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FPLC and liquid-chromatography mass spectrometry identify candidate necrosis-inducing proteins from culture filtrates of the fungal wheat pathogen *Zymoseptoria tritici*

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ABSTRACT

Culture filtrates (CFs) of the fungal wheat pathogen *Zymoseptoria tritici* were assayed for necrosis-inducing activity after infiltration in leaves of various wheat cultivars. Active fractions were partially purified and characterized. The necrosis-inducing factors in CFs are proteinaceous, heat stable and their necrosis-inducing activity is temperature and light dependent. The *in planta* activity of CFs was tested by a time series of proteinase K (PK) co-infiltrations, which was unable to affect activity 30 min after CF infiltrations. This suggests that the necrosis inducing proteins (NIPs) are either absent from the apoplast and likely actively transported into mesophyll cells or protected from the protease by association with a receptor. Alternatively, plant cell death signaling pathways might be fully engaged during the first 30 min and cannot be reversed even after PK treatment. Further fractionation of the CFs with the highest necrosis-inducing activity involved fast performance liquid chromatography, SDS–PAGE and mass spectrometry. This revealed that most of the proteins present in the fractions have not been described before. The two most prominent ZtNIP encoding candidates were heterologously expressed in *Pichia pastoris* and subsequent infiltration assays showed their differential activity in a range of wheat cultivars.

1. Introduction

Phytopathogenic fungi exhibit different lifestyles and modes of interaction with their host plants (Horbach et al., 2011); biotrophs derive their nutrients from living host cells, whereas necrotrophs kill their host tissue presumably by toxic secondary metabolites or necrogenic proteins before feeding (Horbach et al., 2011; Howlett, 2006; Stergiopoulos et al., 2013). Host-selective toxins (HSTs), either proteins or secondary metabolites, are determinants of pathogenicity or virulence of necrotrophs and are produced by a range of fungal genera, particularly in the *Dothideomycetes* (Friesen et al., 2008a; Stergiopoulos et al., 2013). Several HSTs in the cereal necrotrophs *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* induce necrosis and promote disease development in toxin-sensitive wheat plants in a light-dependent manner. In contrast to gene-for-gene (GFG) interactions (Flor, 1971; Keen, 1990; Van den Ackerveken et al., 1992; Jones and Dangl, 2006; Dodds

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et al., 2009; Thomma et al., 2011) that mostly involve dominant resistance genes in the host, susceptibility to necrotrophs was found to depend on the presence of dominant sensitivity genes and these interactions therefore comply with the inverse GFG model (iGFG) (Friesen et al., 2008a; Friesen and Faris, 2010). The underlying mechanism has been named effector-triggered susceptibility (ETS), but irrespective of either GFG or iGFG, the involved effectors operate in a species- and cultivar-specific manner (Wolpert et al., 2002; Friesen et al., 2008a; de Wit et al., 2009).

Zymoseptoria tritici (previously *Mycosphaerella graminicola* (Fuckel) J. Schröt. in *Cohn*) (Quaedvlieg et al., 2011), is a Dothideomycete hemibiotroph (Kema et al., 1996) that has an initial biotrophic phase. During this phase, the fungal biomass hardly increases and is not much different from the biomass produced on resistant plants. It was even suggested that the basic nutrient composition is neither increased nor depleted to any measurable level despite the presence of the fungus (Keon et al., 2007). This is followed by a quick turnover to a destructive phase of pathogenesis where the fungus ramifies the mesophyll, which is accompanied by a reduction in photosynthesis and a massive accumulation of H_2O_2, leading to necrosis. This releases large amounts of nutrients from host cells that facilitate further proliferation of the fungus (Kema et al., 1996; Shetty et al., 2003, 2007, 2009; Keon et al., 2007, Rudd et al., 2008). Very little is known about the mechanism of this switch in lifestyle (Keon et al., 2007; Kema et al., 2008), but several reports suggested that during the biotrophic phase the fungus prepares for the necrotrophic phase by turning on enzymes or pathways for the production of secondary metabolites compounds and proteins (Kema et al., 1996; Perrone et al., 2000; Shetty et al., 2003, 2007, 2009; Rudd et al., 2008). For instance, early chloroplast condensation in wheat mesophyll cells without proximate *Z. tritici* hyphae suggested that toxic fungal compounds affect cell integrity (Kema et al., 1996).

In many pathosystems, necrosis is part of the resistance response that can be very local and restricts the pathogen from further colonization. In *Z. tritici* pathogenesis, however, necrosis is associated with compatibility that seems to facilitate fungal proliferation (Keon et al., 2007; Rudd et al., 2008).

Since HSTs are such prominent pathogenicity factors in related Dothideomycete pathogens, we were interested to investigate whether *Z. tritici* does produce similar proteins that might be involved in the above described biotrophy–necrotrophy switch. Crude culture filtrates (CFs) of many phytopathogenic fungi are known to contain phytotoxic metabolites/proteins and such toxins in CFs can be isolated (Bashan et al., 1995; Avantaggiato et al., 1999; Friesen et al., 2008a). In *P. tritici-repentis* and *P. nodorum*, CFs in combination with fast protein liquid chromatography (FPLC) have been effectively used as tools in the identification of these HSTs (Tomas et al., 1990; Tuori et al., 1995; Effertz et al., 2002; Liu et al., 2004; Friesen et al., 2008b, 2009).

Here, we report the production of *Z. tritici* CFs and the purification and characterization of necrosis-inducing factors. For this purpose, CFs were fractionated by FPLC, SDS–PAGE and the partially purified necrosis-inducing activity containing fraction was subsequently analyzed by liquid chromatography mass spectrometry. We identified a range of candidate NIPs and the two most prominent ZtNIP encoding candidates, which we designate as ZtNIP1 and ZtNIP2, are positioned on chromosomes 5 and 11, respectively, and induce chlorosis and necrosis in different wheat cultivars.

2. Materials and methods

2.1. Fungal and plant materials, and phenotyping assays

The sequenced *Z. tritici* bread-wheat strain IPO323 (Goodwin et al., 2011) was used throughout all experiments according to previously described protocols with minor modifications (Kema and van Silfhout, 1997; Mehrabi, 2006). Phenotyping was conducted on 20 different cultivars that were either susceptible or resistant to *Z. tritici* IPO323. Inoculation assays were conducted on plants grown in controlled greenhouse compartments with 16 h light per day and pre- and post-inoculation temperatures of 18/16 °C vs. 22 °C at day and night, respectively, and a relative humidity (RH) of ⩾85%. Disease symptoms were evaluated 21 days after inoculation as percentages necrosis (N) and pycnidia (P) as described by Tabib Ghaffary et al. (2011). Infiltration assays with CFs or protein fractions were conducted by infiltrating 100 µl in the second leaves of seedlings at growth stage (GS) 13 (Zadoks et al., 1974), which were grown at 22 °C and a RH of 60%, using a 1-ml syringe until water-soaking of the tissue was observed (Liu et al., 2004). The infiltration area was marked with a permanent marker and necrosis-inducing activity was determined at 3–4 days after infiltration (dai) for CFs and at 5 dai for protein tests. All the treated leaves were collected and photographed as well as scanned using a photocopier (RICOH Aficio MPC2500, Tokyo, Japan). The electronic images were subsequently analyzed using Assess software (APS, St. Paul, USA). All infiltrations were repeated at least twice with similar results.

2.2. Culture filtrate production

CF was generated by growing the fungus on V8-potato dextrose agar medium for 5–10 days until yeast-like colonies were formed. MilliQ water was added and 60 µl of the spore suspension from one plate (Petri dish, 9 cm diameter) was added to 60 ml of liquid Fries medium (Liu et al., 2004). The flasks were subsequently placed in a rotary shaker for three days at 27 °C at 100 rpm followed by stationary growth at 21 °C in the dark for one to three weeks. CFs were obtained by filtering these cultures through two layers of cheese cloth and Whatman No. 1 filter (Fisher Scientific, Pittsburg, PA, USA) and subsequent vacuum filtration through a 0.45 µm Durapore PVDF pore size filter (Millipore, MA, USA). The CF was either stored at −80 °C until use or directly used for determination of necrosis-inducing activity.

2.3. Treatments of culture filtrates

We tested the effect of temperature, proteinase K (PK) and light on the necrosis-inducing activity of the CFs. The effect of temperature on the necrosis-inducing activity was determined by incubating the CFs for four hours at room temperature (RT), 37 °C and 50 °C and at 100 °C for 30 min. In addition, the effect of *in vitro* and *in planta* PK treatments (1 mg/ml) (Roche Diagnostics, Almere, Netherlands) on the necrosis-inducing activity of the CFs was tested. CFs were treated with PK and incubated at RT, 37 °C and 50 °C for four hours. The untreated and treated samples were, along with the controls, infiltrated into the leaves of the sensitive cv. Obelisk. The necrosis-inducing activity was assayed by scoring plants either as sensitive or insensitive as reported previously (Liu et al., 2004). *In planta* PK effects of CF necrosis-inducing activity was tested by co-infiltrations of PK (100 µl of 1 mg/ml) and CFs at different time points varying from 0 to 120 min in three replicates. The light effect on necrosis-inducing activity was determined by exposure to normal light conditions or darkness by covering the infiltration zones with aluminum foil for two or three days.

2.4. Culture filtrate fractionation and SDS–PAGE

CF (~400 ml) harvested after three weeks of stationary growth of *Z. tritici* strain IPO323, was dialyzed at room temperature for 4 h against a 20 mM sodium acetate buffer (SAB, pH 5), using

SnakeSkin dialysis tubing (Pierce biotechnology, Rockford, IL) with a 7 kDa molecular weight cut off. Fast Protein Liquid Chromatography (FPLC, Pharmacia Biotech, Piscataway, NJ) was performed at room temperature. A 1 ml HiTrap SP Sepharose™ Fast Flow column (GE Healthcare, Piscataway, NJ, USA) was pre-equilibrated with SAB (pH 5) and 60 ml of dialyzed CF was applied at a flow rate of 1 ml/min and washed with SAB until the baseline was stable. Subsequently, a linear gradient to 0.5 M sodium chloride in SAB was applied at a flow rate of 1 ml/min and 16 fractions of 1 ml were collected. Relative protein concentration was detected by measuring absorbance at 280 nm. All protein-containing fractions were assayed for necrosis-inducing activity following the above mentioned procedure. For further protein purification, three successive runs (60 ml) with the HiTrap SP Sepharose column were performed and the fractions with necrosis-inducing activity were pooled. Three pooled fractions were further purified by FPLC using a Mono-S HR 5/5 cation exchange column (GE Healthcare, Piscataway, NJ, USA) equilibrated in SAB (pH 4.5). Samples were diluted twice in SAB buffer before injection onto the Mono-S column and proteins were eluted at 1 ml/min with a 30 ml linear gradient of 0.0–0.5 M sodium chloride in SAB (pH 4.5). One ml fractions were collected and adjusted to pH 5 with sodium acetate (pH 9.4) and assayed for necrosis-inducing activity. Part of the active fractions was added to Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 8–18% gradient gels (ExcelGel; Amersham Pharmacia Biotech, Sweden) using SDS buffer strips. Separation was performed on an electrophoretic transfer unit (Multiphor II; Amersham Pharmacia Biotech, Sweden) and the separated proteins were visualized by silver staining (Blum et al., 1987; Rabilloud and Chevallet, 1999).

2.5. Protein identification by LC–MS analysis

To identify the proteins present in the (partially) purified fractions with necrosis-inducing activity, samples were freeze-dried, dissolved in 50 µl 0.1% (w/v) RapiGest SF Surfactant (Waters, Milford, USA), 5 mM DTT (Sigma) in 0.1 M ammonium bicarbonate and incubated at 50 °C for 30 min. Alkylation was performed by incubation with 15 mM iodoacetamide (IAA) (GE Healthcare, UK) for 40 min at room temperature (in the dark). Proteolytic digestion was initiated by adding 2 µl of modified porcine trypsin (0.2 µg/µl; Sequence grade modified; Promega, WI, USA) and incubated overnight at 37 °C. After adding trifluoroacetic acid (TFA) (Fluka,-Buchs, GmBH) to a final concentration of 0.5% (v/v), samples were centrifuged at 15,000*g* for 10 min and the supernatant was applied to a SupelClean™ LC-18 1 ml SPE column (Supelco, Bellefonte USA), equilibrated with 0.1% TFA. Bound peptides were eluted with 84% acetonitrile (ACN) (HPLC Supra-gradient, Biosolve, Valkenswaard, NL) containing 0.1% Formic Acid (FA) (Merck, Darmstadt, Germany), dried by vacuum centrifugation, dissolved in 40 µl 0.1% FA and further analyzed by mass spectrometry. The trypsin-digested samples were separated using a nanoAcquity 2D UPLC system (Waters Corporation, Manchester, UK) with orthogonal reversed phase separation at high and low pH. The mixture of peptides was eluted from the first dimension XBridge C_{18} trap column (in 20 mM ammonium formate, ACN, pH 10) with a discontinuous gradient of 13%, 45% and 65% ACN. For the second dimension an acidic ACN gradient was applied using a BEH C_{18} column (75 µm × 25 cm, Waters, UK) and a 65 min linear gradient from 3% to 40% ACN (in 0.1% FA) at 200 nl/min. The eluting peptides were on-line injected into a Synapt Q-TOF MS instrument (Waters Corporation, Manchester, UK) using a nanospray device coupled to the second dimension column output. The Synapt MS was operated in positive mode with [Glu^1] fibrinopeptide B (1 pmol/µl; Sigma) as reference (lock mass) and sampled every 30 s. Accurate liquid chromatography–mass spectrometry (LC–MS) data were collected with the Synapt operating in either the MS/MS or MS^E mode for data-dependent acquisition (DDA) or data-independent acquisition (DIA), respectively, using low (6 eV) and elevated (ramp from 15 to 35 eV) energy spectra every 0.6 s over a 140–1900 *m*/*z* range, respectively. LC–MS/MS was performed by peptide fragmentation on the three most intense multiple charged ions that were detected in the MS survey scan (0.6 s) over a 300–1400 *m*/*z* range and a dynamic exclusion window of 60 s with an automatically adjusted collision energy based on the observed precursor *m*/*z* and charge state. LC–MS/MS and MS^E data were processed using ProteinLynx Global Server software (PLGS version 2.4, Waters Corporation, Manchester, UK) and the resulting list of masses, containing all the fragment information was searched for matching proteins using a merged non-redundant database including all gene models of the *Z. tritici* IPO323 database at the United States Department of Energy – Joint Genome Institute (DOE-JGI, http://genome.jgi-psf.org/Mycgr3/Mycgr3.download.ftp.html). Finally, the LC–MS/MS and MS^E outputs were further merged and since we used all gene models of *Z. tritici*, additional filtering steps were performed for proteins with alternative models (based on additional peptides not covered in the DOE-JGI models) and eventually only best models were used. Furthermore, only proteins with a peptide score $\geqslant$50 and/or >5 with LC–MS/MS and/or MS^E, respectively, were retained for further analyses.

The resulting proteins were characterized, the molecular mass determined, then searched for the presence of signal peptides (SignalP 3.0, http://www.cbs.dtu.dk/services/SignalP/), and cysteine residues and putative functions were identified. In case no putative function was assigned, online software such as BLASTP against the public NCBI non-redundant (NR) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Marchler-Bauer et al., 2009) (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to determine the classifications and possible functions of identified hypothetical proteins. For each identified protein the genomic sequence for the encoding gene, along with its 5′ and 3′ flanking regions were mapped on the *Z. tritici* IPO323 genome sequence (Goodwin et al., 2011) and expression was checked using the EST data base at DOE-JGI. In addition, the molecular masses and isoelectric points of the two most prominent proteins were predicted using the Compute pI/Mw tool at the ExPASy molecular biology server of the Swiss Institute of Bioinformatics (http://www.expasy.org/).

2.6. Heterologous expression of cDNA-encoding candidate proteins in Pichia pastoris

cDNAs encoding candidate proteins were amplified with primers containing Attb1 and Attb2 sites (Table S1) and amplicons were first gel-purified or directly incubated with donor vector pDONR207 and the Gateway® BP clonase (Invitrogen, Carlsbad, USA) and subsequently sequenced to check the reaction. Luria broth (1% tryptone, 0.5% yeast extract and 1% NaCl) with gentamicin (15–20 µg/ml) was used to culture *Escherichia coli* DH10B transformants at 37 °C after the BP reaction. The purified clones were then mixed with a Destination Vector (pMR148, 2.9 kb, containing pGAPZ that was slightly modified for application in both *E. coli* and yeast with zeocin as a selectable marker [*Sh ble*] and recombination sites compatible with the Gateway® system, Mehrabi et al. (2015) in the Gateway® Cloning LR reaction (Invitrogen). The resulting expression constructs were sequenced with primers pGAP and 3′AOX1 (Table S1) for sequence confirmation. After the LR reaction, low salt Luria broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.5) with zeocin (25 µg/ml) was used to culture *E. coli* DH10B transformants at 37 °C. The expression vector from positive clones was linearized with *Rca*I and

competent *P. pastoris* X33 cells (Easy select Pichia Expression system, Invitrogen) were transformed with at least 5 μg of plasmid DNA (Pichia EasyComp Kit manual, Invitrogen). The transformed cells were plated in YPDS agar (1% yeast extract, 2% peptone, 2% sorbitol and 2% dextrose, 2% agar) containing zeocin (100 μg/ml), incubated at 30 °C for three to four days and finally three clones were selected to check gene insertions by colony PCR using specific primers. Protein expression in *P. pastoris* X33 was performed in 50 ml YPD liquid medium (1% yeast extract, 2% peptone and 2% dextrose) in 100 ml Erlenmeyer flasks at 29 °C for two days. Eventually, cells were centrifuged at 4000 rpm for 4 min at 10 °C and the supernatant was checked for necrosis-inducing activity.

2.7. RT-PCR of the genes encoding ZtNIP1 and ZtNIP2

The expression of the genes encoding ZtNIP1 and ZtNIP2 was analyzed by semi-quantitative reverse transcription-PCR (RT-PCR). The susceptible cv. Obelisk was inoculated with *Z. tritici* strain IPO323 in three biological replicates and infected leaves were collected at 2, 4, 8, 12, 16 and 20 days post-inoculation, flash-frozen in liquid nitrogen and kept at −80 °C until use. Total RNA was isolated from ~0.7 ml ground leaf tissue with one ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in 2 ml tubes according to the manufacturer's instruction. To remove contaminated DNA, total RNA was treated with the RNase-free DNase I (Promega, Madison, USA). First-strand cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen, San Diego, CA, USA) and further diluted (5×) and finally used for SYBR® Green qPCR (Applied Biosystems, Foster City, CA). For each reaction, a 2 μl aliquot of cDNA was used in a 25 μl PCR volume with primers at a final concentration of 0.30 μM at an annealing temperature of 60 °C using an ABI 7500 Real-Time PCR System (Applied Biosystems). The expression was normalized with the constitutively expressed *Z. tritici beta-tubulin* gene. The primers used in this study are provided in Table S1.

3. Results and discussion

3.1. Outline of methodology

CFs in combination with FPLC have been effectively used as tools in the identification of HSTs in several Dothideomycetes (Tomas et al., 1990; Tuori et al., 1995; Effertz et al., 2002; Liu et al., 2004; Friesen et al., 2008b, 2009). To investigate whether *Z. tritici* does produce necrogenic proteins, we produced *Z. tritici* CFs that were subsequently fractionated by FPLC (Fig. 1), SDS–PAGE and the partially purified necrosis-inducing activity containing fraction was analyzed by liquid chromatography mass spectrometry.

3.2. Necrosis-inducing activity and preliminary characterization of Z. tritici culture filtrates

Preliminary characterization of the CFs included determination of the effect of different temperatures, light conditions on necrosis inducing activity and the proteinaceous character of the CFs was tested by sensitivity to *in vitro* digestion with Proteinase K (PK). Interestingly, the CFs showed necrosis-inducing activity on a wide range of wheat accessions, including the parental lines of mapping population, irrespective of whether these were resistant or susceptible toward strain *Z. tritici* IPO323 despite some slight quantitative differences (Table S2, Fig. 2). Similar results were observed with CFs from *Z. tritici* other strains such as IPO94269 and IPO95052 that are virulent on bread wheat and durum wheat, respectively (data not shown). This confirmed previous reports that showed no relationship between the toxicity of CFs and virulence of *Z. tritici* strains (Perrone et al., 2000), which contrasts with findings in related phytopathogenic fungi such as *P. nodorum* and *P. tritici-repentis* (Lamari and Bernier, 1989; Effertz et al., 2002; Liu et al., 2006, 2009; Singh and Hughes, 2006; Friesen et al., 2008b). CFs of the latter two fungi, containing necrogenic proteins, showed necrosis-inducing activity mostly only on susceptible cultivars. For *Z. tritici*, it was suggested that resistance is triggered during the early phases of infection rather than during advanced stages of pathogenesis when the mesophyll tissue is colonized. However, the titer of unknown soluble toxic compounds could increase during the course of infection and eventually kill host cells at a later stage of infection as suggested by Perrone et al. (2000).

In addition, necrosis inducing activity appeared sensitive to heat treatment as incubation of CFs at 100 °C strongly reduced its necrosis inducing activity with a proportional loss between 50 and 100 °C (Fig. 3) which is in accordance with observations on phytotoxic proteins isolated from other fungal plant pathogens (Ballance et al., 1989; Lamari and Bernier, 1989; Tomas et al., 1990; Sarpeleh et al., 2008). Furthermore, necrosis inducing activity of CFs was degraded after PK treatment, which confirmed the proteinaceous nature of the active component(s) in partially purified fraction. We also conducted a time-lapse experiment where crude *Z. tritici* CFs were co-infiltrated with PK in leaves of wheat seedlings. Interestingly, the necrosis-inducing activity of CFs could be inactivated until approximately 30 min after infiltration, hence, were apparently still prone to PK degradation in the apoplast (Fig. 3). However, after 30 min the necrosis-inducing activity of CFs was no longer influenced by PK treatments. This suggests that the necrogenic proteins would have either transversed into mesophyll cells, precluding digestion by PK suggesting that the majority of the necrogenic CF components rapidly target intracellular substrates such as the chloroplasts, which complies with the loss of chloroplast integrity during colonization of wheat by *Z. tritici* (Kema et al., 1996) or are protected from the protease by association with a receptor. Alternatively, plant cell death signaling pathways may have kicked in already during the first 30 min after exposure to CF, and cannot be reversed even after PK treatment.

Obviously, further experiments, including localization studies are needed to demonstrate whether or not the necrogenic proteins undergo internalization into wheat cells.

In addition, necrosis inducing activity is light dependent as CF-infiltrated leaves that were incubated in darkness (48 h) did not shown any necrosis but subsequent exposure to light induced necrosis (Fig. 3). This accords with observations of necrogenic proteins in other Dothideomycete–wheat pathosystems (Manning and Ciuffetti, 2005; Friesen et al., 2006, 2007; Sarpeleh et al., 2008; Abeysekara et al., 2009; Stergiopoulos et al., 2013). Manning et al. (2007, 2009) demonstrated that host specificity in the *P. tritici-repentis*–wheat interaction relies on the ability of PtrToxA to traverse the cell membrane and to interact with the chloroplast protein ToxABP1. Once it is translocated into the chloroplast, PtrToxA promotes virulence by interfering with photosystems I and II and finally induces reactive oxygen species accumulation in a light-dependent manner (Manning et al., 2009). Collectively, our data, together with previous histological observations (Kema et al., 1996), and recent studies that demonstrated the importance of chloroplast function on *Z. tritici*-induced cell death (Lee et al., 2014), suggest that a similar system might be active in the wheat–*Z. tritici* interaction.

3.3. Identification of candidate necrosis-inducing proteins through partial purification and mass spectrometry

To characterize the protein(s) responsible for necrosis-inducing activity in more detail, dialyzed CFs were applied to a

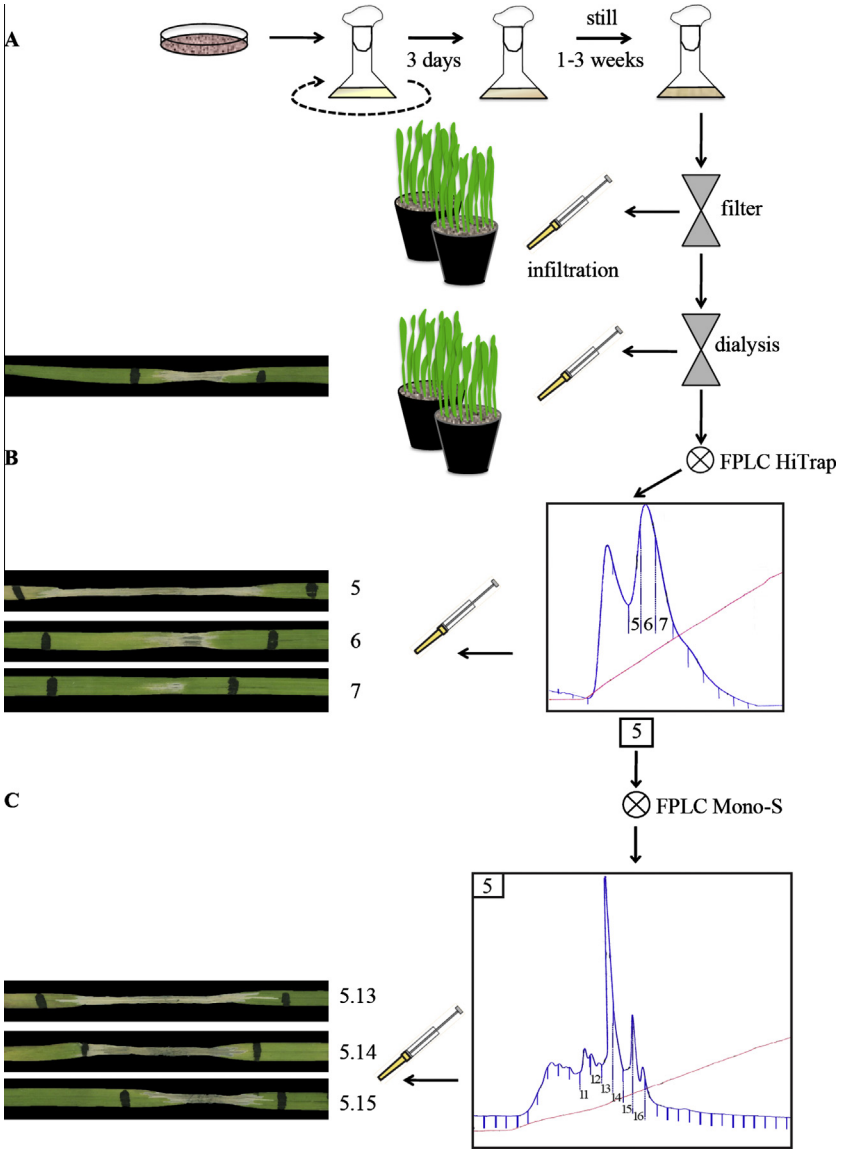

Fig. 1. Schematic representation of the purification procedure of proteins from culture filtrates (CFs) of *Zymoseptoria tritici* strain IPO323 and necrosis inducing activity assays in cv. Obelisk. (A) *Z. tritici* was grown on V8-PDA agar medium for 5–10 days; spores were collected and transferred to liquid Fries medium. Flasks were incubated in a shaker for three days at 27 °C at 100 rpm followed by two to three weeks of stationary growth at 21 °C in the dark. CFs were filtered, dialyzed and infiltrated. (B) Dialyzed CF was applied to a FPLC HiTrap strong cation-exchange column and the bound proteins were eluted in different fractions and assayed for necrosis inducing activity. (C) Fraction 5 was further purified using a FPLC Mono-S strong cation-exchange column, the collected fractions were assayed for necrosis inducing activity and fractions 5.13–5.15 were further analyzed by SDS–PAGE. Fraction 5.14 was further analyzed by mass spectrometry for identification of the proteins. The red lines in the chromatograms are NaCl gradients (0–0.5 M, blue lines are absorbances at 280 nm. The profile of the effluent containing the non-bound proteins is omitted from the chromatogram.

cation-exchange HiTrap column. From the 12 FPLC fractions (Fig. 1), the highest necrosis inducing activity was observed in fraction 5 that was recovered from the HiTrap column at approximately 0.18 M NaCl, with some activity in fractions 6 and 7 (Fig. 1). No significant necrosis inducing activity occurred when seedlings were infiltrated with any of the other eluting fractions or with the unbound proteins present in the flow-through of the HiTrap column.

Further purification of fraction 5 on a Mono-S column yielded a more complex absorption pattern at 280 nm (Fig. 1). Fractions 5.13, 5.14, 5.15 that eluted between 0.16 and 0.2 M NaCl showed the highest necrosis inducing activity in the infiltration assay. These were subsequently analyzed by SDS–PAGE (Fig. S1), which revealed that the necrosis-inducing fractions 5.13–5.15 still contained several protein bands over a broad molecular mass range that clearly differed from the band profiles of fractions 5.12 and 5.16 in the neighboring lanes that did not show activity upon infiltration. This suggests the involvement of a complex mixture of ZtNIPs with either specific differential or general necrosis inducing activities that may have intrinsic avirulence or virulence functions, which may even change during the course of infection.

We subsequently analyzed the partially purified necrosis-inducing activity containing fraction 5.14 by mass spectrometry, which narrowed down candidate ZtNIPs to almost 1%. This yielded peptide matches with 13 proteins (Table S3) in the merged database that matched characteristics of secreted hypothetical proteins. The unknown hypothetical proteins were further characterized and resulted in two prominent ZtNIP encoding candidates that we designated ZtNIP1 and ZtNIP2. The mature proteins appeared to have a mass of 15 and ~16.9 kDa, respectively (Fig. S2). The first protein, ZtNIP1, is a homolog of Ecp2 of *C. fulvum* and is presumably encoded by a fourth paralog of *MgEcp2* (I. Stergiopoulos, personal communication) in addition to the published three others that were previously reported (Stergiopoulos et al., 2010). Interestingly, Ecp2-like effector proteins are also

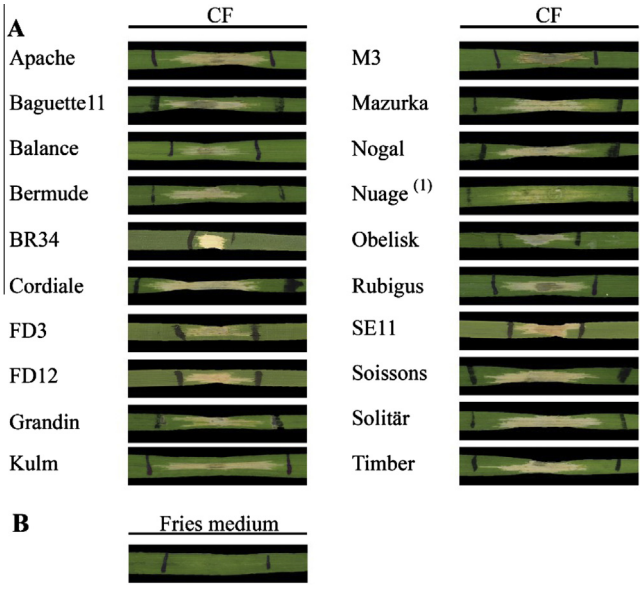

Fig. 2. Necrosis-inducing activity of CFs from *Zymoseptoria tritici* strain IPO323 in 20 wheat cultivars at four days after-infiltration (A) using Fries medium in cv. Obelisk as a control (B). (1) Reduced necrosis-inducing activity compared to other cultivars.

identified in the related Dothideomycete banana pathogen *Pseudocercospora fijiensis* that likely promote virulence by interacting with a putative intracellular host target causing host cell necrosis (Stergiopoulos et al., 2010). Ecp of *C. fulvum* is one of the few effector proteins that can induce necrosis in tomato and tobacco plants irrespective of the presence of the signal peptide indicating that its hosts target is intracellular indeed (Laugé et al., 1997, 2000; de Kock et al., 2004). The second protein, ZtNIP2, contained a putative ML domain (MD-2-related lipid-recognition). Such proteins are subdivided in four groups depending on the sequence similarity, are mostly secreted and consist of multiple β-strands that create β-sheets and regroup multiple proteins of unknown function in plants, fungi and animals (Inohara and Nunez, 2002). ZtNIP2 belongs to the third subgroup that includes the phosphatidylglycerol/phosphatidylinositol transfer protein (PG/PI-TP) of *Aspergillus oryzae*. It has been shown that ML-domain proteins are able to bind lipids and are involved in innate immunity (Kirchhoff et al., 1996; Inohara and Nunez, 2002; Mullen et al., 2003).

3.4. In planta gene expression of the genes encoding ZtNIP1 and ZtNIP2

To investigate whether these proteins might be involved in the above described biotrophy–necrotrophy switch around 10 days after inoculation, we examined the expression of the genes encoding ZtNIP1 and ZtNIP2 during infection in the susceptible wheat cv. Obelisk. The expression of ZtNIP1 correlated with the time point of macroscopical necrotic symptom appearance. Indeed, it was up-regulated at 8 days post-inoculation (dpi) and subsequently down-regulated at 12 dpi, coinciding with the transition between the biotrophic and necrotrophic phases of the fungus (Fig. 4). However no conclusion could be drawn regarding the expression of ZtNIP2 as the data between biological replicates were highly variable (Fig. 4).

3.5. Heterologous expression of the genes encoding ZtNIP1 and ZtNIP2 in P. pastoris

To examine whether ZtNIP1 and ZtNIP2 induce necrosis in wheat cultivars, we expressed the proteins ZtNIP1 and ZtNIP2 in

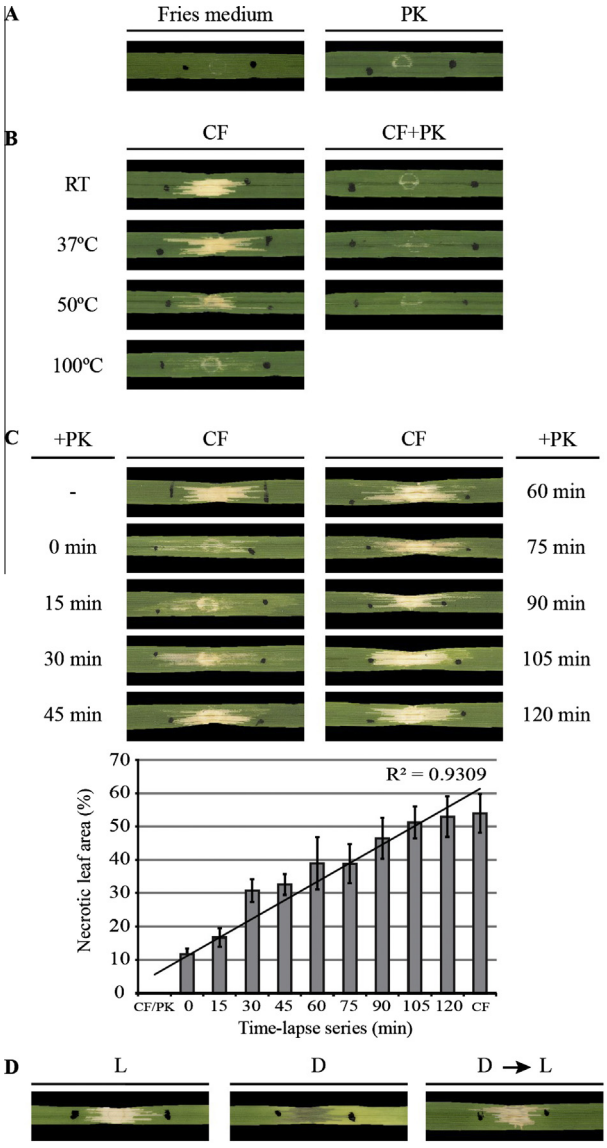

Fig. 3. Necrosis-inducing activity of *Zymoseptoria tritici* strain IPO323 culture filtrates (CFs) in the sensitive wheat cv. Obelisk after different treatments. (A) Fries medium and Proteinase K (PK) controls. (B) The effect of temperature and *in vitro* Proteinase K treatments on necrosis-inducing activity of CFs. (C) The *in planta* effect of proteinase K treatment (100 µl of 1 mg/ml) at different time points on CF-infiltrated leaves from 0 to 120 min. Below: Chart displaying time-lapse series (min) of *in planta* PK infiltration of CFs-infiltrated leaves. (D) The effect of light on necrosis inducing activity of CFs. Assays were placed under ambient light conditions (L), in darkness for 72 h (D), or exposed to ambient light after 48 h of darkness (D → L). Black dots on leaves delimit the infiltrated area.

P. pastoris that has been used extensively and successfully to express recombinant proteins. The CF from *P. pastoris* transformed with the gene encoding ZtNIP1 showed necrosis-inducing activity in wheat cvs. FD3, FD12, Nuage, Solitär and Kulm albeit of different intensity (Fig. 5). The other wheat accessions including SE11, SE3, Apache, Balance, Cordiale, Soisson, Timber, Bermude, Mazurka, Baguette, Nogal, Grandin, BR34 and M3, did not show any necrosis-inducing activity (Fig. S3) and were comparable to those observed with CF from non-transformed control *P. pastoris*. The CF from *P. pastoris* transformed with the gene encoding ZtNIP2 showed strong necrosis-inducing activity in cvs. Nuage, Bermude and FD3, but a weaker response cvs. Kulm and Grandin (Fig. 5). Infiltration with the control *P. pastoris* culture filtrates did not show any necrosis-inducing activity other than the physical

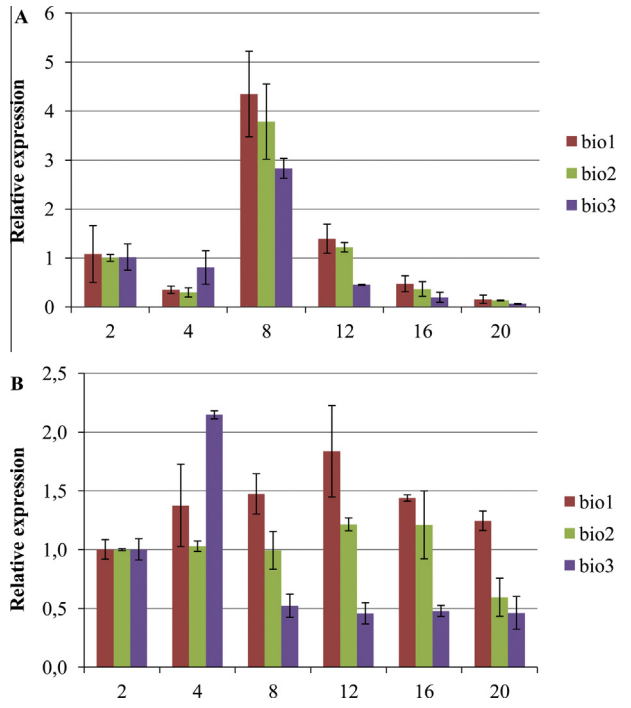

Fig. 4. Real-time qPCR analysis of *Zymoseptoria tritici* gene encoding NIP1 (A) and *Z. tritici* gene encoding NIP2 (B) in leaves of the susceptible cv. Obelisk that were inoculated with *Z. tritici* strain IPO323 and sampled during the course of infection (2, 4, 8, 12, 16, and 20 days post-inoculation) from three biological replicates. Bars and numbers indicate the relative expression levels together with the variation. The *Z. tritici beta-tubulin* gene was used for normalization.

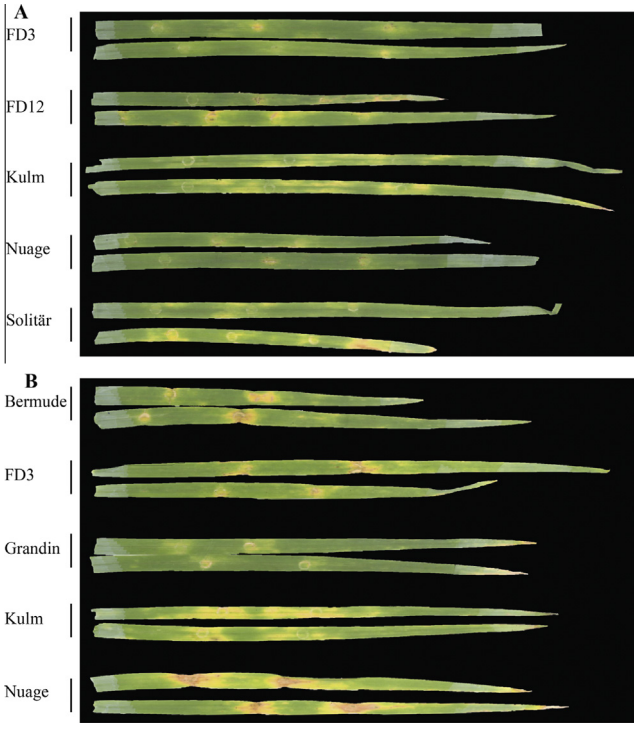

Fig. 5. Necrosis-inducing activity of the ZtNIP1 (A) and ZtNIP2 (B) proteins produced in *Pichia pastoris* cultures on leaves of different wheat cultivars (pictures were taken at five days after infiltration). For ZtNIP1 and ZtNIP2, the necrosis-inducing activity of proteins produced by two different *P. pastoris* transformants were assayed (three clones and two clones for each leaf for ZtNIP1 and ZtNIP2, respectively are shown).

damage caused by the syringe and occasional slight necrosis limited to the site of infiltration (Fig. S4).

Overall, the two proteins produced in CF of the transformed *P. pastoris* cultures showed differential necrosis inducing activity in a range of wheat cultivars, depending on ambient light and temperature conditions (data not shown).

Although the activity of *Z. tritici* NIPs is reminiscent with HSTs in other pathosystems, in *Z. tritici* necrosis occurs much later and also the type of host response is different (from chlorosis to necrosis). This variation could be due to dependence of host responses on environmental factors such as temperature and light. Necrosis-inducing activity of these NIPs of *Z. tritici* appeared indeed light-dependent. Keon et al. (2007) and also more recently Tabib Ghaffary (2011) reported that light intensity has a marked influence on symptom development in wheat cultivars that were inoculated with *Z. tritici* strains. Higher light intensities result in higher disease severities. However, this depended strongly on the specific resistance genes, such as *Stb2* in cv. Veranopolis that seems to be very sensitive (Tabib Ghaffary, 2011). Reduced light intensities resulted in poor disease development, whereas high intensities resulted in fully sporulating susceptible responses. Also, proteolytic degradation or protein expression and concentration differences could vary from one experiment to the other and influence phenotypic expression. It has been shown that PtrToxA, SnToxA, SnTox1, SnTox2, SnTox3, SnTox4 induce maximal necrosis at three days after-infiltration (Strelkov et al., 1999; Liu et al., 2004, 2009; Manning and Ciuffetti, 2005; Friesen et al., 2006, 2007; Abeysekara et al., 2009) whereas activity with ToxC was only visible after five days (Effertz et al., 2002) and the activity of *P. tritici-repentis* ToxB depends strongly on its concentration (Strelkov et al., 1999; Kim and Strelkov, 2007). Further investigations are underway to determine the effect of ZtNIPs concentrations on the phenotype.

We also observed a discrepancy between the appearance of necrosis after infiltration of ZtNIP1 and ZtNIP2 and after inoculation with conidia of *Z. tritici*. It is, therefore, still unclear whether the necrosis-inducing activity observed in resistant cultivars is related to a susceptibility or a resistance response as was also reported for other proteins reviewed by Rep (2005). In this respect, *P. nodorum* HSTs induce cell death in susceptible host plants (Friesen et al., 2007, 2008a) whereas avirulence proteins of *C. fulvum* only induce cell death in tomato plants with the corresponding *Cf* resistance genes (de Wit et al., 2009). Nip1, a small phytotoxic protein from *R. secalis* is an avirulence factor that is required for Rrs1-mediated resistance of barley (Rohe et al., 1995), and stimulates the activity of the barley plasma membrane H+-ATPase in a genotype-unspecific manner as it induces necrotic lesions in leaf tissues of barley and other cereal plant species (Wevelsiep et al., 1991, 1993; van't Slot et al., 2007). Obviously, differential necrosis inducing activity, in the case of *Z. tritici*, would comply with either GFG of the *Z. tritici*–wheat pathosystem (Brading et al., 2002) or with iGFG, which was particularly discovered in the necrotrophic fungal pathogen *P. nodorum* (Friesen et al., 2008a; Friesen and Faris, 2010). At this stage we can only speculate on iGFG since no sensitivity genes to *Z. tritici* have been mapped. Resistant wheat cultivars could be sensitive to a necrosis-inducing protein. However, it is conceivable that its activity threshold is never reached as fungal proliferation is controlled by effective resistance genes and, consequently ZtNIPs produced by the fungus never reach the required concentration in the apoplast. The necrosis-inducing activity observed after infiltration is hardly ever observed in inoculation assays due to the slow build-up of fungal biomass in resistant wheat cultivars. Another hypothesis would be that one and the same protein induces resistance in resistant plants, but functions as a host-selective toxin in susceptible plants.

Further functional analyses of the identified ZtNIPs and their encoding genes are necessary to elucidate the role of these proteins during pathogenesis and their targets need to be identified. Future studies will focus on these aspects and on the diversity and function of the encoding genes in natural *Z. tritici* populations.

4. Conclusions

Overall, this study shows that the production and analysis of crude culture filtrates coupled with FPLC and mass spectrometry is a powerful tool for the identification of candidate necrosis-inducing proteins of *Z. tritici* that should further elucidate its pathogenesis in wheat.

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All materials and protocols are made available upon request.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.03.015.

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