Available online at www.sciencedirect.com
ScienceDirect
Postharvest Biology and Technology xxx (2007) xxx–xxx
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide

W.J. Janisiewicz [a,*], I. Bastos Pereira [b], M.S. Almeida [b], D.P. Roberts [c], M. Wisniewski [a], E. Kurtenbach [b]

[a] *Appalachian Fruit Research Station, USDA-ARS, Kearneysville, WV, United States*
[b] *Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Instituto de Bioquímica Médica, Rio de Janeiro, R.J., Brazil*
[c] *USDA-ARS, Sustainable Agricultural Systems Laboratory, Beltsville, MD, United States*

Received 8 February 2007; accepted 10 June 2007

Abstract

Future expansion of biological control of postharvest diseases will depend largely on improving its effectiveness under a broader range of conditions and expanding its activity to new commodities and new diseases. Plasmid pGAPZαC/*Ps*d1, a binary vector encoding the constitutive expression of the gene for the pea defensin *Ps*d1, was used to transform the yeast *Pichia pastoris*, and transformed strains were evaluated for enhancing biocontrol potential by *Ps*d1. Two *P. pastoris* strains, X-33 and GS115, were successfully transformed by electroporation and produced the active r*Ps*d1 peptide. Nontransformed strain X-33 grew faster than strain GS115 in Golden Delicious apple wounds and was chosen as the host for plasmid pGAPZαC/*Ps*d1 in biocontrol tests. The severity and incidence of blue mold decay caused by *Penicillium expansum* were significantly reduced on apples treated with X-33(pGAPZαC/*Ps*d1/X-33) when compared to apples inoculated with this fungus alone or in combination with the nontransformed parental strain X-33, or the X-33(pGAPZαC/X-33) recombinant containing the empty binary vector. Four selected transformants reduced decay in repeated studies, but were effective only when applied at a lower (6.3×10^5 CFU mL^{-1}) cell concentration. This study demonstrates the potential of *Ps*d1 for enhancing suppression of postharvest diseases. However, the full potential of the *Ps*d1 defensin may be achieved after optimizing its expression and activity on the fruit.
Published by Elsevier B.V.

Keywords: Postharvest decay; Blue mold; Biological control; Defensins

1. Introduction

Biological control of postharvest decays (BCPD) of fruits has been used commercially for 10 years and the use of BCPD in agriculture has steadily increased and expanded to new commodities (Janisiewicz and Jeffers, 1997; Janisiewicz and Korsten, 2002). Biological control is currently used to control various decays on pome, citrus and stone fruits, avocado, seed potatoes, and sweet potatoes (Stockwell and Stack, 2007). The future expansion of BCPD will largely depend on improving its effectiveness under an increased range of conditions and on expanding the spectrum of activity to new commodities and new diseases. This may be accomplished with the discovery of new antagonists (Mercier and Jimenez, 2004), by combining antagonists with different mechanisms of biocontrol (Conway et al., 2005), by combining antagonists with other alternatives to synthetic fungicides (e.g. GRAS substances, heat or UV treatments) (Conway et al., 2004; El-Ghaouth et al., 2000; Janisiewicz et al., 2003; Obagwu and Korsten, 2002; Porat et al., 2002; Smilanick et al., 1999; Wilson et al., 1994), or by improving antagonists through genetic manipulation. Genetic manipulation offers tremendous potential for improving BCPD. For example, antagonists can be manipulated to over-express mechanisms of biocontrol, or foreign genes can be transferred to antagonists to increase tolerance to environmental stresses or to produce antifungal substances (Jones and Prusky, 2002; Wisniewski et al., 2005). In this way, it may be possible to convert microorganisms that can colonize fruit but do not exhibit antagonistic activity into biocontrol agents (Janisiewicz, 1998; Jones and Prusky, 2002).

Genes from edible portions of plant commodities, such as certain defensins, have particular appeal for use in genetic

* Corresponding author. Fax: +1 304 728 2340.
E-mail address: wojciech.janisiewicz@ars.usda.gov (W.J. Janisiewicz).

0925-5214/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.postharvbio.2007.06.010

manipulations of antagonists since consumers have been exposed to the gene product. Many plant defensins have been isolated and characterized over the past 15 years (Broekaert et al., 1995; Thomma et al., 2002). Plant defensins are small peptides consisting of 45–54 amino acids. These peptides are highly basic and rich in cysteines, presumably to stabilize structures through the formation of disulfide bonds. Defensins are not toxic to plants and are inhibitory to a variety of fungi in micromolar concentrations. These peptides are encoded by single genes and can be synthesized by the host with a minimal expenditure of energy (Thomma et al., 2002). In some cases the responsible genes have been characterized and cloned (Gao et al., 2000; Wisniewski et al., 2005). Defensins are found in a variety of organisms including plants, insects, humans and other mammals indicating that production of defensins is a common defense strategy (Broekaert et al., 1995; Raj and Dentino, 2002; Thevissen et al., 2004; Zhang et al., 2002).

Almeida et al. (2000) reported on the purification and characterization of two defensins from pea seeds (*Pisum sativum*), *Ps*d1 and *Ps*d2. The N-terminal sequence of *Ps*d1 had 95% identity with the N-terminus of the translated/putative the Disease Resistance Response Gene (DRR) 230-b reported by Hadwiger and co-workers (Chiang and Hadwiger, 1991; Fristensky et al., 1988) suggested that *Ps*d1 may be derived from the same or similar gene. Transcription of the DRR 230-b gene in pea pods increased rapidly during exposure to the pea pathogen *Fusarium solani* suggesting the participation of this gene in the plant defense mechanism. *Ps*d1 and *Ps*d2 constituted approximately 0.5% of the total protein content of the pea seed. These peptides were present in epidermal tissue and vascular bundles (Almeida et al., 2000). This, together with strong antifungal activity against a variety of fungi, including pea pathogens, suggests their involvement in plant defense mechanisms. High resolution NMR structural data of native *Ps*d1 indicates that the eight-cysteine residues in *Ps*d1 primary structure participate in the formation of the four-disulfide bridges characteristic of its βαββ tertiary structure (Almeida et al., 2002).

To obtain greater quantities of *Ps*d1 for further characterization of this peptide, Cabral et al. (2003) made *Ps*d1 cDNA from total RNA of wet pea seeds by RT-PCR, cloned the corresponding *Ps*d1 cDNA, and expressed it in the methylotrophic yeast *Pichia pastoris* (GS115). The *Ps*d1 peptide did not inhibit growth of *P. pastoris* even at 100 mg/L.

The heterologous expression system of the yeast *P. pastoris* has been successfully used for the production of a variety of microbial, plant and animal proteins (Cereghino and Cregg, 2000). This system has many advantages including (1) post-translational modifications such as the formation of disulfide bonds and glycosylation, (2) secretion of large quantities of heterologous protein into the culture medium, (Larentis et al., 2004), and (3) stable integration of the expression vector into the yeast genome (Cereghino and Cregg, 2000; Lueking et al., 2000).

Discrepancy in activity between the native protein, isolated from pea seeds, and the recombinant peptide r*Ps*d1 was detected and probably related to the presence of an additional STE13 protease cleavage signal at the N-terminus in r*Ps*d1 (Almeida et al., 2001; Cabral et al., 2003). The presence of this protease cleavage signal may have affected folding of the r*Ps*d1 peptide. To overcome this, *Ps*d1 cDNA was cloned directly in frame with a widely used secretion signal from *Saccharomyces cerevisiae* α-mating factor without the STE13 proteolytic signal cleavage sequence (Cabral et al., 2003). The result was the first heterologous expression of a fully active plant defensin in high-yield flask culture. The production of the r*Ps*d1 defensin on a large laboratory-scale was optimized, and purified r*Ps*d1 had antifungal activity comparable to the native *Ps*d1 for *Neurospora crassa* and *F. solani* (Larentis et al., 2004). All of these experiments were conducted using the pPIC9 vector, in which the heterologous protein is under the control of a methanol-induced AOX promoter. Although this resulted in a high level of production of the defensin in culture media, such transformants are useless for conducting experiments on plants because of the high toxicity of methanol to plants in general. To address this problem, Pereira (2006) transformed *P. pastoris* strain GS115, using the pGAPZαC vector, where the defensin expression is under the control of the constitutive GAP promoter.

In this paper, we report on the transformation of *P. pastoris* strain X-33 with pGAPZαC/*Ps*d1 and its use as a biocontrol agent for the control of apple decay caused by *Penicillium expansum*.

2. Materials and methods

2.1. Expression vectors

The binary pGAPZαC vector (Invitrogen, Carlsbad, CA), used in the biocontrol study, uses the glyceraldehyde-3-phosphate-dehydrogenase *GAP* promoter (P_{GAP}) to constitutively express recombinant proteins in *P. pastoris*. It contains the α-mating factor signal sequence from *S. cerevisiae* for excretion of the protein of interest. This vector was linearized with *Bsp*HI restriction enzyme before transformation to improve its integration into the *P. pastoris* genome via homologous recombination. Such integrants are very stable even in the absence of selective pressure. Selection for pGAPZαC is based on Zeocin resistance, which is inhibitory to both *P. pastoris* and *Escherichia coli*. The pGAPZαC vector does not contain a yeast origin of replication, thus yeast transformants with Zeocin resistance can only be isolated if pGAPZαC has been integrated into the *P. pastoris* genome (Cregg and Higgins, 1985; Cregg et al., 1989; Invitrogen).

2.2. P. pastoris strains

P. pastoris host strain X-33 is the wild-type and can be used for expression of recombinant proteins from vectors, such as pGAPZα, with selection based on Zeocin resistance. *P. pastoris* strain GS115 has a mutation in the gene encoding histidinol dehydrogenase (*his4*) which prevents synthesis of histidine. Strain GS115 can be used for the expression of recombinant proteins from vectors using HIS4 and/or Zeocin resistance selection.

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

2.3. Pathogen

The *P. expansum* isolate (MD-8) used in this study is an aggressive pathogen isolated from a decayed apple. The pathogen was grown on potato-dextrose-agar (PDA) and virulence maintained by periodic transfer through apple fruit. Conidial suspensions of 1×10^3 mL^{-1} and 5×10^3 conidia mL^{-1} used for fruit inoculation were prepared from a 10-days-old culture as previously described (Janisiewicz and Marchi, 1992).

2.4. Transformation of P. pastoris

P. pastoris GS115 and X-33 strains were transformed with pGAPZαC/*Ps*d1 plasmid as briefly described below (Pereira, 2006). An overnight culture of *P. pastoris* grown on YPD medium was diluted to $OD_{660\,nm}$ 0.7 and 250 mL of the culture incubated on a rotary shaker at 300 rpm until it reached $OD_{660\,nm}$ 1.34. The culture was washed five times in sterile cold water and the final pellet resuspended in 750 μL 1 M sorbitol. Plasmid pGAPZαC/*Ps*d1was linearized by digestion with *Bsp*HI, purified and added to the cell suspension at a final concentration of 5 μg DNA per 100 μL cells. Electroporation was performed in a Gene Pulser with pulse controller (Bio-Rad, Hercules, CA) in 0.2 cm gap cuvets, at 25 μF, 400 Ω, at 2.43 kV. Shocked cells were incubated in YPD broth for 1 h at 28 °C prior to selection for Zeocin resistance (100 mg/L). Cells were plated on YPDA with or without Zeocin and incubated for 72 h at 28 °C in darkness. Colony PCR using *Ps*d1 specific primers P1N′ (5′-CTC GAG AAA AGA AAG ACT TGC GAA CAC TTA GCT GAC ACC TAC AGG GGA GTA TCT TCA CG-3′) and C-PRI (5′-GGA ATT CCT AAC AGT TTT GAG TAC AGA AAC ACT TCC AGT TGT GAC A-3′) followed by agarose gel electrophoresis was performed to confirm the presence of *Ps*d1 in transformed strains (Cabral et al., 2003). The initial *P. pastoris* transformation was conducted with strain GS115 using Zeocin selection. However, this strain grew poorly in apple wounds and subsequent transformations were conducted with strain X-33.

2.5. Purification of Psd1 defensin

Four positive recombinant *P. pastoris* clones were analyzed for the amount of secreted *Ps*d1 through small-scale expression trials in 4-mL cultures. The highest *Ps*d1-expressing colony was grown in YPD medium in baffled 2 L Erlenmeyer flasks for 5 days and aliquots were collected every 24 h for *Ps*d1 content analysis on 18% SDS-PAGE. Purification of *Ps*d1 from *P. pastoris* pGAPZαC/*Ps*d1/GS115 recombinant was conducted with modifications as described by Almeida et al. (2001). The 5 days growth culture was harvested by centrifugation at 14,000 × *g* for 30 min, the supernatant was collected and protein content precipitated by adding ammonium sulfate at a rate of 1 g/min to 0–34% (F1) and 35–65% (F2) of saturation. The individual precipitated fractions were collected by centrifugation at 14,000 × *g* for 30 min and dissolved in 20 mL of distilled water. The F2 sample, enriched with *Ps*d1 as previously shown by Almeida et al., 2001, was subjected to gel filtration on a Sephadex G-50 Fine column (100-cm-long × 2.5-cm diameter) equipped with a Pharmacia LKB-FRAC-100 fraction collector set to collect 6.5 mL fractions. The $OD_{280\,nm}$ of the fractions was determined and fractions containing proteins were combined, freeze dried, and resuspended in 2.5 mL of water. Protein molecular mass was estimated by SDS-PAGE in 18% (w/v) polyacrylamide gels (Laemmli, 1970) stained with Silver Stain (Bio-Rad) and MALDI-TOF mass spectroscopy. Precision Plus Protein Standards were run as standards (Bio-Rad, Hercules, CA).

2.6. MALDI-TOF MS analysis of Psd1 sample

*Ps*d1 samples obtained from gel filtration on Sephadex G-50 Fine chromatography were co-crystallized with matrix (α-cyano-4-hydroxycinnamic acid 10 g/L) on a stainless steel target using 1:1 ratio between matrix and sample with 50% of acetonitrile and 0.3% of trifluoroacetic acid. A 4700 Applied Biosystems Voyager-DE time-of-flight mass spectrometer, operating in reflector mode, was used to obtain peptide mass value. Samples were externally calibrated with a peptide mixture of des-Arg-bradykinin (904.46), angiotensin I (1296.68), Glu ^{1}-fibrinopeptide B (1570.67), ACTH-(1–17) (2093.08), and ACTH-(18–39) (2465.19).

2.7. Inhibitory activity of Psd1 against P. expansum in vitro

Tests for inhibitory activity of *Ps*d1 against *P. expansum* were conducted in tissue culture plastic plates with membrane cylinder inserts as described previously (Janisiewicz et al., 2000) and on regular microscope slides covered with PDA containing 25 μL drops of *Ps*d1 and *P. expansum* conidia suspension (2×10^5 conidia/mL) side by side. The slides were incubated at 24 °C for 72 h and examined under a microscope after staining with lactophenol-cotton-blue. The concentration of *Ps*d1 ranged from 10 to 100 mg/L.

2.8. Growth of P. pastoris strains in apple wounds

To determine the suitability of *P. pastoris* for biocontrol tests on apples, the growth of nontransformed strains GS115 and X-33 in apple tissue was evaluated. Strains GS115 and X-33 were grown overnight in 250 mL Erlenmeyer flasks on a rotary shaker at 250 rpm at 28 °C, harvested by centrifugation at 7000 × *g*, resuspended in sterile tap water, and adjusted to a concentration of 50% transmittance (3×10^6 CFU mL^{-1}) at 420 nm. 'Golden Delicious' apples were wounded with a cylindrical tool and a piece of tissue 3-mm high × 3-mm diameter was removed. These wounds were inoculated with 25 μL of the yeast suspension. Fruit were placed on fruitpack trays in plastic boxes and one set of fruit was incubated at 24 °C and the other set at 1 °C. Samples for recovery of yeast were taken over time intervals up to 9 days on fruit stored at 24 °C and up to 28 days on fruit stored at 1 °C. Apple tissue that included wounded areas was removed with a cork borer (10 mm diameter) to a depth of 10 mm, placed in 4.5 mL phosphate buffer (pH 6.8) in Stomacher bags and extracted in a Stomacher 80 blender (Tekmar, Cincinnati, OH) for 2 min at normal speed. The resulting slurry was filtered through a syringe packed with glass wool, and the filtrate

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

dilution-plated on nutrient yeast dextrose agar (NYDA) medium (Janisiewicz, 1987) using an Automated Spiral Plater, Autoplate 4000 (Spiral Biotech, Inc. Norwood, MA). NYDA plates were incubated at 28 °C and colonies counted using a QCount colony counter system (Spiral Biotech). There were five replications per temperature × time treatment, with each replicate consisting of one apple. This experiment was performed after a preliminary experiment showed poor recovery of GS115 from apple wounds.

2.9. Biological control tests on apple

Yeasts were grown in NYDA medium overnight on a rotary shaker (200 rpm) at 26 °C. Cultures were harvested by centrifugation at 7000 × *g*, resuspended in sterile tap water, and the yeast concentration adjusted with a spectrophotometer to 80% (6.3×10^5 CFU mL^{-1}) and 50% (3×10^6 CFU mL^{-1}) transmittance at 420 nm. 'Golden Delicious' apples were placed on fruitpack trays in plastic boxes, wounded as described above, and the wounds inoculated with 50 μL of an aqueous suspension of the transformed or nontransformed yeast. After drying (~1 h) the wounds were inoculated with 25 μL of the conidial suspension of *P. expansum* and the fruit incubated at 24 °C. Decay development was recorded after 5 and 7 days. There were 111 transformants tested, and there were three replicates of three fruit each for every pathogen × antagonist concentration combination. The experiment was repeated with the 10 best performing transformants.

3. Results

3.1. Construction of recombinant P. pastoris and expression of Psd1

Both strains of *P. pastoris*, GS115 and X-33, transformed well with pGAPZαC and pGAPZαC/*Ps*d1. A total of 111 transformants were obtained. Colony PCR and subsequent agarose gel electrophoresis confirmed the presence of an amplified fragment of 159 bp corresponding to the *Ps*d1 in all putative pGAPZαC/*Ps*d1 transformants of both strains (Fig. 1).

The temporal expression and secretion into the culture medium of the 5.2 kDa *Ps*d1 by *P. pastoris* pGAPZαC/*Ps*d1GS115 was confirmed by purification of *Ps*d1 followed by SDS-PAGE. The result showed an expressive amount of a 5.2 kDa secreted peptide after the first 24 h of culture growth that increased with a longer expression periods of 48 and 72 h (Fig. 2).

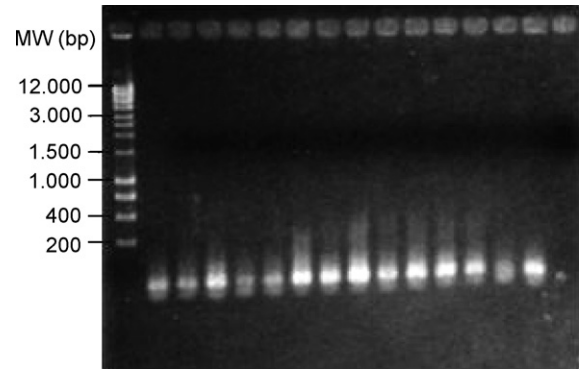

Fig. 1. PCR screening of 14 *Pichia pastoris* strains transformed with pGAPZαC/*Ps*d1 plasmid. The presence of the specific *Ps*d1-159 bp PCR amplicon was detected by 2% agarose gel electrophoresis (ethidium bromide staining), confirming the insertion of pGAPZ αC/*Ps*d1 plasmid to yeast genome. Lane 1: molecular markers and lane 16: PCR negative control.

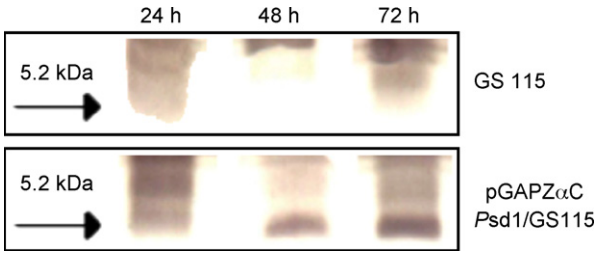

Fig. 2. Small-scale expression of *Ps*d1 protein from *Pichia pastoris* host strain GS115. Aliquots from 24, 48 and 72 h cultures (supernatant of the yeast culture media) were subjected to 18% SDS-PAGE gel electrophoresis and revealed by silver staining. Arrows indicate a single 5.2 kDa band corresponding to *Ps*d1 defensin. The recombinant peptide could be detected even in the first 24 h of cell growth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

The purification of *Ps*d1 on Sephadex G-50 Fine gel-filtration chromatography is shown in Fig. 3. Fractions corresponding to recombinant *Ps*d1 were identified by $OD_{280\,nm}$ at retention volume around 529 mL (Fig. 3, fraction F2) and confirmed by SDS-PAGE (see inset in Fig. 3). The position of these fractions

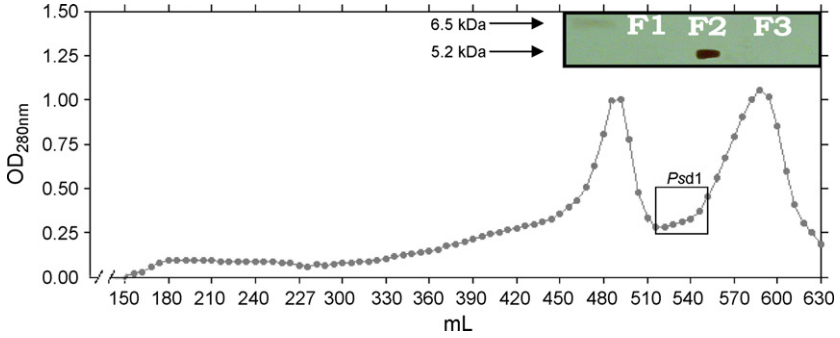

Fig. 3. Purification of *Ps*d1 defensin by gel filtration on a Sephadex G-50 Fine column. Fractions (F1, F2 and F3), indicated by the box, were submitted to SDS-PAGE electrophoresis for *Ps*d1 detection (inset). Arrows indicate 6.5 and 5.2 kDa protein bands compatible with aprotinin and *Ps*d1 peptides, respectively (silver staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

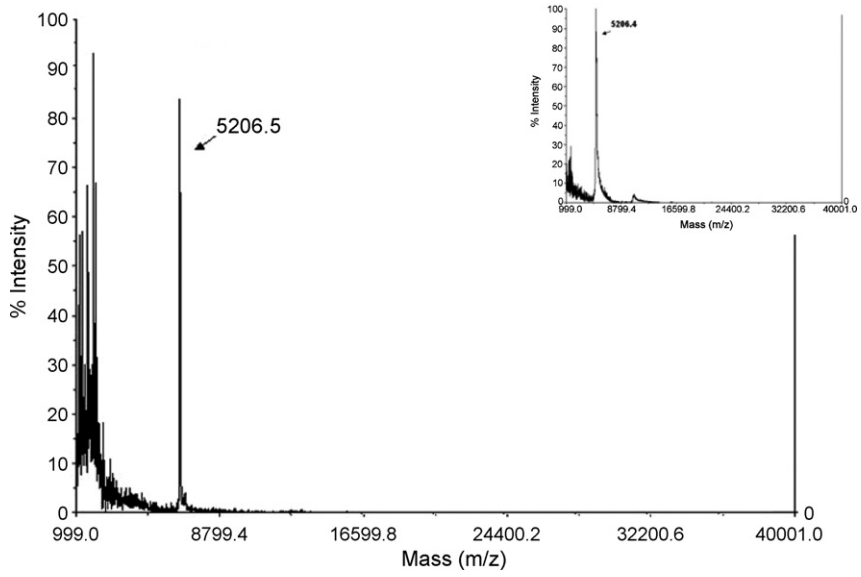

Fig. 4. MALDI-TOF mass spectra of the native (insert) and the recombinant *Ps*d1 sample purified from a Sephadex G-50 Fine column. A main peak of 5.2 kDa could be detected in both samples demonstrating that the recombinant and native proteins share the same molecular mass.

in relation to the other peaks was similar to the pattern reported by Cabral et al. (2003). Fraction F2 was subjected to mass spectrometry analysis, giving a main peak at *m/z* 5206.5 Da (Fig. 4) that is consistent with the peak obtained for native *Ps*d1 (Fig. 4, inset).

3.2. In vitro activity of Psd1

Neither test resulted in measurable inhibition of conidia germination or inhibition of hyphal growth for all strains used in biocontrol tests, although the hyphae subjected to *Ps*d1 defensins appeared to be thinner and often internally decompartmentalized as compared to the control treatments (data not shown).

3.3. Growth of nontransformed P. pastoris strains in apple

Populations of strain X-33 in apple wounds increased rapidly, by almost 3 log units, during the first 3 days after inoculation at 24 °C. This was followed by a 1 log decline and then a gradual increase during the rest of the experiment (Fig. 5). At 1 °C, the increases in populations were gradual and reached a maximum (∼1.2 log increase) after 21 days. In contrast, strain GS115 grew poorly and the populations declined by ∼2.5 log units 3 days after inoculation. This was followed by population increases, and by day 7 populations reached the original level. At 1 °C, the populations were stable and increased by no more than 0.3 log units half way through the experiment and by the end of the experiment were about 0.2 log units below the original level.

3.4. Biocontrol tests

The evaluation of transformed yeast strains for biocontrol activity against blue mold decay on apples revealed a broad range of biocontrol activity (data not shown). Further evaluation of the 10 best performing strains showed that only 4

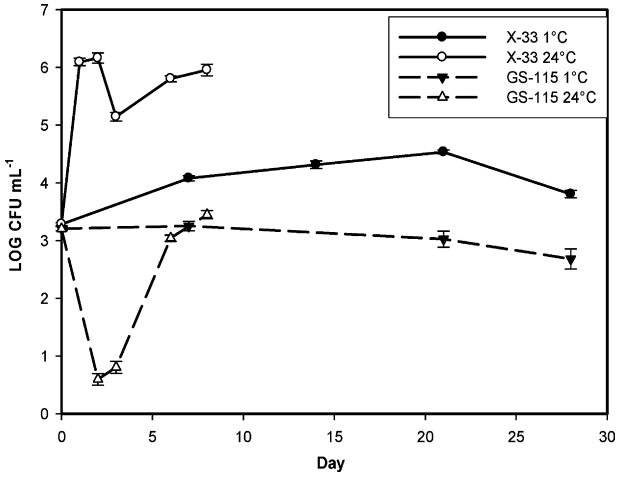

Fig. 5. Growth rate of *Pichia pastoris* strains X-33 and GS115 in wounds of 'Golden Delicious' apple. The fruit were wound-inoculated with both *P. pastoris* strains and stored on fruitpack trays in plastic boxes at 24 or 1 °C. Error bars represent standard error from the mean.

strains consistently controlled severity and incidence of decay on 'Golden Delicious' apple (Figs. 6 and 7) at 6.3×10^5 CFU mL^{-1} concentration of the antagonist, and 1×10^3 conidia mL^{-1} concentration of the pathogen. No reduction in decay severity or incidence was observed at the higher (5×10^3 conidia mL^{-1}) concentration of *P. expansum* pathogen inoculum. The wild-type strain, X-33, and X-33 transformed with vector but no *Ps*d1 gene did not reduce decay severity at either pathogen concentration. However, both had a lower incidence of decay than the control with the pathogen alone.

4. Discussion

The *P. pastoris* system has been very useful for the transformation of the pGAPZαC vector containing *Ps*d1 cassette and

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

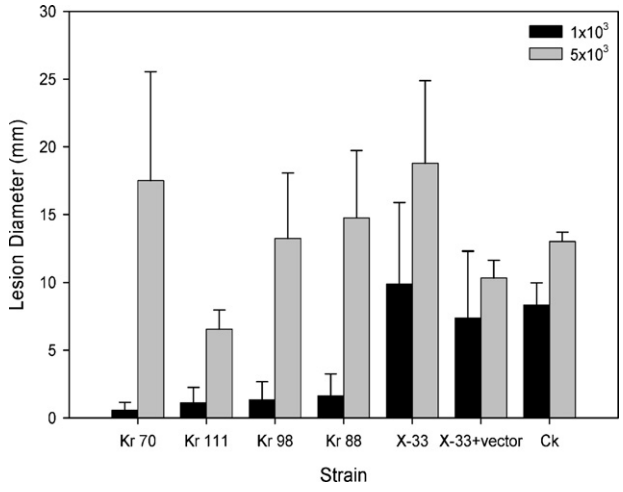

Fig. 6. Severity of blue mold decay caused by *Penicillium expansum* on 'Golden Delicious' apples inoculated with nontransformed wild-type *Pichia pastoris* X-33 or transformed with the *Ps*d1 defensin cDNA encoding sequence. The fruit were wound-inoculated with *Penicillium expansum* conidia alone (CK) or in combination with nontransformed *P. pastoris* cells (X-33), transformed with the empty pGAPZαC vector (X-33 + vector) or transformed with the vector containing *Ps*d1 cDNA sequence (colonies Kr 70, Kr 88, Kr 98, and Kr 111), and stored on fruitpack trays in plastic boxes at 24 °C for 5 days before evaluating for decay development. Error bars represent standard error from the mean.

expression of this defensin. Colony PCR confirmed the presence of the *Ps*d1 gene in all transformants. Interestingly, the level of resistance to Zeocin was similar among the Kr 70, Kr 88, Kr 98, and Kr 111 transformants in the agar Zeocin gradient (2000 mg/L) square 10 cm plates (Szybalski, 1952), where the growth streak of these strains was approximately 3 cm long, with no growth for the untransformed control (data not shown). This suggests a single copy of the expression cassette

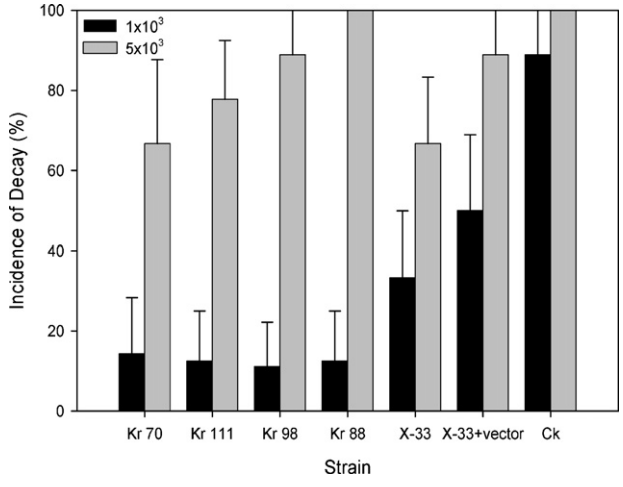

Fig. 7. Incidence of blue mold decay caused by *Penicillium expansum* on 'Golden Delicious' apples inoculated with the nontransformed wild-type *Pichia pastoris* X-33 or transformed with *Ps*d1 gene. The fruit were wound-inoculated with *P. expansum* conidia alone (CK) or in combination with untransformed *P. pastoris* cells (X-33), transformed with the empty pGAPZαC vector (X-33 + vector) or transformed with the vector containing *Ps*d1 cDNA sequence (colonies Kr 70, Kr 88, Kr 98, and Kr 111), and stored on fruitpack trays in plastic boxes at 24 °C for 5 days before evaluating for decay development. Error bars represent standard error from the mean.

with the *Ps*d1 defensin integrated into genome of the transformants (Cereghino and Cregg, 2000). The expression level of *Ps*d1 in *P. pastoris* under GAP promoter was about five times higher than under methanol inducible AOX1, which also provided a more economical approach for the mass production of *Ps*d1 (Waterham et al., 1997; Zhang et al., 2007). Purification of the *Ps*d1 defensin on Sephadex 50 Fine column and subsequent SDS-PAGE elecrophoresis and MALDI-TOF analysis confirmed the production of the 5.2 kDa *Ps*d1 defensin.

Strain X-33 is the parental strain for GS115. Poor growth of GS115, possibly due to histidine auxotrophy, and excellent growth of X-33 in apple wounds at both 1 and 24 °C temperatures, caused us to focus on the transformation of the X-33, and use these transformants in biocontrol tests. Biocontrol tests on apples against blue mold decay revealed significant differences in the effectiveness and consistency among transformants. Four transformants, Kr 70, Kr 88, Kr 98, and Kr 111 provided consistent control of blue mold at the lower inoculum level of the pathogen (1×10^3 conidia mL^{-1}) and the antagonist concentration of 6.3×10^5 CFU mL^{-1}. At the higher pathogen inoculum level, the pathogen was able to overcome any control provided by *P. pastoris* strains transformed with *Ps*d1 defensin. It is well established that there is a quantitative relationship between the concentration of the pathogen and the effectiveness of biocontrol, and as the concentration of the pathogen inoculum increases the effectiveness of the yeast in controlling fruit decay decreases (Janisiewicz, 1987). Also, in general, as antagonist concentration increases, at a given pathogen level, biocontrol also increases. Surprisingly, this was not the case with the four transformants used in this study, where their increase in concentration from 6.3×10^5 to 3×10^6 CFU mL^{-1}, at the same pathogen concentration (1×10^3 conidia mL^{-1}), resulted in less biocontrol (data not shown). This may have been caused by changes in growth dynamics in apple wounds where application of the higher concentration of the antagonist resulted in a shorter period of growth and less expression of the *Ps*d1 defensin.

Nontransformed wild-type *P. pastoris* X-33 and X-33 transformed with the vector alone significantly reduced decay indicating the potential of this yeast for biocontrol. This is not surprising because other *Pichia* spp. have been reported to control various diseases. For example, *P. guillermondi* controlled postharvest decays on citrus fruit (Droby et al., 1993) and *Botrytis cinerea* on strawberries in the field (Guetsky et al., 2002). *P. membranefaciens* controlled gray mold of grapevine (Santos and Marquinq, 2004) and postharvest Rhizopus rot of nectarines (Qing and Shiping, 2000), while *P. anomala* controlled spoilage in long-term storage of moist feed grain (Druvefors et al., 2002).

Developing new biocontrol agents against postharvest fruit diseases by genetically modifying microbes and using them as a delivery system for various biocontrol traits isolated from other microorganisms or other foreign genes responsible for antifungal activity have been suggested in the past (Janisiewicz, 1998). Efficient colonization of the site of entry for the pathogen (fruit wound) was considered to be a prerequisite for a potential microbe candidate. Jones and Prusky (2002) used this approach to make *S. cerevisiae* a biocontrol agent by cloning the gene for cecropin production, an antifungal defensin from insects. This

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

genetically modified yeast expressed the cloned gene and controlled postharvest decay caused by *Colletotrichum coccodes* on tomatoes. Wisniewski et al. (2005) cloned and expressed in *P. pastoris* a defensin gene isolated originally from peach bark that had antifungal activity against *B. cinerea* and *P. expansum in vitro*, the major postharvest pathogens of various fruits. The biocontrol of fruit decays by this transformant was not reported.

The use of antagonists producing defensins on consumable products, apart from other regulatory issues, will have to overcome the hurdles of potential mammalian toxicity and allergic reactions before approval can be anticipated. The main appeal of the *Ps*d1 defensin is that it was isolated from pea seeds that are part of the human diet and therefore the possibility of toxic or allergic reactions is less likely than from defensins originating from insects or from bark tissue. Nevertheless, all these cases confirmed the validity of this approach in improving biological control of fruit decays. The *Pichia* system has an additional appeal because this yeast has been approved as a dietary supplement to feed animals [58 FR 59170, November 8, 1993; 21 CFR Chapter 1 (4-1-02 Ed.) Section 573.750]. Optimization of *Ps*d1 expression under a variety of conditions (e.g. fruit wounds, storage conditions, etc.) appears to be the next major challenge in determining the commercial potential of this system.

Acknowledgements

We thank Roger Lewis and Tony Rugh for technical assistance in the transformation of *P. pastoris*, biocontrol tests and summarizing data.

References

Almeida, M.S., Cabral, K.M.S., Kurtenbach, E., Almeida, F.C.L., Valente, A.P., 2002. Solution structure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. J. Mol. Biol. 315, 749–757.

Almeida, M.S., Cabral, K.M.S., Zingali, R.B., Kurtenbach, E., 2000. Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds. Arch. Biochem. Biophys. 378, 278–286.

Almeida, M.S., Cabral, K.M.S., de Medeiros, L.N., Valente, A.P., Almeida, F.C.L., Kurtenbach, E., 2001. cDNA cloning and heterologous expression of functional cysteine-rich antifungal protein *Ps*d1 in the yeast *Pichia pastoris*. Arch. Biochem. Biophys. 395, 199–207.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., Osborn, R.W., 1995. Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 108, 1353–1358.

Cabral, K.M.S., Almeida, M.S., Valente, A.P., Almeida, F.C.L., Kurtenbach, E., 2003. Production on the active antifungal *Pisum sativum* defensin 1 (*Ps*d1) in *Pichia pastoris*: overcoming the inefficiency of the STE13 protease. Protein Expr. Purif. 31, 115–122.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45–66.

Chiang, C.C., Hadwiger, L.A., 1991. The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. Mol. Plant-Microbe Interact. 4, 324–331.

Conway, W.S., Leverentz, B., Janisiewicz, W.J., Blodgett, A.B., Saftner, R.A., Camp, M.J., 2004. Integrating heat treatment, biocontrol and sodium bicarbonate to reduce postharvest decay of apple caused by *Colletotrichum acutatum* and *Penicillium expansum*. Postharvest Biol. Technol. 34, 11–20.

Conway, W.S., Leverentz, B., Janisiewicz, W.J., Saftner, R.A., Camp, M.J., 2005. Improving biocontrol using antagonist mixtures with heat and/or sodium bicarbonate to control postharvest decay of apple fruit. Postharvest Biol. Technol. 36, 235–244.

Cregg, J.M., Higgins, D.R., 1985. Production of foreign proteins in the yeast *Picha pastoris*. Can. J. Bot. Supp. 73, 5981–5987.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G., Stillman, C.A., 1989. Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. Mol. Cell. Biol. 9, 1316–1323.

Droby, S., Hofstein, R., Wilson, C., Wisniewski, M., Fridlender, B., Cohen, L., Wiess, B., Daus, A., Timar, D., Chalutz, E., 1993. Pilot test of *Pichia guillermondii*: a biocontrol agent of postharvest diseases of citrus fruit. Biol. Control 3, 47–52.

Druvefors, U., Jonsson, N., Boysen, M.E., Schnurer, J., 2002. Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. FEMS Yeast Res. 2, 304–389.

El-Ghaouth, A., Smilanick, J.L., Wilson, C.L., 2000. Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. Postharvest Biol. Technol. 19, 103–110.

Fristensky, B.W., Hadwiger, L.A., Horovitz, D., 1988. cDNA sequences for pea disease resistant response genes. Plant Mol. Biol. 11, 713–715.

Gao, A.-G., Hakimi, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J., Rommens, C.M.T., 2000. Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. 18, 1307–1310.

Guetsky, R., Elad, Y., Shtienberg, D., Dinoor, A., 2002. Establishment, survival and activity of the biocontrol agent *Pichia guillermondii* and *Bacillus mycoides* applied as a mixture on strawberry plants. Biocontrol Sci. Technol. 12, 705–714.

Janisiewicz, W.J., 1987. Postharvest biological control of blue mold on apples. Phytopathology 77, 481–485.

Janisiewicz, W.J., Jeffers, S.N., 1997. Efficacy of commercial formulation of two biofungicides for control of blue and gray mold of apples in cold storage. Crop Prot. 16, 629–633.

Janisiewicz, W.J., 1998. Biocontrol of postharvest diseases of temperate fruits. Challenges and opportunities. In: Boland, G.J., Kuykendall, L.D. (Eds.), Plant-Microbe Interactions and Biological Control. Marcel Dekker, Inc., New York, pp. 171–198.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. 40, 411–414.

Janisiewicz, W.J., Leverentz, B., Conway, W.J., Saftner, R.A., Reed, A.N., Camp, M.J., 2003. Control of bitter rot and blue mold of apples by integrating heat and antagonist treatments on 1-MCP treated fruit stored under controlled atmosphere conditions. Postharvest Biol. Technol. 29, 129–143.

Janisiewicz, W.J., Marchi, A., 1992. Control of storage rots on various pear cultivars with a saprophytic strain of *Pseudomonas syringae*. Plant Dis. 76, 555–560.

Janisiewicz, W.J., Tworkoski, T.J., Sharer, C., 2000. Charactering mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. Phytopathology 90, 1196–1200.

Jones, R.W., Prusky, D., 2002. Expression of an antifungal peptide in Saccharomyces: a new approach for biological control of the postharvest disease caused by *Colletotrichum coccodes*. Phytopathology 92, 33–37.

Larentis, A.L., Almeida, M.S., Carbal, K.M.S., Medeiros, L.N., Kurtenbach, E., Coelho, M.A.Z., 2004. Expression of *Pisum sativum* defensin 1(*Ps*d1) in shaking flask and bioreactor cultivation of recombinant *Pichia pastoris* at different pHs. Braz. J. Chem. Eng. 21, 155–164.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 681–685.

Lueking, A., Holz, C., Gotthold, C., Lehrach, H., Cahill, D., 2000. A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. Protein Expr. Purif. 20, 372–378.

Mercier, J., Jimenez, J.I., 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. Postharvest Biol. Technol. 31, 1–8.

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010

Obagwu, J., Korsten, L., 2002. Integrated control of citrus green and blue molds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water. Postharvest Biol. Technol. 28, 187–194.

Pereira, I.B., 2006. Expressão heteróloga constitutiva da defensina de ervilha *Ps*d1 em *Pichia pastoris* e seu uso como bioantagonista no controle da doença de maçãs causada pelo fungo *Penicillium expansum*. M.S. Thesis. Universidade Federal do Rio de Janeiro, Instituto de Bioquímica Médica, Programa de Biologia Molecular e Biotecnologia, Rio de Janeiro, Brazil, 109 pp.

Porat, R., Daus, A., Weiss, B., Cohen, L., Droby, S., 2002. Effects of combining hot water, sodium bicarbonate and biocontrol on postharvest decay of citrus fruit. J. Hort. Sci. Biotechnol. 77, 441–445.

Qing, F., Shiping, T., 2000. Postharvest biological control of Rhizopus rot of nectarine fruits by *Pichia membranefaciens*. Plant Dis. 84, 1212–1216.

Raj, P.A., Dentino, A.R., 2002. Current status of defensins and their role in innate and adaptive immunity. FEMS Microbiol. Lett. 206, 9–18.

Santos, A., Marquinq, D., 2004. Kiler toxin of *Pichia membranefaciens* and its possible use as a biocontrol agent against mould diseases of grapevine. Microbiology 150, 2527–2534.

Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J., Michael, M.F., 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Dis. 83, 139–145.

Stockwell, V.O., Stack, J.P., 2007. Using *Pseudomonas* spp. for integrated biological control. Phytopathology 97, 244–249.

Szybalski, W., 1952. Gradient plate technique for study of bacterial resistance. Science 116, 46–48.

Thevissen, K., Warnecke, C.D., Francois, I.E.J.A., Leipelt, M., Heinz, E., Ott, C., Zahringer, U., Thomma, B.P.H.J., Ferket, K.K.A., Cammue, P.A., 2004. Defensins from insects and plants interact with fungal glucosylceramides. J. Biol. Chem. 279, 3900–3905.

Thomma, B.P.H.J., Cammue, B.P.A., Thevissen, K., 2002. Plant defensins. Planta 216, 193–202.

Wilson, C.L., El Ghaouth, A., Chalutz, E., Droby, S., Stevens, C., Lu, J.Y., Khan, V., Arul, J., 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Dis. 78, 837–843.

Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Gregg, J.M., 1997. Isolation of the *Pichia pastoris* glyceraldehydes-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186, 37–44.

Wisniewski, M.E., Bassett, C.L., Artlip, T.S., Janisiewicz, W.J., Norelli, J.L., Droby, S., 2005. Overexpression of a peach defensin gene can enhance the activity of post harvest biocontrol agents. Acta Horti. 682, 1999–2006.

Zhang, A.L., Zhang, T.Y., Luo, J.X., Chen, S.C., Guan, W.J., Fu, C.Y., Peng, S.Q., Li, H.L., 2007. Constitutive expression of human angiostatin in *Pichia pastoris* by high-density cell culture. J. Ind. Microbiol. Biotechnol. 34, 117–122.

Zhang, L., Yu, W., He, T., Yu, J., Caffrey, R.E., Dalmasso, E.A., Fu, S., Pham, T., Mei, J., Ho, J.J., Zhang, W., Lopez, P., Ho, D.D., 2002. Contribution of human α-defensin 1, 2 and 3 to the anti-HIV-1 activity of CD8 antiviral factor. Science 298, 995–1000.

Please cite this article in press as: Janisiewicz, W.J. et al., Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing *Ps*d1 antifungal peptide, Postharvest Biol. Technol. (2007), doi:10.1016/j.postharvbio.2007.06.010