Diagnosis of primary aldosteronism in the hypertension specialist centers in Italy: a national survey

Published version:

DOI:10.1038/s41371-018-0094-6

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Postharvest application of a novel chitinase cloned from *Metschnikowia fructicola* and overexpressed in *Pichia pastoris* to control brown rot of peaches

Short running head:

Chitinase against brown rot of peaches

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Abstract

Metschnikowia fructicola strain AP47 is a yeast antagonist against postharvest pathogens of fruits. The yeast was able to produce chitinase enzymes in the presence of pathogen cell wall. A novel chitinase gene *MfChi* (GenBank accession number HQ113461) was amplified from the genomic DNA of *M. fructicola* AP47. Sequence analysis showed lack of introns, an open reading frame (ORF) of 1,098 bp encoding a 365 amino acid protein with a calculated molecular weight of 40.9 kDa and a predicted pI of 5.27. *MfChi* was highly induced in *Metschnikowia fructicola* after interaction with *Monilinia fructicola* cell wall, suggesting a primary role of MfChi chitinase in the antagonistic activity of the yeast. The *MfChi* gene overexpressed in the heterologous expression system of *Pichia pastoris* KM71 and the recombinant chitinase showed high endochitinase activity towards 4-Nitrophenyl β-D-N, N', N''-triacetylchitotriose substrate. The antifungal activity of the recombinant chitinase was investigated against *Monilinia fructicola* and *Monilinia laxa in vitro* and on peaches. The chitinase significantly controlled the spore germination and the germ tube length of the tested pathogens in PDB medium and the mycelium diameter in PDA. The enzyme, when applied on peaches cv. Redhaven, successfully reduced brown rot severity. This work shows that the chitinase MfChi could be developed as a postharvest treatment with antimicrobial activity for fruit undergoing a short shelf life, and confirms that *Pichia pastoris* KM71 is a suitable microorganism for cost-effective large-scale production of recombinant chitinases.

Keywords: Cloning, Quantitative real-time PCR (RT-qPCR), recombinant expression, *Pichia pastoris, Monilinia fructicola, Monilinia laxa*

1. Introduction

Considerable losses are caused by postharvest diseases during transportation and storage of fruit (Sharma et al., 2009). Brown rot caused mainly by *Monilinia laxa* (Aderh. et Rulh.) Honey and *Monilinia fructicola* (G. Wint.) Honey is considered the main postharvest disease of stone fruit (De Cal and Melgarejo, 1999; De Cal et al., 2009). *M. fructicola* is the most destructive pre- and postharvest pathogen in all stone fruit-growing regions of the United States (Janisiewicz et al., 2013). In the European Union, neither additional cultural measures nor increased fungicide treatments are sufficient to control brown rot in the orchard and in postharvest after the introduction of *M. fructicola* (EFSA, 2011; Pellegrino et al., 2009), and no chemical fungicides are allowed for postharvest treatment of stone fruit. Moreover, the public demands to reduce pesticide use on fruit and to improve environmental protection and human health have increased the need to develop alternative control methods (Lopez-Reyes et al., 2013; Sisquella et al., 2014). Biological control using antagonistic yeasts has been explored as one of several promising alternatives to chemical fungicides (Liu et al., 2013a). Antagonistic yeasts deserve particular attention and are considered promising biocontrol candidates, as their activity neither involves production of toxic metabolites nor negative impact on the environmental safety (Spadaro et al., 2002; 2008).

Among different antagonistic yeasts, *Metschnikowia fructicola* Kurtzman and Droby is an important yeast species which has been successfully applied to control a number of pathogens on fruits and vegetables, such as *Penicillium expansum* on apple (Liu et al., 2011; Spadaro et al., 2013), *Botrytis cinerea* on grape (Karabulut et al., 2003; Kurtzman and Droby, 2001) and on strawberries (Karabulut et al., 2004). Moreover, one strain of *Metschnikowia fructicola* was registered and commercially available in Israel to control storage diseases of fruits and vegetables (Kurtzman and Droby, 2001, Macarisin et al., 2010). The strain AP47 of *Metschnikowia fructicola* (Zhang et al., 2010a) was obtained from the carposphere of an apple grown in organic orchard in North Italy. Under semi-commercial conditions, *Metschnikowia fructicola* strain AP47 showed a high efficacy in controlling brown rot caused by *Monilinia* spp. on stone fruits, however its mechanism against postharvest pathogens is still unclear (Zhang et al., 2010a).

Various mechanisms of action of antagonistic yeasts have been described, such as competition for nutrients and niche exclusion (Li et al., 2008; Liu et al., 2012a), induction of host defense mechanisms (Jiang et al., 2009; Xu et al., 2013) and the production of hydrolases such as chitinase, protease and glucanase, which is proposed as an important mode of action against fungal pathogens, due to its role in breaking down pathogens cell wall and inhibiting spore germinations (Masih and Paul, 2002; Smits et al., 2001; Zhang et al., 2011; 2012).

Cloning, expression and characterisation of new chitinase genes from microorganisms is useful for antagonism activity as well as for developing new potential chitin biological degraders. Compared with the extensive research into the chitinases from some antagonistic fungi, such as *Trichoderma* spp. (Nakahara et al., 2001; Silva et al., 2011) and bacteria such as *Bacillus* spp. (Shivakumar et al., 2014; Yang et al., 2009), few studies have been carried out on chitinases produced by yeasts with molecular tools. To our knowledge, there is no published report on cloning and phylogenetic analysis and expression of chitinase from the antagonistic yeast species *Metschnikowia fructicola*.

Recently *Pichia pastoris* has emerged as an important yeast host for heterologous protein expression (Cregg et al., 1993; Macauley et al., 2005), since it has many of the advantages of higher eukaryotic expression systems, such as protein processing and folding and posttranslational modifications (Balamurugan et al., 2007). Therefore it was used in this study for chitinase expression.

The objectives of this research were: i) to study the chitinolytic activity of the antagonistic yeast *Metschnikowia fructicola* strain AP47 *in vitro*; ii) to clone and characterize the chitinase gene *MfChi* from AP47; iii) to analyse *MfChi* gene expression in AP47 after exposure to pathogen cell wall preparation through reverse transcription quantitative PCR (RT-qPCR); iv) to express the chitinase MfChi in the methylotrophic yeast *Pichia pastoris;* v) to study the antifungal activity of the expressed chitinase *in vitro* and *in vivo* and the effect of the enzyme concentration on the control of *M. laxa* and *M. fructicola*.

2. Materials and Methods

2.1. Microorganisms, growth media, plasmids and molecular kits

Metschnikowia fructicola Kurtzman and Droby strain AP47 (Zhang et al., 2010a) was isolated from the carposphere of apple cv. Golden delicious, harvested in an organic orchard located in Piedmont, Northern Italy and identified by using molecular and morphological tools. The microorganism culture was stored at −80 °C in cell suspension with 65% (v/v) glycerol and 35% (v/v) of a solution of 100 mM $MgSO_4$ and 25 mM Tris (pH 8.0). Yeast subcultures were grown in YEMS (30 g/L yeast extract, 5 g/L D-mannitol, 5 g/L l-sorbose (Spadaro et al., 2010). Five strains of *Monilinia fructicola* (G. Wint.) Honey and five strains of *Monilinia laxa (*Aderhold & Ruhland) Honey isolated from rotted peaches were used as a mixture throughout this work after being selected for their virulence by inoculation in artificially wounded peaches.

Oligonucleotides, pGEM-T vector and *Escherichia coli* strain JM109 used in this study were purchased from Promega (Madison, WI, USA). The kits of DNA and RNA extraction (DNeasy and RNeasy). QIAquik PCR purification, Reverse-transcript PCR, Plasmid-extraction, QIAquik Gel extraction and one step RT-PCR kit as well as the materials for PCR were purchased from Qiagen (Hilden, Germany). The kit "Gene Walking Made Easy" and other materials for enzyme assays were purchased from Sigma-Aldrich (St. Louis, MO,USA). *Pichia pastoris* KM71 strain used as host for transformations with the plasmid pPIC9 and *Escherichia coli* strain DH5α used as host for the plasmids were obtained from Invitrogen (Life Technologies, Carlsbad, USA). TURBO DNase was purchased from Ambion (Ambion, Foster City, CA, USA). iScript cDNA Synthesis Kit and 2× Power syber green supermix were purchased from Bio-Rad (Richmond, CA, USA) for RT-qPCR.

2.2. Chitinase activity of the strain AP47 grown in vitro

To study the chitinase enzyme production from the strain AP47, and the effect of different substrates on its chitinolytic activity, the yeast strain was cultured in modified Lilly-Barnett minimal salt (LBMS) medium (Lilly and Barnett, 1951) containing 2 mg/mL *Monilinia fructicola* cell wall preparation (CWP), glucose or 5 mg/mL colloidal chitin as sole carbon source. CWP of the pathogen *Monilinia fructicola* was prepared as described by Saligkarias *et al*., (2002), and colloidal chitin was prepared according to the method described by Roberts and Selitrennikoff (1988) from shrimp shell chitin (C9752, Sigma–Aldrich). In preliminary experiments, the yeast strain produced the highest chitinase activity when grown for 48 h. Therefore, we just measured the chitinase activity of the strain when grown for 48 h. The spectrophotometric assay of chitinase activity was carried out according to the procedure developed by Miller (1959), with small modifications. Chitinase activity was determined colorimetrically by using colloidal chitin as substrate. The reaction mixture, consisting of 500 µL colloidal chitin (0.5% w/v) and 500 µL enzyme solution, was incubated at 50 °C in a water bath for 30 min. The reaction was stopped by centrifugation at 3,000×*g* for 3 min. An aliquot of the supernatant (0.8 mL) was pipetted into a new sterile tube followed by adding 500 µL dinitrosalicylic acid. The reaction mixture was immediately boiled for 5 min. After cooling, the reducing sugars released as chitinase activity were measured at 540 nm. One unit of chitinase activity was defined as the amount of enzyme which produced 1 µM/min reducing *N*-acetyl-D-glucosamine.

2.3. Cloning the chitinase gene MfChi from the genomic DNA

The strain AP47 was grown in liquid medium YPD (20 g D-glucose, 20 g peptone casein, and 10 g yeast extract per litre) at 25 °C for 48 h, then centrifuged at 5,000×g for 10 min. DNA was extracted from the pellet with DNeasy extraction kit (Qiagen), according to the manufacturer’s instruction.

To clone the first partial sequence of the chitinase gene from genomic DNA of *Metschnikowia fructicola* strain AP47, PCR amplification of the extracted DNA was performed by using the degenerate primers 5’-CTNCTNTCNCTNGTNGTN-3’ (Forward primer DPf) and 5’-CARTARTTRTTRTARAAYTG-3’ (reverse primer DPr). DPf and DPr were designed according to the conserved protein sequences (LLSLGG and QFYNNYC) obtained with DNAMAN 7.0 by using the alignment of the deduced amino acid sequences of 8 yeasts chitinase genes deposited (Suppl. Fig. 1). After loading on agarose gel**,** PCR products were purified with QIAquick gel extraction kit (Qiagen) according to the supplier’s instructions, then ligated into pGEM-T cloning vector (Promega), followed by transformation into chemically competent cells of *E. coli* strain DH5α (Invitrogen) and selection of positive transformants with blue / white screening technique. The sequencing and BLAST analysis showed that a fragment of 350 bp was obtained. To amplify and identify the 5’ and 3’ flanking regions of the chitinase gene from the genomic DNA of *Metschnikowia fructicola* strain AP47, special restriction digestion enzymes and primers were designed according to the obtained sequence and the kit “Gene Walking Made Easy” (UVS1, Sigma–Aldrich, USA): AP47-5UTR: 5'-TCAGTCAAGAACGACAAGATCACAGTGTCC-3' and AP47-3UTR: 5'-TGATATGGACAAGAAGAAGCCTTTTGACTTGAACAAG-3' together with Vectorette *Cla* I library of “genomic walking kit”. The specific process was performed according to the supplier’s instructions. The fragment from Vectorette *Cla* I library of the strain AP47 was purified, ligated into pGEM-T cloning vector and sequenced as described above. Finally the whole sequence of the targeted gene was assembled, designated as *MfChi* and deposited in GenBank (accession number: HQ113461.1).

2.4. Cloning the chitinase gene MfChi from the cDNA

Total RNA was extracted from the strain AP47 grown for 48 h in YPD broth at 25 °C by using an RNeasy Mini Kit (Qiagen), then treated with TURBO DNase (Ambion) according to the manufacturer's instructions. The absence of genomic DNA contamination was confirmed by PCR amplification of the housekeeping gene Actin1 (Li et al., 2006) using One Step RT-PCR Kit (Qiagen).

First-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. To amplify the chitinase gene *MfChi* from the cDNA of *Metschnikowia fructicola* strain AP47, specific primers were designed according to the chitinase gene sequence obtained from the genomic DNA of *Metschnikowia fructicola* strain AP47: forward primer (FP) 5’-ATGTTGATGCAACCATTTTTATGC -3’ and reverse primer (RP) 5’-TCAGACTTTGAACTTTGGCTTG-3’, then PCR products were purified and sequenced as described above.

2.5. Analysis of MfChi gene expression

AP47 was cultured in LBMS medium containing 2 mg/mL of *Monilinia fructicola* CWP at 23 °C by shaking at 100 rpm, then collected after 6 h, 12 h, 24 h and 48 h of incubation. AP47 grown without CWP served as a control. Each treatment consisted of three replicates at each time point and the experiment was repeated three times. Total RNA and cDNA synthesis were performed as described previously, then the resulting cDNA was used as a template for RT-qPCR to quantify the *MfChi* transcript expression under different time points. RT-qPCR was performed in triplicate on the cDNA obtained from each biological replicate using the 2× Power syber green supermix (Bio-Rad) for the reaction mix according to the manufacturer’s instructions. Amplification and detection were carried out in an iCycler (Bio-Rad), set up with initial denaturation at 95 °C for 10 min followed by 40 cycles comprising a denaturation step at 95 °C for 15 s and an annealing step at 60 °C for 1 min. The primers *MfChi*-F (5’-TGATTTCCCCAAGATGAAGC-3’) and *MfChi*-R (5’-AAAGTCACGAGCCTCTGCAT-3’) were designed to optimally amplify *MfChi* gene sequence, and transcript levels of Actin1 served as an internal standard. The primers used were Act1 F (5’-CCTGAGGAACACCCAGTCTT-3’) and Act1 R (5’-GAGTTGTAAGTGGTTTGGTCG-3’) according to Liu et al. (2011). The expression ratio was calculated from equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$

represents the $\Delta CT_{sample} - \Delta CT_{control}$ (Livak and Schmittgen, 2001), and values were normalized to the control at 6 h, arbitrarily set to unity.

2.6. Heterologous expression of MfChi in Pichia pastoris

The chitinase gene *MfChi* was amplified from *Metschnikowia fructicola* cDNA by PCR using forward primer (5’-TCAGAATTCATGTTGATGCAACCATTTTTATGC-3’) and reverse primer (5’-CAGGAATTCTCAGACTTTGAACTTTGGCTT-3’); bases underlined encode *Eco*RI restriction sites. The resulting DNA fragment (1098 bp) was digested with *Eco*RI before being ligated into the corresponding sites of the *expression* vector pPIC9 and designated as pPIC9-*MfChi*. The ligation product was transformed into *E. coli* JM109 (Promega) and the plasmid was sequenced at BMR Genomics (Padova, Italy). Transformation of *MfChi* gene into *P. pastoris* KM71 was performed as recommended by the manufacturer (Invitrogen). Briefly, pPIC9-*MfChi* was linearized using *Stu I* then transformed into competent *P. pastoris* KM71 cells via electroporation. The empty vector pPIC9 was also transformed as a negative control. Finally His^{+} transformants of *P. pastoris* KM71 were purified on minimal medium plates without histidine to ensure pure clonal isolates.

Transformed *P. pastoris* isolates were grown in 100 mL of Buffered Complex Glycerol Medium (BMGY) until the culture reached an OD 600 nm of 2-6, then the pellet was resuspended in 20 mL of Buffered Complex Methanol Medium (BMMY). Methanol was added at every 24 h interval to a final concentration of 1% to maintain the induction.

To analyze expression levels and the optimal time post-induction for harvest, supernatants were collected at different time points (0, 24, 48, 72, 96, 120 and 144 h) and secreted proteins were analyzed by SDS-PAGE (Laemmli, 1970) (Amersham ECL Gel 10%, GE Healthcare Life Science, Uppsala, Sweden). The recombinant protein MfChi was purified following the method of Liu et al., (2013b), then protein concentration was determined according to Bradford (1976) by using bovine serum albumin (Sigma–Aldrich) as a standard.

2.7. Recombinant chitinase activity assay

Recombinant MfChi chitinase activity was determined using a colorimetric Chitinase assay kit (CS0980, Sigma-Aldrich) following manufacturers instruction. The absorbance was measured at 405 nm, then the specific activity of chitinase was expressed as U/mg, where one unit will release 1.0 micromole of *p*-nitrophenol from the substrate at pH 4.8 and 37 °C in one minute for each milligram of protein. Supernatant from cell culture of transformed *P. pastoris* with empty vector pPIC9 was used as a negative control. Three replicates in each treatment were performed, and the experiment was repeated three times.

2.8. Effect on pathogen mycelium growth in vitro

The activity of the recombinant chitinase MfChi against *Monilinia fructicola* was assayed in Petri dishes containing PDA according to Banani et al. (2014) with some modifications. In brief, the recombinant chitinase MfChi was streaked into a PDA plate. A *M. fructicola* mycelial plug (5 mm diameter) was corked from a PDA culture and fixed in Petri dish at the same distance from the enzyme streak and the Petri dish border (control). After 6 days of pathogen growth at 25 °C, mycelial inhibition was measured and direct interaction *in vitro* was observed using an optical microscope (Eclipse 55i, NIKON, Tokyo, Japan).

2.9. Effect on pathogen spore germination and germ tube elongation

The effect of the recombinant chitinase MfChi was tested on conidia germination of *M. fructicola* and *M. laxa* using the method of Zhang et al. (2012) with some modifications. In brief, tubes containing 2.4 mL potato dextrose broth medium (PDB, Merck), 300 µL of *Monilinia* spp. conidial suspension (1×10^6 conidia/mL) and 300 µL of the recombinant chitinase were co-incubated at 25 °C on a rotary shaker (200 rpm). Two chitinase concentrations were assayed: C1 (7 ng/µL) and C2 (70 ng/µL). The control treatment consisted of water added to the tubes instead of the enzyme solution. After 9 h and 18 h of incubation, 100 conidia of *Monilinia* spp. per replicate were observed, and their germination rate (%) and germ tube length (µm) were measured by using an optical microscope. For each treatment, three replications of three tubes were performed and the experiment was repeated three times.

2.10. Efficacy against Monilinia spp. in vivo

The biocontrol activity of the recombinant chitinase in controlling *M. fructicola* and *M. laxa* on peaches cv. Redhaven was evaluated using the method of Yan et al. (2008) with some modifications. Three wounds (4 mm deep × 3 mm wide) were made at the equator of each fruit. 20 µL of recombinant chitinase were applied into each wound. The yeast AP47 was applied at 10^8 cells /mL (20 µL) in order to compare its activity with the chitinase. Peaches inoculated with *Monilinia* spp. spore suspension acted as untreated control and peaches inoculated and treated with 2.5 mL/L of tebuconazole (Folicur, Bayer Crop Science, Monheim, Germany; a.i.: 25.0%) were the chemical control. Two hours later, 20 µL of pathogen suspension (10^5 conidia/mL) was inoculated into each wound. Two chitinase concentrations **(**C1: 7 ng/µL and C2: 70 ng/µL) were used. The treated fruits were incubated at 23 °C, and the rot diameter was measured 3 and 5 days after inoculation (DAI). Each treatment consisted of three replicates with ten fruits per replicate and the experiment was performed three times.

2.11. DNA sequence and phylogenetic analysis of the chitinase gene

BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for the nucleotide sequence analysis, the deduction of the amino acid sequence and database searches. Multiple sequence alignments of DNA and amino acid sequence were performed using the programs of DNAMAN 7.0 and CLASTALW. The phylogenetic tree of the chitinase gene was generated by MEGA6 using neighbour-joining method.

2.12. Statistical analysis

All statistical analyses were performed with SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Data obtained in all the experiments were analysed using analysis of variance (ANOVA). The treatment means were separated at 5% significance level by using Duncan’s multiple range tests. Values are presented as the mean ± SD (standard deviation of the mean). The results are the mean of three independent experiments.

3. **Results**

3.1. Production of chitinase by Metschnikowia fructicola AP47 and its activity

The strain AP47 showed chitinase activity when grown in different media. The chitinase activity of AP47 was higher when grown in LBMS with *Monilinia fructicola* CWP as sole carbon source (0.35 U/mL) than with glucose (0.21 U/mL). The highest chitinase activity (0.46 U/mL) was observed when grown in LBMS with colloidal chitin.

3.2. Cloning the chitinase gene from DNA and cDNA of the yeast strain AP47

PCR amplification of the partial sequence of chitinase gene from *Metschnikowia fructicola* AP47 DNA with the degenerate fragments DPf and DPr produced a 350 bp fragment of the putative chitinase gene containing the consensus motif (DGXDFXXE) as signature pattern of Family 18 hydrolases. The signature pattern is highly conserved among most known chitinases from bacteria and yeasts in its deduced amino acid sequence. PCR amplification of the flanking regions of the fragment of the chitinase gene was performed from the genomic DNA of AP47, then the whole chitinase gene sequence was assembled and designated as *MfChi* (GenBank accession number HQ113461) with 1,098 bp (Suppl. Fig. 2). PCR amplification of the cDNA of *Metschnikowia fructicola* strain AP47 and sequence analysis revealed that the ORF from the cDNA of *Metschnikowia fructicola* strain AP47 shared the same nucleotide sequence with that of the genomic DNA, showing the lack of introns inside the gene *MfChi*.

3.3. Characterization of the chitinase gene MfChi

The prediction of the signal peptide of the chitinase gene *MfChi* was performed according to Bendtsen et al. (2004), and showed the presence of 19 amino acid signal peptide (positions from 1 to 19). Often, the first 20 amino acids serve as a typical cleavable signal sequence for secreted proteins (Kuranda and Robbins. 1991). Sequencing and nucleotide analysis confirmed the lack of introns inside *MfChi* gene, and an ORF of 1,098 bp encoding a 365 amino acid protein with predicted molecular weight of 40.9 kDa and pI of 5.27 were calculated (Suppl. Table 1). Prediction and analysis of the deduced amino acids from the gene showed that MfChi has 14 putative phosphorylation sites at positions of 34, 49, 103, 108, 115, 233, 285, 296, 125, 288, 92, 159, 204 and 304, respectively. In addition, MfChi has 4 putative *N*-glycosylation sites at positions of 67, 314, 318 and 324, respectively (Suppl. Fig. 2), while no *O*-glycosylation sites were observed. Sequence alignment of the deduced amino acids from *MfChi* with other related chitinases of yeasts retrieved from NCBI database showed that *MfChi* has only high similarity (97.0%) with *MpChi* chitinase of *Metschnikowia pulcherrima* strain MACH1 (GenBank accession number HQ113462, Saravanakumar et al., 2009), but low similarity to other yeast chitinases (Suppl. Fig. 3). However, when alignment was performed with the N-terminal regions of the chitinase MfChi and other yeast chitinases, a high homology was observed between these chitinases (Suppl. Fig. 4). Moreover MfChi shared a common putative catalytic domain which conformed to the signature motif (DXXDXXXE) of family 18 of chitinases proposed by Watanabe et al. (1993), suggesting to belong to family 18 of chitinases. In addition, six conserved cysteine residues required for substrate-binding by the chitinase were identified (Suppl. Fig. 4). To reveal the relationship of MfChi with the chitinases from other yeast and other organisms, a phylogenetic analysis was performed on the nucleotide sequences. MfChi belong to GH family 18 and it is included into subgroup II including yeast chitinases (Suppl. Fig. 5 and Suppl. Table 2). Among the chitinases of the subgroup II, MfChi is the closest to the yeast chitinase MpChi *of Metschnikowia pulcherrima* strain MACH1 (Saravanakumar et al., 2009).

3.4. Expression of MfChi in Metschnikowia fructicola in response to pathogen cell wall

Quantitative real-time PCR (RT-qPCR) was conducted to analyze the expression of *MfChi* in AP47 after exposure to CWP of *Monilinia fructicola in vitro***.** The time-points included in the analysis were 6 h, 12 h, 24 h and 48 h of co-incubation. The expression of the *MfChi* gene was upregulated at an early stage of incubation and then it was downregulated after 24 h of incubation. The results indicated that *Monilinia fructicola* CWP directly induced *MfChi* expression in *Metschnikowia fructicola,* especially at 12 hours of incubation, when the gene expression was threefold higher than without CWP (Fig. 1). At longer incubation times, *MfChi* expression gradually decreased.

3.5. Expression and purification of recombinant chitinase MfChi

The recombinant chitinase expressed in different *P. pastoris* isolates was analysed with SDS-PAGE (Fig. 2). After 120 h induction, a wide band appeared in some transformed isolates, with a size of about 40.9 kDa, which corresponds to the same molecular weight predicted, while no band was observed in the negative control (non-insert control: lane number 0). After small-scale production, the best producer colonies (isolate 2 and 4, Fig. 2) were selected for large-scale chitinase expression and purification.

3.6. Identification of MfChi enzyme activity

Chitinase activity was performed to assess whether or not the expression of the chitinase MfChi in the *P. pastoris* expression system resulted in a functional protein, and to evaluate its chitinolytic activity. The recombinant chitinase MfChi showed high endochitinase activity towards the chitin pseudosubstrate 4-Nitrophenyl β-D-N, N', N''-triacetylchitotriose p-(GlcNAc)3, which is a suitable substrate for endochitinase activity detection . Additionally, no chitinase activity was detected in the culture medium of *P. pastoris* KM71 transformed with the empty vector pPIC9 after methanol induction, indicating that chitinase displayed in the transformed yeast cells was due to the expression of the foreign gene *MfChi*.

3.7. Effect on Monilinia spp. mycelium growth in vitro

After 6 days of *Monilinia fructicola* growth in PDA plates streaked with the chitinase MfChi, the effect of the recombinant enzyme on pathogen mycelium growth was observed. MfChi chitinase significantly inhibited *M. fructicola* mycelial growth, in addition, no conidia sporulation was observed in the growing side of the pathogen mycelium closer to the chitinase treatment -.

This result was confirmed by observation under optical microscope which showed that the presence of chitinase caused swelling of *M. fructicola* hyphae (Data not shown).

3.8. Effect on Monilinia spp. spore germination and germ tube elongation

By co-culturing both pathogens and the enzyme in liquid medium (PDB), the recombinant chitinase MfChi efficiently controlled the conidial germination and germ tube length of *M. fructicola* and *M. laxa* compared to the control treatments (pathogen+water) either at 9 h or at 18 h of incubation (Fig. 3). Moreover, the chitinase applied at higher concentration (C2: 70 ng/µL) showed better results in reducing the spore germination (Fig. 3A) and the germ tube elongation, than applied at lower concentration (C1: 7 ng/µL). At 70 ng/µL, the chitinase almost completely blocked the germ tube development of *M. fructicola* and *M. laxa* either at 9 h or 18 h of incubation (Fig. 3B). Higher germ tube lengths were observed for *M. fructicola* than for *M. laxa*, when incubated with water as control (Fig. 3B).

3.9.Antifungal activity of recombinant chitinase against Monilinia spp. on peaches

The antifungal activity of the recombinant chitinase was investigated on peaches stored at room temperature. After 3 DAI (Fig. 4), the chitinase treatment significantly reduced *Monilinia* spp. rot diameter compared to *M. fructicola* (33 mm) and (24 mm) *M. laxa* untreated controls. The chitinase at 70 ng/µL (C2) significantly controlled the lesion diameter on peaches (about 13 mm for both pathogens), similarly to the antagonistic cells of *Metschnikowia fructicola* AP47 (10 mm), and better than the protease at 7 ng/µL (C1) for *M. fructicola* (23 mm) and *M. laxa* (20 mm). At 5 DAI for *M. fructicola*, the chitinase was still more efficient than the untreated control (68 mm) especially at 70 ng/µL (50 mm), but its efficacy was lower than AP47 cell suspension (32 mm). For *M. laxa* (Fig. 4), the rot diameter of peaches treated with the chitinase was similar to the untreated control.

4. Discussion

This is the first study to characterize the role of a chitinase from *Metschnikowia fructicola* in the control of brown rot.. Though several studies have been performed by a large number of laboratories on the antagonistic activity of *Metschnikowia fructicola*, its mechanism of action against postharvest pathogens is still unclear, and the production of lytic enzymes, especially chitinases, is proposed as an important mode of action of antagonistic yeasts. Hydrolases attack the cell wall of phytopathogenic fungi to cause cell lysis and subsequent death (Tseng et al., 2008).

A better understanding of the modes of action of yeast biocontrol agents is essential for developing appropriate commercial formulations and application methods, to maximize their potential use to manage postharvest diseases (Droby et al., 2009; Zhang et al., 2010b).

In this research, the antagonistic yeast *Metschnikowia fructicola* strain AP47 showed to produce higher chitinase activity in the presence of *Monilinia fructicola* CWP and colloidal chitin, compared to glucose as sole carbon source in the medium.Therefore, we cloned,characterized, and expressed a novel endochitinase gene *MfChi* from *Metschnikowia fructicola* AP47 and we studied its antifungal activity and potential use against *Monilinia* spp. *in vitro* and *in vivo*.

The presence of a 19-residue putative signal peptide confirmed that MfChi is an extracellular protein, a feature common to the majority of endochitinases expressed by mycoparasites (Hayes et al., 1994; Morissette et al., 2003; Viterbo et al., 2001) and its activity towards p-(GlcNAc)3 substrate confirms its endochitinase activity.

RT-qPCR expression analysis clarified that *MfChi* gene of *Metschnikowia fructicola* is highly induced by cell wall fragments of *Monilinia fructicola* during the first 24 h of contact then the gene was downregulated. Similar results were reported for the endochitinase chi46 from the fungus *Chaetomium globosum*, which was highly upregulated at the early stage of interaction with different pathogens cell wall, and then it was downregulated (Liu et al., 2008). It is important to note that *MfChi* gene expression was altered dramatically within 12 h of exposure to cell walls, indicating a rapid physiological response pathway in *Metschnikowia fructicola* AP47.

As expected, the recombinant chitinase expressed in *P. pastoris* was directly secreted into the medium, with a size of 40.9 kDa, it confirmed to have a high endochitinase activity, and it was the most abundant protein in the medium. These results confirmed MfChi characteristics, previously calculated by sequence analysis tools, and proved that *P. pastoris* is a successful system for yeast protein expression.

The expressed chitinase was able to cause swelling of the hyphae of *Monilinia fructicola* under optical microscope, confirming the reliability of dual culture procedure to evaluate the presence of active hydrolases *in vitro*.

Previous studies confirmed that chitinase can decompose fungal cell walls (Li et al., 2005; Liu et al., 2008) since the chitin is the essential cell wall component of many fungal pathogens (Liu et al., 2012b). This study demonstrated that the recombinant chitinase MfChi expressed in *P. pastoris* is highly effective in reducing spore germination and germ tube length of *Monilinia* spp. *in vitro,* but its antifungal activity mainly depends on the chitinase concentration.

In vivo trials confirmed the high efficacy shown *in vitro* by the recombinant chitinase, and demonstrated the capacity of the chitinase to keep its activity for some days in the unfavourable environment of the fruit wounds and on fruit stored at room temperature. However, the efficacy was dependent on the enzyme concentration and the temporal distance from the chitinase treatment which could be explained by the loss of chitinolytic activity with increasing the number of storage days. Our results are in accordance with the results obtained by previous studies, which demonstrated that the efficacy of recombinant enzymes expressed in *P. pastoris* against pathogens in fruits is dependent on the concentration of the enzyme and the time between enzyme treatment and pathogen inoculation (Banani et al., 2014; Yan et al., 2008).

Interestingly, though *M. fructicola* has bigger conidial dimension, more abundant sporulation, longer germ tube length and higher growth rates than *M. laxa* (EPPO, 2009), the recombinant chitinase similarly controlled both species, either *in vitro* or *in vivo,* showing that its efficacy is not dependent on the pathogen species.

To our knowledge, it is the first time that a chitinase gene from the yeast *Metschnikowia fructicola* was cloned and characterized. The present work provides the clarification of the chitinase role in the antagonistic activity of the biocontrol agent *Metschnikowia fructicola* AP47.

Moreover, our results confirm that MfChi chitinase has an excellent antifungal activity to control *Monilinia* species, present as postharvest pathogens not only on stone fruits, but also onother fruits such as apples and pears. *Pichia pastoris* KM71 is a suitable strain for the expression of foreign chitinase genes, which could facilitate the development of a new cost-effective technique for large-scale production of recombinant chitinases for biocontrol of fungal postharvest pathogens of fruit.

This work shows that the chitinase MfChi could be developed as a postharvest treatment with antimicrobial activity for fruit undergoing a short shelf life, since it is able to keep its enzymatic activity for some days on the fruit surface and in the wounds. The storage conditions tested in the experiments are highly favourable to the development of brown rot, while when peaches are stored at cold storage temperature, the disease development is slower and the efficacy of chitinase could be for longer periods. Further work will aim at determining the best conditions of activity and stability of this enzyme to obtain the maximum efficacy against the pathogens.

Acknowledgements

This research was funded by the project “SafeFoodControl - Development of innovative systems and technologies for the production, storage, processing and valorization of Piedmontese fruit and vegetables” granted by the Piedmont Region.

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Supplementary Tables

Supplementary Table 1 Amino acid characterization of *MfChi* of *Metschnikowia fructicola* AP47.

Parameters**	MfChi	
Number of amino acids	365	
Molecular weight (kDa)	40.92	
Theoretical pI	5.27	
Amino acids and composition*****	Nr	Cp
Ala (A)	25	6.8%
Arg (R)	16	4.4%
Asn (N)	29	7.9%
Asp (D)	34	9.3%
Cys (C)	7	1.9%
Gln (Q)	15	4.1%
Glu (E)	13	3.6%
Gly (G)	27	7.4%
His (H)	4	1.1%
Ile (I)	10	2.7%
Leu (L)	31	8.5%
Lys (K)	24	6.6%
Met (M)	12	3.3%
Phe (F)	23	6.3%
Pro (P)	17	4.7%
Ser (S)	21	5.8%
Thr (T)	16	4.4%
Trp (W)	3	0.8%
Tyr (Y)	14	3.8%
Val (V)	24	6.6%

***** Nr: number of residues; Cp: composition (percentage) of each amino residue.

******The parameters of MpChi and MfChi were charachterized with ExPASy Proteomics Server at the website: http://www.expasy.org

Supplementary Table 2 Full name and accession number of the **c**hitinases used for the phylogenetic tree analysis of MfChi, retrieved from NCBI and UniProt database.

Abbreviation	**Full names of the chitinases**
B. bassiana endo-Chi	*Beauveria bassiana*, endochitinase gb|AAN41260.1|
B. circulans ChiA	*Bacillus circulans* chitinase A1 (chiA) gb|M57601.1|BACCHIA3
B. licheniformis Chi	*Bacillus licheniformis*, chitinase gene, gb|AY205293.1|
B. subtilis Chi	*Bacillus subtilis* chitinase (chi) gene, gb|AF069131.1|
C. albicans Chi	*Candida albicans*, chitinase gb|AAS66201.1|
C. albicans |S65110| Chi	*Candida albicans*, chitinase (EC 3.2.1.14) gb|S65110|
C. albicans SC5314 Chi	*Candida albicans* SC5314, chitinase ref|XP_719348.1|
C. albicans SC5314 Cht2	*Candida albicans* SC5314, chitinase Cht2 ref|XP_721807.1|
C. albicans SC5314 Cht2(2)	*Candida albicans* SC5314, chitinase Cht2 ref|XP_721966.1|
E. americana Chi	*Ewingella americana*, chitinase emb|X90562.1|
I. farinosa Chi	*Isaria farinosa*, chitinase gb|ABD64606.1|
I. fumosorosea endo-Chi	*Isaria fumosorosea*, bacterial-type endochitinase gb|AAX19146.1|
L. lecanii acidic-Chi	*Lecanicillium lecanii*, acidic chitinase gb|AAX56960.1|
L. lecanii basic-Chi	*Lecanicillium lecanii*, basic chitinase gb|AAV98691.1|
M. anisopliae Chi	*Metarhizium anisopliae*, chitinase gb|AAY32603.1|
M. flavoviride Chi	*Metarhizium flavoviride*, chitinase emb|CAB44709.1|
Malus x *domestica* class II CHTMA	*Malus* x *domestica*, class II chitinase (CHTMA) gb|HQ416905.1|
N. rileyi Chi	*Nomuraea rileyi* , chitinase |AAP04616.1|
N. tabacum endo-Chi	*Nicotiana tabacum*, Acidic endochitinase sp|P17514|CHIQ_TOBAC Q
O. sativa CHI11	*Oryza sativa* subsp. *japonica*, Chitinase 11 sp|Q10S66|CHI11_ORYSJ
S. cerevisiae endo-Chi 2	*Saccharomyces cerevisiae* endochitinase gb|AAA34539.1|
S. cerevisiae endo-Chi1	*Saccharomyces cerevisiae* endochitinase gb|AAA34538.1|
S. cerevisiae S288c Cts1p	*Saccharomyces cerevisiae* S288c, Cts1p ref|NP_013388.1|
S. cerevisiae RM11-1a endo-Chi	*Saccharomyces cerevisiae* RM11-1a, endochitinase gb|EDV08610.1|
S. cerevisiae YJM789 endo-Chi	*Saccharomyces cerevisiae* YJM789, endochitinase gb|EDN59372.1|
S. stipitis Chi	*Scheffersomyces stipitis* CBS 6054, chitinase ref|XP_001386607.2|
Streptomyces sp. ChiN	*Streptomyces* sp. ABRIINW 18 ChiN gene, gb|HM748586.1|
T. aureoviride endo-Chi	*Trichoderma aureoviride*, 42 kDa endochitinase gb|AY850032.1|
U. dioica endo-Chi	*Urtica dioica*, Lectin/endochitinase 1 sp|P11218| AGI_URTDI

Figure captions

Fig. 1 Relative expression levels (transcript accumulation) determined by Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) of the chitinase gene *MfChi* in *Metschnikowia fructicola* AP47 cultured with *Monilinia fructicola* CWP and without CWP (control) at each time point (6 h, 12 h, 24 h and 48 h). Values were normalized to the control at 6h arbitrarily set to unity. Vertical lines represent the standard error for an average of three biological replicates. Different letters above the columns indicated a significant difference determined by Duncan's Multiple comparison Test ($p < 0.05$).

Fig. 2 SDS-PAGE analysis of the recombinant chitinase expressed in *P. pastoris.* Supernatants of the yeast culture were taken from different isolates after 120 h of induction. Lanes: M: molecular weight marker (Precision Plus Protein Dual Color Standards, BIO RAD); 0: *P. pastoris* KM71 isolate transformed with pPIC9 (Control); 1, 2, 3 and 4: some transformed *P. pastoris* isolates with pPIC9-*MfChi.*

Fig. 3 Effect of the recombinant chitinase MfChi on spore germination (**A**) and germ tube length (**B**) of *M. fructicola* and *M. laxa* after 9 h and 18 h of incubation at 25 °C in potato dextrose broth medium. The chitinase was applied at 7 ng/µL (C1) and 70 ng/µL (C2). Treatments followed by different letters are statistically different following the Duncan's multiple range test ($p < 0.05$).

Fig. 4 Antifungal activity of the recombinant chitinase MfChi in controlling the decay development of *M. fructicola* and *M. laxa* in wound-inoculated peaches. The chitinase was applied at 7 ng/µL (C1) and 70 ng/µL (C2). The results are the mean of three independent experiments. Treatments followed by different letters are statistically different following the Duncan's multiple range test ($p < 0.05$).

Supplementary Fig. 1 Alignment of the deduced amino acid sequence of eight chitinase genes from yeasts. The data were retrieved from NCBI database: *Candida albicans* chitinase (S65110); *Candida albicans* chitinase (AAS66201.1); *Candida albicans* SC5314 chitinase (XP_719348.1); *Candida albicans* SC5314 chitinase Cht2 (XP_721807.1); *Candida albicans* SC5314 chitinase Cht2 (XP_721966.1); *Candida tropicalis* MYA-3404 chitinase 1 precursor (XP_002546283.1); *Saccharomyces cerevisiae* endochitinase (AAA34538.1); *Scheffersomyces stipitis* CBS 6054 chitinase (XP_001386607.2). Multiple sequence alignment of proteins was performed by using DNAMAN 7.0. Identical residues are highlighted in black background. The conserved region (LLSLGG and QFYNNYC) marked with asterisks were used to design the degenerate primers to amplify the partial sequence of chitinase genes from the antagonistic yeasts *Metschnikowia fructicola* strain AP47.

Supplementary Fig. 2 Nucleotide and deduced amino acid sequences of *MfChi*. The entire DNA sequence of *MfChi* is shown together with the corresponding amino acid sequence displayed below it. Nucleotides and amino acids are numbered on the left side of the sequence. The start codon (ATG) is underlined with a single line; the stop codon (TGA) is marked with an asterisk; the signal peptide is highlighted with the arrows; four putative *N*-glycosylation sites (NFSN, NLTN, NLTV and NLTN) are underlined with double lines, the chitinase catalytic activity site (DGYDFNME) is bolded and underlined with a single line, and the two repeated regions in the 3 prime terminal of the open reading frame (ORF) of *MfChi* are shadowed with grey colour.

Supplementary Fig. 3 Alignment of *MfChi* amino acids with those of chitinase genes from the yeasts, *Metschnikowia pulcherrima*, *Candida albicans*, *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* with DNAMAN 7.0 and CLASTALW. Chitinase genes for alignments were retrieved from NCBI database. Amino acids that are identical between MfChi and other sequences are shadowed with black color. Non-coding amino acids were shown in dashed line.

Supplementary Fig. 4 Alignment of the N-terminal regions of MfChi with those of other known yeast chitinases retrieved from NCBI and UniProt databases. Alignment was performed with DNAMAN 7.0 and CLASTALW. Identical residues are shadowed with black color. Non-coding amino acids are shown in dashed line. Numbers mean the position of selected peptide fragments starting from their corresponding start codons. Six cysteine residues highly conserved are marked with an asterisk. The chitinase family 18 active site is highlighted with a box. The proposed aspartic and glutamic catalytic residues are highlighted with a full-black triangle.

Supplementary Fig. 5 Phylogenetic analysis of MfChi and other chitinases from different microorganisms and plants. The amino acid sequences of other chitinases were retrieved from NCBI and UniProt database as seen in Supplementary Table 2. The phylogenetic tree of MfChi was generated using MEGA6 by neighbour-joining method. The numbers at node indicate the bootstrap percentages of 1000 resamples.