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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

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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 *Metschnikowia fructicola* AP47. Sequence analysis showed lack of introns, an open reading frame (ORF) of 1098 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 *P. pastoris* KM71 is a suitable microorganism for cost-effective large-scale production of recombinant chitinases.

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 the stone fruit (De Cal and Melgarejo, 1999; De Cal et al., 2009). *Monilinia 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 postharvest after the introduction of *Monilinia 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 grapes (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 an organic orchard in North Italy. Under semi-commercial conditions, *Metschnikowia*

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fructicola strain AP47 showed 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 (Masih and Paul, 2002; Smits et al., 2001; Zhang et al., 2011, 2012).

Cloning, expression and characterization of new chitinase genes from microorganisms are useful for exploring antagonistic 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-Patrick 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 analyze *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 *P. 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 *Monilinia 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 the 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 *M. 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.

The oligonucleotides, pGEM-T vector and *Escherichia coli* strain JM109 used in this study were purchased from Promega (Madison, WI, USA). The kits for DNA and RNA extraction (DNeasy and RNeasy), QIAquik PCR purification, reverse-transcript PCR, plasmid-extraction, QIAquik Gel Extraction and One Step RT-PCR 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). The *P. pastoris* KM71 strain used as host for transformations with the plasmid pPIC9 and *E. 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). An iScript cDNA Synthesis Kit and 2× Power SYBR 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 a 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 the 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 the 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 3000 ×*g* for 3 min. An aliquot of the supernatant (0.8 mL) was pipetted into a new sterile tube followed by the addition of 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 liter) at 25 °C for 48 h, then centrifuged at 5000 ×*g* for 10 min. DNA was extracted from the pellet with the DNeasy Extraction Kit (Qiagen), according to the manufacturer's instruction.

To clone the first partial sequence of the chitinase gene from genomic DNA of the *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 yeast chitinase genes deposited (Suppl. Fig. 1). After loading on an agarose gel, PCR products were purified with the QIAquik Gel Extraction Kit (Qiagen) according to the supplier's instructions, then ligated into the pGEM-T cloning vector (Promega), followed by transformation into chemically competent cells of *E. coli* strain DH5α (Invitrogen) and selection of positive transformants with a 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 the 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).