP1 and FgCPP2 proteins with chitin, filter paper, CMC and 1,3-β-glucan. After incubation, the mixtures were centrifuged and the proteins remaining in the supernatant were analyzed by SDS-PAGE. Compared to control sample, consisting of FgCPPs incubated without the polysaccharides, CMC and to a lesser extent 1,3-β-glucan were able to sequester the FgCPPs from the incubation mixture (Fig. 2).

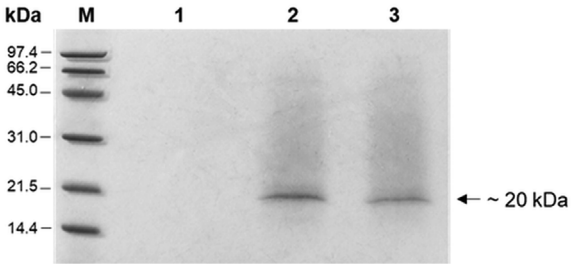

Fig. 1. SDS-PAGE analysis of the *Pichia pastoris* secretome of the colonies transformed to express the *Fusarium graminearum* CPPs. After induction with methanol for 72 h, aliquots of *P. pastoris* liquid cultures were loaded on a 16% polyacrylamide gel and then stained with Coomassie Brilliant Blue G250. Lane M: 10 μL of molecular weight standards (Low Range; Bio-Rad Laboratories); lane 1: 5 μL of the empty *pPICZαA* vector (CK) culture filtrate, used as negative control; lane 2: 5 μL of culture filtrate containing the recombinant FgCPP1; lane 3: 5 μL of culture filtrate containing the recombinant FgCPP2. Size of the molecular mass standards is reported on the left.

Table 1
Viscosimetric assay performed with *Fusarium graminearum* CPPs on carboxymethyl cellulose (CMC) with different viscosity. The assay was performed at 30 °C in a 2 mL reaction mixture containing 0.25% (w/v) high, medium or low viscosity CMC dissolved in 50 mM sodium phosphate buffer (pH 6.0) and 0.1 μM recombinant native or boiled (B) FgCPPs. An equal volume of the CK preparation or 0.1 μM of BSA were used as negative control. At least three independent experiments were performed. Viscosity reduction data represent the average ± standard deviation of the percentage (%) of decrease of the initial relative viscosity after 30 min of incubation. T_{15} or T_{10} data represent the average ± standard deviation of the time (expressed in sec) necessary to decrease the initial relative viscosity by 15% or 10%, respectively.

High viscosity CMC		
Sample	Viscosity reduction (%)	T_{15} (s)
BSA	0.0 ± 0.0	∞
CK	0.8 ± 0.7	∞
FgCPP1	27.2 ± 0.7	714.3 ± 75.1
FgCPP2	43.7 ± 0.7	338.7 ± 24.7
FgCPP1B	3.8 ± 0.1	11812.5 ± 1412.1
FgCPP2B	7.9 ± 0.3	4470.0 ± 161.2

Medium viscosity CMC		
Sample	Viscosity reduction (%)	T_{15} (s)
BSA	0.0 ± 0.0	∞
CK	0.9 ± 1.1	∞
FgCPP1	24.4 ± 0.3	909.0 ± 11.4
FgCPP2	40.8 ± 0.7	357.7 ± 22.1
FgCPP1B	3.6 ± 0.6	13414.7 ± 308.1
FgCPP2B	7.1 ± 0.7	5354.8 ± 442.2

Low viscosity CMC		
Sample	Viscosity reduction (%)	T_{10} (s)
BSA	0.0 ± 0.0	∞
CK	0.0 ± 0.0	∞
FgCPP1	5.7 ± 0.6	4360.0 ± 427.1
FgCPP2	13.3 ± 1.1	1216.0 ± 84.8

3.4. Effect of the FgCPPs on chitin polysaccharide

Since the double mutant *ΔΔfgcpp*$_{1,2}$ was more sensitive to chitinase treatment than the WT (Quarantin et al., 2016), we investigated whether the FgCPPs in solution can protect chitin from chitinase enzymatic activity. The amount of N-acetylglucosamine equivalents released from chitin by chitinase in the presence of the FgCPPs was comparable to that released in the presence of the CK preparation (Fig. 3), suggesting that the protective role on chitin is not caused by the FgCPPs in solution. In addition, as expected, the FgCPPs did not show any hydrolytic activity on chitin (Fig. 3).

3.5. The *ΔΔfgcpp*$_{1,2}$ mutant produces higher cellulase activity than WT in a CMC containing medium

The cellulase activity produced by the *F. graminearum* WT and the *ΔΔfgcpp*$_{1,2}$ mutant grown for 4 days in liquid culture containing high, medium or low viscosity CMC as the sole carbon source was measured viscosimetrically by using 0.25% (w/v) medium viscosity CMC as substrate. WT and double mutant produced respectively about 3.5 and 2 folds more cellulase activity when grown in low viscosity CMC than

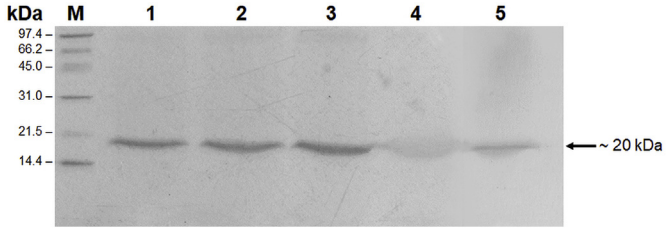

Fig. 2. SDS-PAGE analysis of the carbohydrate binding assay. The *Fusarium graminearum* CPPs were incubated with different polysaccharide substrates at 38 °C for 48 h with shacking (250 rpm). The samples were then centrifuged at 12000 × *g* for 2 min and the unbound proteins present in the supernatants were precipitated, resuspended in 100 µL sample buffer and 50 µL were loaded on a 16% polyacrylamide gel. Staining was performed with Coomassie Brilliant Blue G250. Lane M: molecular weight standards (Low Range; Bio-Rad Laboratories); lane 1: FgCPPs incubated with 50 mM sodium acetate buffer (pH 5), as control; lane 2: FgCPPs incubated with 0.25% (w/v) chitin; lane 3: FgCPPs incubated with 1-cm diameter Whatman 3 MM filter paper; lane 4: FgCPPs incubated with 0.25% (w/v) medium viscosity carboxymethyl cellulose (CMC); lane 5: FgCPPs incubated with 0.25% (w/v) 1,3-β-D-glucan. Size of the molecular mass standards is reported on the left.

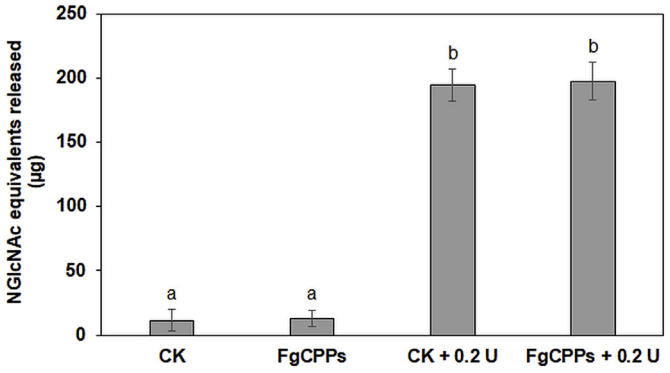

Fig. 3. N-acetylglucosamine (NGlcNac) equivalents released from a colloidal chitin solution by chitinase in the presence of *Fusarium graminearum* CPPs (FgCPPs). Five hundred µL reaction mixtures containing 0.5% (w/v) colloidal chitin dissolved in 0.1 M sodium phosphate buffer (pH 6) supplemented with 0.2 U of *Streptomyces griseus* chitinase were incubated in the presence of 0.3 µM FgCPPs or an equal volume of the CK preparation used as negative control at 37 °C for 2 h. Mixtures containing FgCPPs or an equal volume of the CK preparation without chitinase were also tested. Total amount of NGlcNac equivalents released (expressed in µg) was measured. Data were obtained from three independent experiments and statistically analyzed by the ANOVA Tukey-Kramer test. Different letters (a, b) indicate significant differences at $P < 0.05$. Error bars correspond to the standard error.

on medium and high viscosity CMC (Fig. 4). Compared to WT, the double mutant produced significantly higher cellulase activity in all the three CMC media. In particular, on medium and high viscosity CMC the mutant secreted about 4-folds more cellulase activity than WT, while the difference was lower (about 2-folds) on low viscosity CMC (Fig. 4).

3.6. The FgCPPs increase the activity of a fungal cellulase on CMC and wheat cell walls

Since the FgCPPs bind to CMC and affect the viscosity of this polysaccharide, we investigated whether these proteins affect the fungal cellulase activity. To this aim, a β-1,4-glucanase from *T. longibrachiatum* was incubated in presence or absence of the FgCPPs and the activity was measured viscosimetrically on medium viscosity CMC substrate. The fungal β-1,4-glucanase became ~2.5-folds more active in the presence of the FgCPPs compared to the control in which the FgCPPs were replaced by the CK preparation (Table 2).

Cellulase activity was also determined by measuring the release of

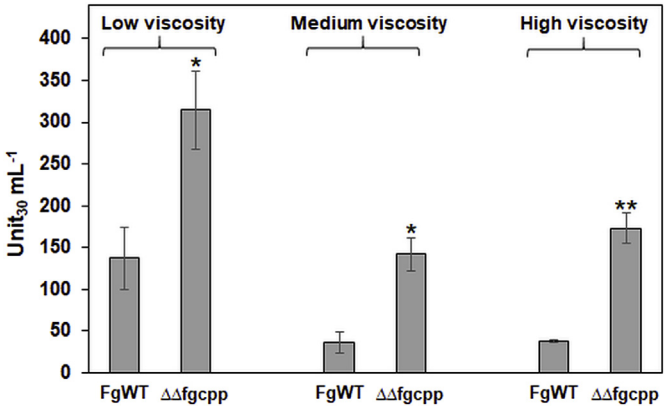

Fig. 4. Cellulase activity produced by *Fusarium graminearum* wild type (FgWT:8/1) and *ΔΔfgcpp*$_{1,2}$ mutant grown in low, medium and high viscosity carboxymethyl cellulose (CMC) media. After 4 days of growth at 25 °C, 500 µL of culture supernatants, previously filtered, were assayed by viscosimetry in a 2 mL reaction mixture containing 0.25% (w/v) medium viscosity CMC dissolved in 50 mM phosphate buffer (pH 6.0) at 30 °C. One unit of activity was defined as the amount of enzyme that decreases the initial relative viscosity of the substrate by 30% in 1000 s (Unit$_{30}$). Three independent experiments were performed. Data are expressed as mean ± standard error and were statistically analyzed by the Student's t-test. * indicates that the difference is significant at $P < 0.05$, ** indicates that the difference is significant at $P < 0.01$.

Table 2
Viscosimetric assay performed to investigate the effect of the *Fusarium graminearum* CPPs on fungal cellulase activity. The two recombinant native FgCPPs (0.1 µM) or an equal volume of the CK preparation as negative control were incubated in the presence or absence of 0.04 U of β-1,4-glucanase from *Trichoderma longibrachiatum* (Sigma-Aldrich) at 30 °C in a 2 mL reaction mixture containing 0.25% (w/v) medium viscosity carboxymethyl cellulose (CMC) dissolved in 50 mM phosphate buffer (pH 6.0). One unit of activity was defined as the amount of enzyme that decreases the initial relative viscosity of the substrate by 50% in 1000 s (Unit$_{50}$). At least three independent experiments were performed. Data are expressed as mean ± standard deviation and were statistically analyzed by the ANOVA Tukey-Kramer test. Different letters (a, b, c) indicate significant differences at $P < 0.05$.

Sample	Unit$_{50}$
CK	∞
FgCPPs	39.6^{b} ± 3.4
CK + 0.04 U	27.6^{a} ± 4.0
FgCPPs + 0.04 U	66.1^{c} ± 1.7

reducing sugars. In the presence of the FgCPPs, the β-1,4-glucanase released about 25% more reducing sugars, expressed as glucose equivalents, compared to the control with the CK preparation (Fig. 5). The amount of glucose equivalents released by the FgCPPs alone was as low as the control CK preparation (Fig. 5).

The capability of the FgCPPs to increase the hydrolytic activity of cellulase was also verified on *Triticum aestivum* cell walls. After 24 h of incubation, the β-1,4-glucanase released about 20% more reducing sugars in the sample containing the FgCPPs than in that containing the control CK preparation (Fig. 6). The cell walls incubated with CK or FgCPPs in absence of β-1,4-glucanase produced negligible or undetectable reducing sugars, respectively (Fig. 6).

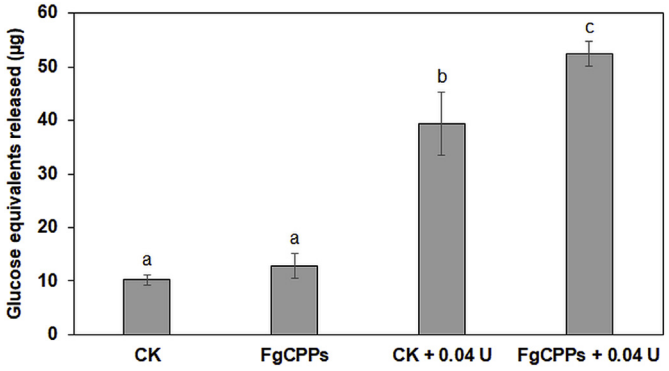

Fig. 5. Glucose equivalents released from carboxymethyl cellulose (CMC) substrate by β-1,4-glucanase in combination with the *Fusarium graminearum* CPPs (FgCPPs). Five hundred µL reaction mixtures containing 0.25% (w/v) CMC dissolved in 50 mM phosphate buffer (pH 6.0) supplemented with 0.04 U of β-1,4-glucanase from *Trichoderma longibrachiatum* were incubated in combination with 0.1 µM of the FgCPPs or with an equal volume of the CK preparation as negative control at 30 °C for 1 h. Mixtures containing FgCPPs or an equal volume of the CK preparation without β-1,4-glucanase were also tested. Samples were assayed as reported by Nelson (1944) using D-(+)-glucose as standard. Data were obtained from three independent experiments and were statistically analyzed by the ANOVA Tukey-Kramer test. Different letters (a, b, c) indicate significant differences at $P < 0.05$. Error bars correspond to the standard error.

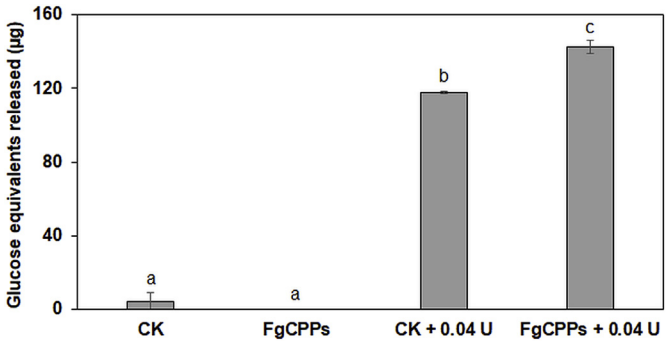

Fig. 6. Synergistic effect of the *Fusarium graminearum* CPPs and cellulase on wheat cell walls. *Triticum aestivum* cell walls (1% w/v) were incubated in a 500 µL reaction mixture containing 50 mM sodium acetate buffer (pH 6.0) supplemented with 0.2 mg mL^{-1} streptomycin, 0.04 U of a ß-1,4-glucanase from *Trichoderma longibrachiatum* and 0.3 µM of the recombinant FgCPPs or with an equal volume of the CK preparation as negative control. Mixtures containing FgCPPs or an equal volume of the CK preparation without β-1,4-glucanase were also tested. Samples were incubated at 30 °C and 250 rpm for 20 h and µg of reducing sugars equivalents released were measured by the method of Nelson (1944) using D-(+)-glucose as standard. The experiment was repeated three times. Error bars indicate the standard deviation. Data were statistically analyzed by the ANOVA Tukey-Kramer test and different letters (a, b, c) indicate significant differences at $P < 0.05$.

3.7. Effect of the co-incubation of FgCPPs and a fungal cellulase on filter paper

The expansin-like effect of the FgCPPs was also tested on filter paper. After incubating the filter paper with FgCPPs for 72 h at 38 °C and for further 24 h with the β-1,4-glucanase from *T. longibrachiatum*, the cellulosic fragments released in the mixture were determined spectrophotometrically. FgCPPs increased by about 45% the absorbance of the suspension compared to the negative controls containing the BSA or CK (Fig. 7). In the samples containing FgCPPs or CK or BSA without β-1,4-glucanase no significant change of absorbance was detected during the experiment (Fig. 7). From these experiments, it appears that the FgCPPs positively affect the enzymatic activity of the β-1,4-glucanase with an expansin-like mechanism.

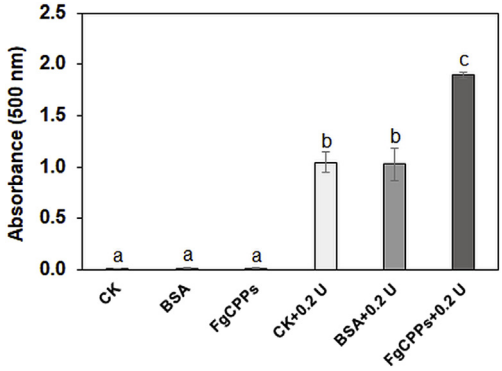

Fig. 7. Synergistic effect of the *Fusarium graminearum* CPPs and cellulase on Whatman 3 MM filter paper. Filter papers (1-cm-diameter discs) were incubated for 72 h at 38 °C and 320 rpm in 1 mL of 50 mM sodium acetate buffer (pH 5.0) containing 3 µM of FgCPPs or an equal volume of the CK preparation or 3 µM BSA used as negative controls. Subsequently, 0.2 U of β-1,4-glucanase from *Trichoderma longibrachiatum* were added to each tube and a 24 h incubation at 38 °C and 320 rpm was performed. Mixtures containing FgCPPs or an equal volume of the CK preparation or BSA without β-1,4-glucanase were also tested. The absorbance at 500 nm was then measured to quantify the turbidity due to the release of paper fragments. Absorbance values of the control samples were subtracted to values of the enzyme treated samples. Error bars correspond to the standard error. Data obtained from three independent experiments were statistically analyzed by the ANOVA Tukey-Kramer test and different letters (a, b, c) indicate significant differences at $P < 0.05$.

3.8. FgCPP2 elicits H_2O_2 accumulation and necrotic symptoms in A. thaliana leaves

To investigate the ability of the FgCPPs to elicit defense responses and/or necrosis, *A. thaliana* leaves were infiltrated with different concentrations of the recombinant FgCPP proteins or with an equal volume of the CK preparation, used as negative control. Potential H_2O_2 production was visualized as a brown precipitation following DAB treatment. Twenty µM FgCPP1 and FgCPP2 induced a faint and a strong brown precipitation, respectively, 4 h after infiltration (Fig. 8a). FgCPP concentrations of 1.5 or 5 µM did not induce any browning, typical of H_2O_2 accumulation, following the DAB treatment (Fig. 8a). Five days after infiltration appeared chlorotic and necrotic symptoms in the leaves infiltrated with 20 µM FgCPP2 (Fig. 8cd). The leaves infiltrated with the CK preparation did not show any H_2O_2 accumulation nor necrotic symptoms (Fig. 8).

3.9. The FgCPPs treatment increases resistance to B. cinerea in A. thaliana leaves and induce defence-signalling pathways

To test the FgCPPs ability to protect plants from fungal infection, the abaxial surface of *A. thaliana* leaves was treated with the FgCPP1 or FgCPP2 and, after 72 h, infiltrated leaves were inoculated with *B. cinerea* mycelium. After 3 dpi, treated leaves showed a significant reduction (about 30–40%) of disease symptoms caused by *B. cinerea* compared to untreated leaves or leaves treated with the CK preparation only (Supplementary Fig. 3a). In particular, the lesions remained more restricted around the infection site (Supplementary Fig. 3b).

To identify the signalling pathways possibly activated by the FgCPPs, the expression of PR1, PDF1.2, COI1 and ERF1b genes was analyzed in *A. thaliana* infiltrated leaves. The infiltration with FgCPP1 and FgCPP2 induced the expression of SA- and Et-dependent genes. The pattern of up-regulation of the PR1 gene was similar for both FgCPPs, although the expression level was higher in the leaves infiltrated with FgCPP2. In particular, the expression of the PR1 gene was induced 4- and 11-folds at 16 h and 25- and 45-folds at 48 h after infiltration with

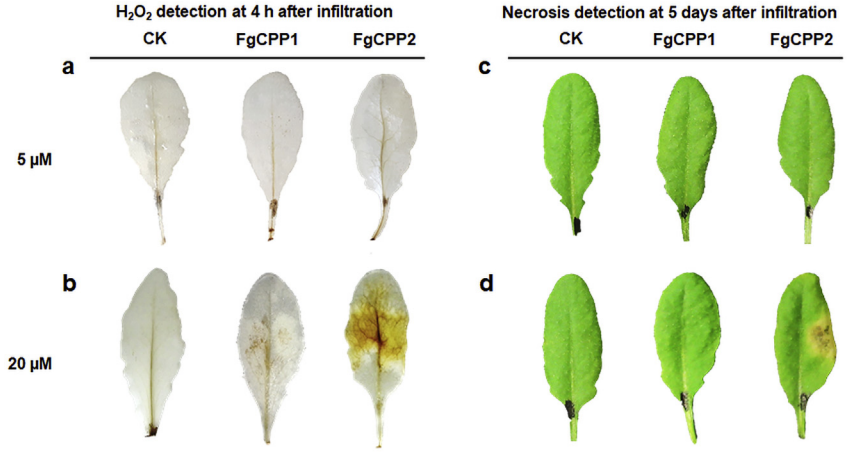

Fig. 8. Detection of H_2O_2 production and necrotic symptoms in *Arabidopsis thaliana* (cv. Col-0) leaves infiltrated in the lower surface with 5 µM or 20 µM of the *Fusarium graminearum* CPPs (FgCPP1 and FgCPP2), or with an equal volume of the CK preparation as negative control. To reveal the production of H_2O_2 leaves were treated with 3,3′-diaminobenzidine (DAB). (a) H_2O_2 detection 4 h after infiltration with 5 µM of FgCPPs; (b) H_2O_2 detection 4 h after infiltration with 20 µM of FgCPPs; (c) detection of necrotic symptoms 5 days after infiltration with 5 µM of FgCPPs; (d) detection of necrotic symptoms 5 days after infiltration with 20 µM of FgCPPs.

FgCPP1 or FgCPP2, respectively (Supplementary Table 2). The ERF1b gene was also more induced by FgCPP2 than by FgCPP1 at both 16 and 24 h after infiltration, while the expression levels were similar at 48 h (Supplementary Table 2). PDF1.2 and COI1 genes showed only a low basal level of expression at each time point considered (Supplementary Table 2).

4. Discussion

The two CPPs secreted by *F. graminearum* share important amino acid residues with the so far characterized CPPs (Baccelli, 2015), in particular those related to the main functional characteristics of this protein family. The conserved structure of these proteins anticipates possible interactions with plant or fungal cell wall polysaccharides. Indeed, FgCPPs bind to the cellulose derivative CMC and this binding is likely responsible for the observed alterations of the viscous properties of this substrate. However, FgCPPs do not cleave CMC and decrease the viscosity of this substrate also after protein denaturation, clearly demonstrating that the viscosity reduction is not a consequence of enzymatic activity. The lack of a glycoside hydrolase activity is consistent with the presence of the amino acid Asn-84 in the primary structure of FgCPPs, as previously suggested for the CPP of *C. platani* (de Oliveira et al., 2011). The alteration of the viscous properties of CMC in absence of any glycosidase activity is probably due to the capacity of CPPs to displace the ion binding and hydrogen bonding that are responsible for the electrostatic interaction among the polysaccharide chains (Yang and Zhu, 2007).

The capacity of FgCPPs to bind to a cellulose substrate is shared with the *B. subtilis* EXLX1 protein (Kerff et al., 2008) but is uncommon among the fungal CPPs. However, similarly to FgCPPs, the *C. platani* CP, *C. populicola* Pop1, *T. atroviride* Epl1 and *V. dahliae* VdCP1 are unable to bind to filter paper (de Oliveira et al., 2011; Frischmann et al., 2013; Baccelli et al., 2014b; Zhang et al., 2017), while their binding affinity to the soluble derivative CMC has never been investigated.

Interestingly, the fungal CPPs so far characterized bind to chitin (de Oliveira et al., 2011; Frischmann et al., 2013; Frías et al., 2014; Zhang et al., 2017), predicting a role in fungal growth (Baccelli, 2015) and/or in fungal cell wall protection against chitinases of plant or microbial origin. Instead, FgCPPs do not bind to chitin and then differ from the other fungal CPPs, nevertheless they were previously demonstrated to reduce the detrimental effect of chitinase on *F. graminearum* hyphal growth (Quarantin et al., 2016), a feature also reported for the *V. dahliae* VdCP1 (Zhang et al., 2017). Since here we show that FgCPPs do not inhibit chitinase activity in solution and seem to partially bind to 1,3-β-glucan, we can hypothesize that the protective effect of FgCPPs on cell wall polysaccharides (Quarantin et al., 2016) is related to a weak binding to the fungal cell wall, a feature observed also for the *B. cinerea* BcSpl1 (Frías et al., 2014).

The loosening of cellulose due to FgCPPs is also likely responsible for the increase of the fungal β-1,4-glucanase activity as determined on various cellulosic substrates. This synergistic activity has been demonstrated viscosimetrically on CMC, colorimetrically on CMC and wheat cell walls and turbidimetrically on filter paper. To date, a synergistic activity in cellulose hydrolysis has been observed only for the *B. subtilis* EXLX1 protein incubated with low-dose cellulases (Yan et al., 2012), but not for other fungal CPPs (Baccelli et al., 2014b). Taken together, these results suggest that the FgCPPs act as expansin proteins and by weakening cellulose, including the native polysaccharide present in plant cell walls, favor an increased enzymatic activity, potentially facilitating tissue colonization by fungal hyphae. Interestingly, a synergistic effect between two different plant cell wall degrading enzymes was recently shown in *F. graminearum*. Indeed, while the virulence of single gene disruption mutants impaired in polygalacturonase or xylanase activity is unaffected, the virulence of a double gene disruption mutant with both enzymatic activities compromised is reduced (Paccanaro et al., 2017).

Although the expansin-like activity of FgCPPs suggests an important role played by the FgCPPs during wheat infection, the *F. graminearum* double knock-out mutant $\Delta\Delta fgcpp_{1,2}$ resulted as virulent as the WT strain (Quarantin et al., 2016). We have found here that this mutant produces significantly more cellulase activity than WT and this increased enzymatic activity could compensate for the loss of expansin like activity. Therefore, the FgCPPs have a distinct role during plant infection, even if the disruption of the corresponding encoding genes does not reduce the virulence of the mutants under the tested conditions. Generally, a distinction is made between pathogenicity genes, essential for the success of infection, and virulence genes, whose disruption only partially reduces virulence (Schäfer, 1994). Here we propose a new distinction for virulence genes: essential virulence genes, whose disruption causes a reduction in virulence, and accessory virulence genes, whose disruption does not change the overall virulence of the fungal pathogen because mechanisms compensating the defective function are activated. Following this distinction, the FgCPPs as well as the aforementioned *F. graminearum* polygalacturonase and xylanase enzymes (Paccanaro et al., 2017) would be classified as accessory virulence factors. Similarly, many fungal effectors highly expressed during plant infection but not clearly contributing to fungal virulence, likely due to functional redundancy or the inability to accurately detect small changes in phenotype (Selin et al., 2016), could be considered accessory virulence genes.

The CP of *C. platani* and those of *B. cinerea*, *Magnaporthe grisea* and *V. dahliae* induce HR on leaves of several plants (Baccelli et al., 2014a; Frías et al., 2011; Wang et al., 2016; Zhang et al., 2017). Similarly, the FgCPPs induced H_2O_2 accumulation and necrotic symptoms on leaves

of the model plant *A. thaliana*. This effect was clearly evident with FgCPP2 at the highest concentration used (20 μM) and was just detectable with FgCPP1. This result suggests that plant responses induced by FgCPP2 could potentially hamper the *F. graminearum* advancement in the plant tissue, but considering that the encoding gene is barely expressed during the first stages of wheat spike infection (Quarantin et al., 2016), according to the late necrotrophic lifestyle of this fungus, the magnitude of the activated defenses can be moderate.

Frías et al. (2014) identified at the surface of the *B. cinerea* BcSpl1 two conserved regions (PepA and PepB) interacting with each other and involved in the necrosis-inducing activity. Both these regions are present in the CPPs known to elicit necrosis in plants and are conserved in the FgCPPs. However, the PepA and PepB of FgCPP1 and FgCPP2 differ for a single amino acid and then we can hypothesize that these amino acid substitutions could affect the different elicitor activity of the two FgCPPs. Similarly, a single point mutation able to influence the elicitor activity has recently been demonstrated with the *C. platani* CP (Luti et al., 2017).

Some authors suggested that the CPPs of *B. cinerea* and *C. platani* could be exploited to enhance plant resistance to bacterial and fungal pathogens (Frías et al., 2013; Baccelli et al., 2014a). We have here verified that treatments of *A. thaliana* leaves with the FgCPPs enhanced plant resistance to *B. cinerea*. Similarly to what observed for the *B. cinerea* and *C. platani* CPPs (Frías et al., 2013; Baccelli et al., 2014a), these treatments strongly activate the defensive signalling mediated by SA and, to a lesser extent, that mediated by Et, which could be crucial for the resistance induction against the necrotrophic fungal pathogen *B. cinerea* (Baccelli et al., 2014a).

In conclusion, the FgCPPs share common structural and functional features with plant expansin proteins; as expansins (Georgelis et al., 2015; Sampedro and Cosgrove, 2005), FgCPPs are trapped by a cellulose soluble derivate, loosen its viscosity without glycoside hydrolase activity, and enhance fungal cellulase activity on various cellulose containing substrates making them more accessible to the enzyme. These features could be further investigated in order to verify if these proteins can find a biotechnological application in the production of bioethanol from cereal straw, a potential biofuel resource abundantly produced worldwide. This plant waste tissue is particularly rich in lignocellulose materials, consisting mainly of cellulose, hemicellulose and lignin polymers, which are particularly recalcitrant to digestion by cellulosolytic enzymes (Liu et al., 2015). Since, many production costs are related to the pre-treatment of these lignocellulose materials, proteins with expansin-like activity such as the FgCPPs could improve this production step contributing to cellulose degradation by increasing the activity of cellulolytic enzymes.

Contribution

A.Q. contributed to conceive the experimental design, performed *in silico* analysis, cloning and heterologous protein expression, enzymatic assays, plant infections, gene expression, data analysis and paper writing.

C.C. performed proteins preparation and enzymatic assays.

W.S. performed critical revision of the data and contributed to write the final version of the paper.

F.F. supervised the experimental design, performed critical revision of the data and contributed to write the final version of the paper.

L.S. conceived and designed the experiments, supervised the work with data evaluation and paper writing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.025.

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