Characterization of the Exoglucanase-Encoding Gene *PaEXG2* and Study of Its Role in the Biocontrol Activity of *Pichia anomala* Strain K

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ABSTRACT

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The *PaEXG2* gene, encoding an exo-β-1,3-glucanase, was isolated from the biocontrol agent *Pichia anomala* strain K. *PaEXG2* has the capacity for coding an acidic protein of 427 amino acids with a predicted molecular weight of 45.7 kDa, a calculated pI of 4.7, and one potential N-glycosylation site. *PaEXG2* was disrupted by the insertion of the *URA3* marker gene, encoding orotidine monophosphate decarboxylase in strain KU1, a uracil auxotroph derived from strain K. Strain KU1 showed inferior biocontrol activity and colonization of wounds on apples, compared to the prototrophic strain. Antagonism and colonization were recovered after the restoration of prototrophy by transformation with the *URA3* gene. Integrative transformation was shown to be mostly ectopic in strain K descendants (only 4% of integration by homologous recombination). *PaEXG2* disruption abolished all detectable extracellular exo-β-1,3-glucanase activity in vitro and in situ but did not affect biocontrol of *Botrytis cinerea* on wounded apples.

Additional keywords: biocontrol mechanisms, biological control, gray mold, hydrolytic enzyme, mycoparasitism, postharvest apples.

Pichia anomala (Hansen) Kurtzman strain K, a yeast isolated from the surface of apple, has been reported to have a high level of reliable biocontrol activity against *Botrytis cinerea* Pers.:Fr. (the gray mold agent) on wounded Golden Delicious apples (18). One of the prerequisites of the use of biocontrol agents is an understanding of their protection mechanism or mechanisms in order to suitably select, produce, formulate, register, and use these microorganisms.

The mode of action of *P. anomala* strain K has been investigated without being fully elucidated. Mycoparasitism by this yeast has been presumed to explain inhibition of *B. cinerea* on apples on the basis of the study of its hydrolytic enzyme system. Endo- and exo-β-1,3-glucanase activities, but no chitinolytic activities, were detected in culture filtrates of strain K. Specific activities were higher when *B. cinerea* cell wall preparation (CWP) constituted the sole source of carbon, rather than glucose or laminarin (16).

Exo-β-1,3-glucanases (or glucan 1,3-β-D-glucosidase, EC 3.2.1.58) act by successive hydrolysis of the β-*O*-glucosidic bonds at the nonreducing ends of 1,3-β-D-glucans, releasing a-glucose, whereas endo-β-1,3-glucanases (or glucan endo-1,3-β-D-glucosidase, EC 3.2.1.39) cleave inner bonds of the polymer chain, releasing oligosaccharides. β-1,3-Glucanase activities of strain K were found to degrade *B. cinerea* CWP, and the relative proportion of exolytic β-1,3-glucanase activity was higher on that substrate than on laminarin (16). Two bands of enzyme activity were detected in native polyacrylamide gels. The exo-β-1,3-glucanase presenting the highest specific activity (Exoglc1, named in this study PaExg2) was purified from culture filtrates of strain K grown on CWP. The enzyme showed an inhibitory effect on germ tube growth and conidial germination (up to 29% inhibition) of *B. cinerea,* causing morphological changes in germ tubes. Exo-β-1,3-glucanase activity was also detected in apple wounds treated with strain K. Overall results suggested that exo-β-1,3-glucanase activity may be involved in the protective effect of *P. anomala* strain K against *B. cinerea* (16,17).

The production of hydrolytic enzymes that degrade fungal cell walls has been widely reported as a mechanism of suppression of soilborne and foliar pathogens by some biocontrol agents, such as *Trichoderma* spp. (8), but only few reports focus on those enzymes in the biological control of postharvest diseases by antagonistic yeasts (6,9,16).

Our research aimed at providing insights into the mechanisms of the strain K protective effect against gray mold through the study of the PaExg2 enzyme. The chosen strategy involved the creation of mutants defective in the production of PaExg2 and observation of their efficacy in biocontrol.

MATERIALS AND METHODS

Strains and culture conditions. *P. anomala* strain K was isolated from an apple (18). It is a prototroph. Strain KH6 is a segregant of strain K obtained by ascus microdissection. All yeasts were stored on yeast extract–peptone–dextrose (YPD) agar (1) at 4°C. Cells suspensions were prepared after three successive subcultures at intervals of 24 h on YPD agar at 25°C. Yeast colonies were flooded with isotonic water (0.85% NaCl) and scraped with a glass rod, and the cell concentration was adjusted to the desired level as determined by optical density at 595 nm. When required, yeasts cells were grown on 5′-fluoroorotic acid (FOA) agar plates, SD minimal agar medium, or SD agar supplemented with 0.002% uracil (wt/vol) (SD agar + ura) (1).

B. cinerea strain V was isolated from a rotting strawberry and stored on oatmeal agar, consisting of 4.5% oatmeal (wt/vol) and 2% agar (wt/vol), at 4°C in the dark. It was cultivated on oatmeal agar at 21°C for 8 to 12 days with 16 h of light per day. Spore suspensions were obtained by flooding the culture with sterile water containing 0.05% Tween 20. Spores were dislodged with a glass rod. The spore suspension was filtered through sterile nylon gauze. The spore concentration was determined with a Bürcker cell. *B. cinerea* CWP was obtained by scaling up the method described by Jijakli and Lepoivre (16).

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Escherichia coli strains INVaF′ (TA Cloning Kit, Invitrogen, Groningen, The Netherlands), TOP10F′ (Zero Background Cloning Kit, Invitrogen), and XL1-Blue MRA and XL1-Blue MRA (P2) (Lambda EMBL3/*Bam*HI Vector Kit, Stratagene, Cambridge, U.K.) were used as hosts for the maintenance and propagation of phages and plasmids. Bacterial cultures were maintained and grown following the manufacturer's instructions and standard methods (30).

Standard DNA manipulations. Transformation of *E. coli,* plasmid preparation, DNA ligation, phage DNA extraction, plaque hybridization, and other DNA manipulations were accomplished by standard methods (30). Transformation of yeast cells was performed by an adapted lithium acetate–dithiothreitol–Tris-EDTA electroporation method (32) using an electric pulse at 0.75 kV, 25 μF, and 200 Ω. Double-stranded DNA sequencing by the dideoxy chain termination method (31) was performed with the ^{T7}Sequencing Kit as described by the manufacturer (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Isolation of yeast chromosomal DNA was carried out following Ausubel et al. (1), except that the disruption of cell walls by shaking with glass beads was prolonged to 45 min. DNA elutions from electrophoresis agarose gels were performed with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). A *P. anomala* strain K genomic library was constructed by ligating *Sau*3AI DNA fragments of 9 to 23 kb in the lambda EMBL3 phagic vector. Construction, packaging, titration, and amplification of the library were performed with the Lambda EMBL3/*Bam*HI Vector Kit and Gigapack II Gold Packaging Extract (Stratagene), following the manufacturer's instructions. For Southern blots, approximately 1 μg of digested yeast genomic DNA per lane was transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech Benelux) after electrophoresis by standard methods (30). Digoxigenin (DIG) labeling was achieved by polymerase chain reaction (PCR) amplification with the DIG Probe Synthesis Kit (Boehringer Mannheim, Freiburg, Germany), following the manufacturer's instructions. *PaEXG2* probes encompassed most of the gene-coding region (nucleotides 146 to 1,206). Phagemid pBluescript KS (Stratagene) was DIG-labeled with the DIG-High Prime Kit (Boehringer Mannheim). This probe was used to detect an inner region of the *PaEXG2* open reading frame (ORF) included in pZero (see "Construction of plasmids"). Hybridization with DIG-labeled DNA probes was performed with DIG Easy Hyb (Boehringer Mannheim) overnight at 42°C. Washes and detection of the probe were achieved with the DIG Luminescent Detection Kit (Boehringer Mannheim), as described by the manufacturer, with CDP-Star as the substrate of the chemiluminescent reaction.

Isolation of the *PaEXG2* gene. *PaEXG2*a, a probe specific for fungal genes encoding exo-β-1,3-glucanase, was isolated from strain K genomic DNA by PCR amplification with primers 3as and 7. Conserved regions were identified by multiple amino acid alignment of a number of known exo-β-1,3-glucanase genes. These regions were used to design the degenerated reverse primer, 3as (5′-GARTTRTCRAANCCRTTYTG-3′, where R = A + G, Y = C + T, and N = A + C + G + T), coding for the conserved region QNGFDN. The organisms used included the yeasts *Pichia angusta* (Q12626), *Kluyveromyces lactis* (Q12628), *Debaryomyces occidentalis* (Q12700), *Yarrowia lipolytica* (Q12725), *Saccharomyces cerevisiae* (P23776, P52911, and P32603), and *Candida albicans* (P29717) and a filamentous fungus, *Agaricus bisporus* (Q12539 and Q12540). The N terminus of the protein PaExg2, an exo-β-1,3-glucanase (previously referred as exoglc1) purified from strain K culture filtrate (17), has been sequenced by R. Wattiez (Université de Mons-Hainaut, Mons, Belgium). The degenerated forward primer, 7 (5′-GATAARTTTCGGGGNGTN-3′), corresponds to the amino acid sequence DKIRGV from the N terminus of PaExg2. Before PCR cycles, strain K genomic DNA was denatured at 94°C for 5 min. The following thermal cycling scheme was used for 40 reaction cycles: 94°C for 1 min, 45°C for 30 s, and 72°C for 30 s. A final 10-min elongation step at 72°C was performed at the end of the cycles. The *PaEXG2*a PCR product was cloned and sequenced. *PaEXG2*a was used as a P^{32}-labeled probe to isolate the corresponding full-length gene, *PaEXG2,* from the strain K genomic library in EMBL3 according to standard methods (30). A 2,173-bp fragment containing *PaEXG2* was sequenced on both strands.

Construction of plasmids. The plasmid pZ-URA (Fig. 1) was constructed by ligation of the 1,516-bp *URA3* gene at *Xba*I and *Apa*I sites of the plasmid pZero-1 (Invitrogen). *URA3* was amplified from strain K genomic DNA by the use of the primers ura1 (5′-CGCTCTAGAATCGGTGAAACAACGTTCAA-3′, forward) and ura2 (5′-CATGGGCCCGCGATTAGGTAATATTCAGG-3′, reverse), designed on the base of the *URA3* gene of *P. anomala* strain H-1 (26). Primers introduced an *Xba*I site and an *Apa*I site at the 5′ and 3′ ends of the amplicon. PCR was performed with the Expand High Fidelity PCR System (Boehringer Mannheim), after a denaturation step of 5 min at 94°C. The following thermal cycling scheme was used for 45 reaction cycles: 94°C for 30 s, 45°C (cycles 1 to 5) or 50°C (cycles 6 to 45) for 30 s, and 72°C for 45 s, followed by elongation for 8 min at 72°C.

Plasmid pORO (Fig. 1) was generated by ligation of the ORO PCR product at the *Eco*RI and *Xho*I sites of pZero. ORO, which spans the *URA3* ORF from bases 746 to 1,053, was amplified by the use of the plasmid pZ-URA as the template, the Expand High Fidelity kit, and the primers oro1 (5′-TGAGAATTCCTCAAAGAGCGGAAGCTCAC-3′, forward) and oro2 (5′-TCACTCGAGGCACCACCTGAATATTG-3′, reverse). The primers introduced an *Eco*RI site and an *Xho*I site at the 5′ and 3′ ends of the amplicon, respectively. After a denaturation step of 5 min at 94°C, the following thermal cycling scheme was used for 45 reaction cycles: 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min 30 s, followed by a 7-min elongation step at 72°C, performed at the end of the cycles.

Plasmid pURA-EXG2 (Fig. 1) was constructed as follows: EXG2, a 352-bp inner region of the *PaEXG2* ORF (nucleotides 344 to 696, Fig. 2), was amplified by PCR on phage clone DNA using the primers EXG2-1 (5′-CGCGGATCCTTGTTCGTATTCCAATCGG-3′, forward) and EXG2-2 (5′-ACGCTGCAGCCCATATCCAAGACAGAACC-3′, reverse), which added *Bam*HI and *Pst*I sites at the 5′ and 3′ ends of the amplicon, respectively. Amplification was carried out with the Expand High Fidelity kit and the following thermal cycling scheme for 45 reaction cycles: 94°C for 30 s, 45°C (cycles 1 to 5) or 50°C (cycles 6 to 45) for 30 s, and 72°C for 45 s, followed by a final 8-min elongation step at 72°C. The PCR product was finally ligated in the *Bam*HI and *Pst*I sites of the plasmid pZ-URA (Fig. 1).

Mitotic stability of mutants. The phenotypic mitotic stability of mutant strains was estimated by cultivation in 10 ml of YPD broth at 25°C, shaken at 120 rpm overnight to stationary phase. Flasks containing fresh YPD were inoculated with the overnight culture and grown at 25°C while being shaken at 120 rpm for several generations of nonselective growth. After a spectrophotometric determination of cell concentration, an estimated 1×10^9 CFU was collected by centrifugation and plated (three plates per mutant) on selective medium (SD agar for uracil auxotrophic mutants and FOA agar for *paexg2* mutants) as described by Boeke et al. (3). Dilutions of cells were also plated (three plates per dilution) on nonselective medium (SD agar + ura) to refine the estimate of the number of plated cells on selective medium. Reversion frequencies were calculated by dividing the number of colonies on the selective plate by the estimated number of plated CFU on nonselective medium.

Biocontrol assays. Golden Delicious apples were surface-disinfected with 10% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water, and wounded with a cork borer. Two wounds, 8 mm in diameter and 3 mm deep, were made at the equator of each fruit. Each wounded site was treated with 50 μl of a suspen-

sion of the biocontrol agent (yeast) or sterile distilled water (control). After drying, the fruits were stored in closed plastic boxes with a humid filter paper for 24 h at 21°C in the dark, and then 50 µl of a suspension of *B. cinerea* spores (1×10^6 spores per ml, or 5×10^4 spores per wound) was applied to the wound. The apples were stored under the same conditions until the mean diameter of lesions on the control reached about 30 mm (a period of about 7 days). The percentage of protection (*P*) was calculated as follows: $P = 100 \times (D_t - D_y)/D_t$, where D_t is the mean diameter of lesions on apples in the control group, and D_y is the lesion diameter in the presence of the biocontrol agent. Assays were arranged in a completely randomized design. The biocontrol activity of uracil auxotrophic mutants was assessed twice independently, with 5×10^4 yeast CFU per wound. pURA-EXG2 transformants and wild-type strains (K and its descendant KH6) were tested at 5×10^3, 5×10^4, and 5×10^5 yeast CFU per wound (one concentration per trial). Each concentration was assayed in three independent trials.

Exo-β-1,3-glucanase activity and growth rate measurements. A suspension of 2×10^6 yeast CFU per ml was used to inoculate 50 ml of yeast-nitrogen broth (YNB) (Difco Laboratories, Detroit, MI) (6.7 g/liter) supplemented with 0.2% CWP (wt/vol) (three independent cultures per yeast strain), which was incubated at 25°C in 100-ml flasks shaken at 100 rpm for 24 h. Three flasks were supplemented with water as a reference point. Cultures were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were filtered through an Acrodisc filter (0.45 µm) (Gelman Sciences, Ann Arbor, MI) and were stored in aliquots at –20°C. Cell concentrations were estimated by dilution plating of the culture on YPD agar plates (three plates per dilution). The plates were left at 30°C for 2 days. The growth rate was expressed as the number of generations produced in 24 h, calculated as follows: $G_{24h} = \log_2(C_{24h}/C_0)$, where G_{24h} is the number of generations between time 0 and time +24 h, C_{24h} is the number of CFU per ml after 24 h of cultivation, and C_0 is the number of CFU per ml at the time of inoculation. The whole assay was repeated three times.

Detection of the secretion of exo-β-1,3-glucanase in situ was performed as follows: apple wound sites, 18 mm in diameter and 4 mm deep on one side of the fruit (three wounds per apple, in a triangle), were inoculated with 0.45 ml of a suspension of 1×10^7 yeast CFU per ml or 0.45 ml of distilled water. Three apples (with a total of nine wounds) were used per treatment. After incubation for 24 h at 21°C, one of the three yeast-treated apples (three wounds) was inoculated with 0.45 ml of CWP (2 mg/ml), and a second apple (three wounds) was inoculated with 0.45 ml of a suspension of *B. cinerea* spores (1×10^6 spores per ml), the third apple receiving neither CWP nor spores (inoculated with yeast alone). The concentrations of yeast cells and *B. cinerea* spores were chosen so that gray mold development was totally inhibited. The apples were left at 21°C for 24 h, and each wounded site was rinsed with 1 ml of sterile water. The rinse water from the three wounds on the same apple were pooled and passed through an Acrodisc filter (0.45 µm). Samples were extensively dialyzed against 0.05 M potassium acetate buffer (pH 5.5) at 4°C. The experiment was conducted twice.

Exo-β-1,3-glucanase (EC 3.2.1.58) activity was determined by following the release of glucose monomers from laminarin, derived from *Laminaria digitata* (Sigma-Aldrich, Bornem, Belgium). A reaction mixture containing 100 µl of the sample in 1 ml of potassium acetate buffer (pH 5.5) supplemented with 2% laminarin and 0.02% NaN_3 was shaken at 120 rpm for 24 h at 37°C. The reaction was stopped by mixtures for 10 min. Glucose release was measured with a commercial Glucose HK Kit (Sigma) as described by manufacturer, on 100 µl of each reaction mixture (two measures per reaction mixture). One unit (U) of exo-β-1,3-glucanase activity was defined as the amount of enzyme releasing 1 µg of glucose equivalent per hour.

Measure of wound colonization. Colonization of apple wound sites by yeast strains was assessed by treating apple wounds (five wounds, 8 × 3 mm, at the equator of each fruit) with 5×10^4 yeast CFU. Three apples were used per strain. After drying of the applied drop (time 0), the apples were incubated at 21°C. At 0, 24,

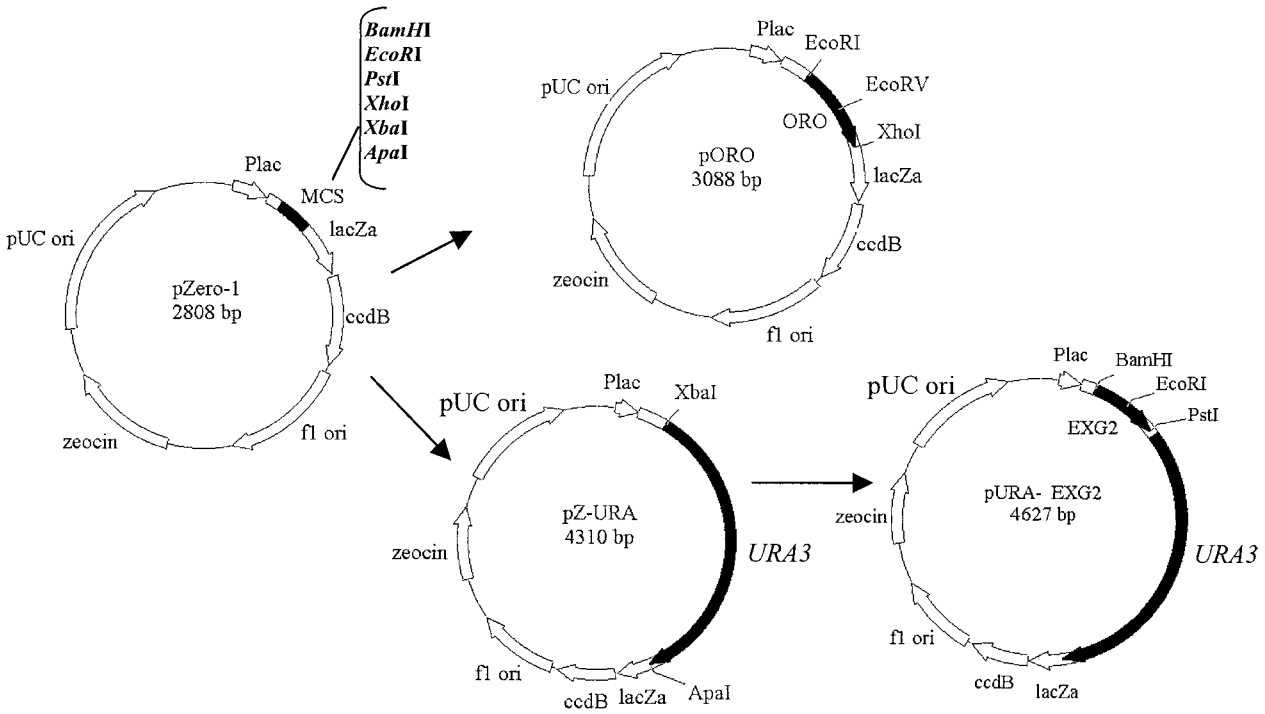

Fig. 1. Construction of the plasmids pORO, pZ-URA, and pURA-DEXG2. pORO was obtained by the insertion of the polymerase chain reaction (PCR) fragment ORO, derived from the *URA3* gene of *Pichia anomala* (nucleotides 746 to 1,053), at *Eco*RI and *Xho*I sites of plasmid pZero-1. pZ-URA was generated by cloning the *URA3* gene of *P. anomala* (coding for a orotidine-5′-phosphate decarboxylase) at *Xba*I and *Apa*I sites of pZero-1. Plasmid pURA-EXG2 was constructed by ligating the PCR fragment EXG2 (derived from the *PaEXG2* open reading frame coding for the exo-β-1,3-glucanase PaExg2, nucleotides 344 to 696), at the *Bam*HI and *Pst*I sites of pZ-URA.

48, 72, and 96 h, one wound per apple was cut with a scalpel and crushed with a pestle in 10 ml of distilled water. Then the washings from three wounds per strain were pooled. Dilutions of the juice were plated on YPD agar (three plates per dilution). The plates were incubated at 30°C for 2 days, and the colonies were then counted. The experiment was arranged in a completely randomized design and conducted three times.

Statistical analysis. SYSTAT 5.02 (Systat Software, Richmond, CA) was used for statistical analysis. The absence of interaction between replicates and treatment was tested before data from several assays were pooled for variance analysis. Fisher's test was used to compare means.

RESULTS

Features of *PaEXG2*. A PCR product, *PaEXG2*a (390 bp), obtained from *P. anomala* strain K genomic DNA showed a significant similarity, in both nucleotide and deduced amino acid sequences, with previously characterized yeast exo-β-1,3-glucanases. *PaEXG2*a used as probe to screen a strain K genomic library

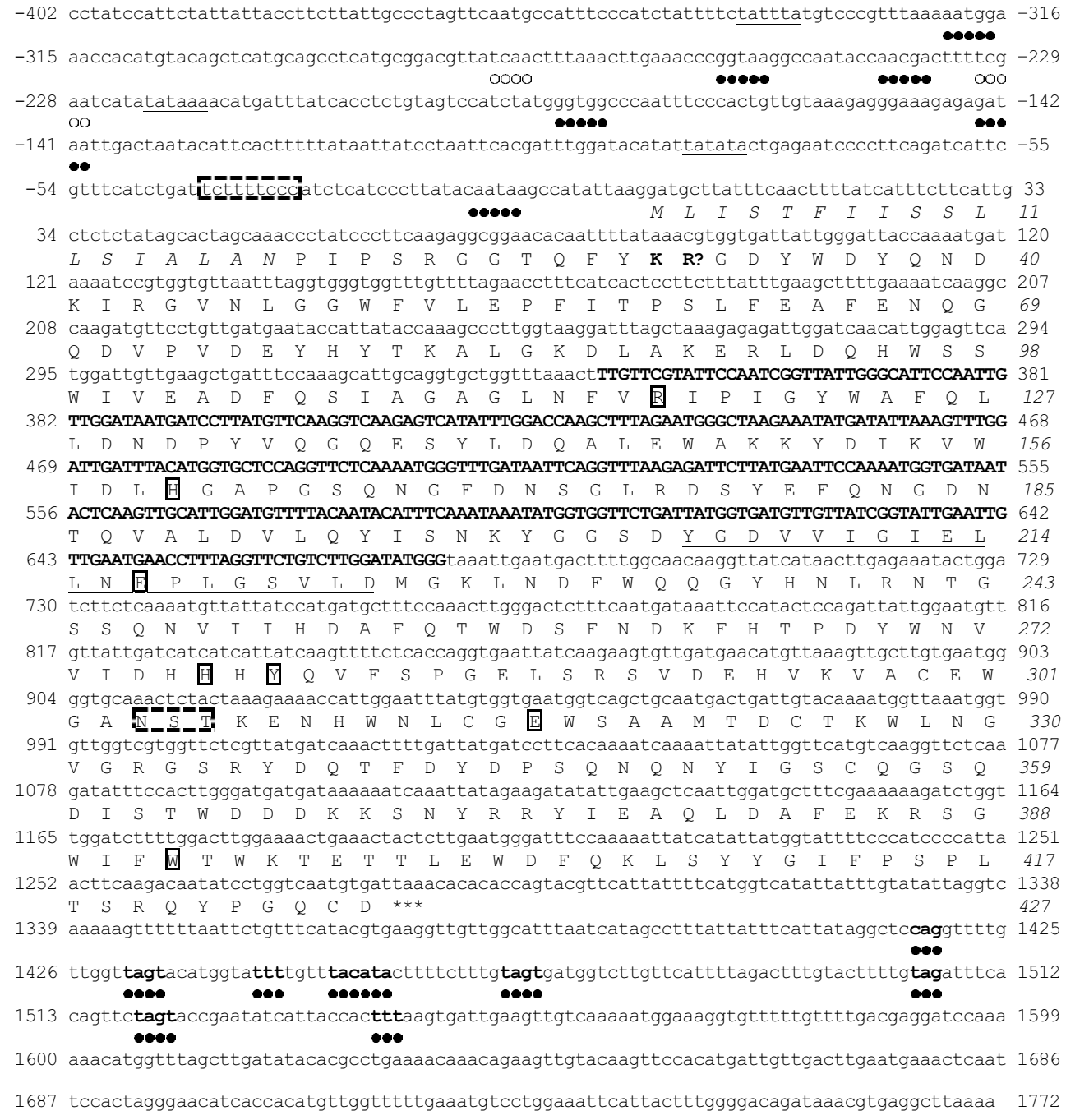

Fig. 2. Sequence of the *PaEXG2* gene from *Pichia anomala* strain K and its primary translation product (upper-case letters). In the 5′-flanking region, TATA-like elements are underlined, a CT box is boxed by a dotted line, and potential transcription initiation sites are indicated by open circles (TC(G/A)A motif) (28) or solid dots (RRYRR motif) (12). In the 3′-flanking region, the sequences matching the consensus transcription termination elements (TAG…TA(G)GT…TTT, TACATA, and AATAAA) are indicated by bold letters underlined with solid dots (2,35). Amino acids are numbered in italic on the right. Three stars indicate the stop codon. The putative signal peptide (25) is italicized, and a dibasic potential processing site for a KEX2-type endoproteinase is indicated by a question mark (5). The hatched box shows a potential N-glycosylation site, which in eukaryotic proteins occurs at the tripeptide sequences Asn-X-Thr and Asn-X-Ser, where X may be any amino acid, with the possible exception of aspartate (15). The glycosyl hydrolase family 5 signature is underlined. Strictly conserved amino acid residues on cellulases classified as family 5 glycosyl hydrolases are boxed. The EXG2 fragment used for disruption experiments in plasmid pURA-EXG2 is in uppercase bold letters.

allowed the isolation of the *PaEXG2* gene. The *PaEXG2* sequence is registered in the EMBL Nucleotide Sequence Database under accession number AJ222862.

The analysis of the nucleotide and amino acid sequences of *PaEXG2* is presented in Figure 2. A Kyte and Doolittle hydropathy profile (21), search for signal peptides (25), and recognition sequences for a Kex2-like protease (5) have been performed on the primary translation product. It suggests a mature protein of 45.7 kDa (pI = 4.68) secreted through entrance into the endoplasmic reticulum. The cleavage of the protein at the predicted site (K_{30}–N_{31}) is supported by the determined amino-terminal sequence of PaExg2, which starts just after that site.

The *PaEXG2* translation product exhibits similarities with exo-β-1,3-glucanases from diverse yeasts and the filamentous fungus *A. bisporus* (Table 1). Like all characterized yeast exo-β-1,3-glucanases, it presents the family 5 glycosyl hydrolases signature and the eight strictly conserved residues in all known family 5 cellulases thought to be responsible for the catalytic activity of those enzymes (Fig. 2) (13,14).

Isolation of uracil auxotrophic mutants. Disruption of the chromosomal copy of the orotidine-5′-phosphate decarboxylase gene (*URA3*), leading to uracil auxotrophy, was achieved by transformation of KH6, a segregant of strain K, with 100 ng of plasmid pORO (Fig. 1). Prior to transformation, pORO was linearized by *Eco*RV (inside ORO) to enhance integration into the *URA3* locus. Ten transformants were selected on FOA agar plates (after 4 days at 30°C). Their subsequent inability to grow without exogenous uracil was confirmed by streaking on both SD agar and SD agar + ura. The reversion frequency of the uracil auxotrophy was assessed after a first growth to saturation in YPD (nonselective medium) and a second cultivation for seven generations in fresh YPD. Mutant KU1 displayed a reversion frequency under 1.8×10^{-9}, which is suitable for the selection of transformants using *URA3* as the marker gene.

Disruption of *PaEXG2.* Preliminary Southern blot analysis of strain K and its descendants (10 strains) using various restriction enzymes and DIG-labeled *PaEXG2*a as a probe showed that *PaEXG2* is present as a single copy per segregant genome (data not shown). Plasmid pURA-EXG2 carries a fragment of *PaEXG2,* which codes for some of the conserved residues of family 5 glycosyl hydrolases, as shown in Figure 2, in view to disrupt the enzymatic activity by its integration in the unique *PaEXG2* locus by homologous recombination. Strain KU1 was electroporated with 100 ng of pURA-EXG2 linearized into EXG2. Transformants were selected on SD medium (after 7 days at 30°C) for expression of the *URA3* marker gene. Southern analysis of 49 potential pURA-EXG2 transformants (digested by *Pst*I) revealed only two mutants (a targeting efficiency of 4%) disrupted in *PaEXG2* (*paexg2* mutants B1-1 and B1-2). Profiles of the *paexg2* mutants are presented in Figure 3.

The stability of the disruption was assessed through the reversion of the uracil prototrophy of transformants B1-1 and B1-2 after an overnight culture in YPD (nonselective medium) followed by 10 generations in fresh YPD. No revertants were detected (the reversion rate of the *URA3* marker was under 10^{-9}). In apple wounds inoculated with 5×10^3 CFU per wound, yeast cells undergo fewer than 11 generations to reach the saturation level of 10^7 CFU per wound (17). Therefore, the probability of getting a population of reverting cells in the biocontrol assays was null.

Exo-β-1,3-glucanase activity in vitro. The disruption of *PaEXG2* (in the B1-2 mutant) abolished the production of detectable exo-β-1,3-glucanase activity in the culture filtrates of the yeast grown on CWP as the sole source of carbon, while its growth remained unchanged when compared to the parental prototrophic strain KH6 (Table 2). The secretion of exo-β-1,3-glucanase by strains K, KH6, and KU1 was directly correlated with growth,

TABLE 1. Sequence identities and similarities between PaExg2 and other fungal exo-β-1,3-glucanases[z]

	Identity (%)	Similarity (%)	Accession number
Agaricus bisporus (Exg1)	44	53	Q12539
Agaricus bisporus (Exg2)	48	61	Q12540
Candida albicans	65	71	P29717
Pichia angusta	61	67	Q12626
Kluyveromyces lactis	61	71	Q12628
Saccharomyces cerevisiae (Exg1)	58	66	P23776
Saccharomyces cerevisiae (Exg2)	42	53	P52911
Saccharomyces cerevisiae (Spr1)	55	64	P32603
Debaryomyces occidentalis	61	68	Q12700
Schizosaccharomyces pombe (Exg1)	44	52	T39282
Schizosaccharomyces pombe (Exgh)	33	43	Q10444
Trichoderma harzianum	30	39	O14402
Yarrowia lipolytica	45	59	Q12725

[z] The degree of relatedness between protein pairs is shown as the percentage of identity or similarity calculated from pairwise sequence alignment of the whole length of the primary translation products, with gaps to maximize identity and similarity. The calculation of the degree of similarity takes into account conservative substitutions.

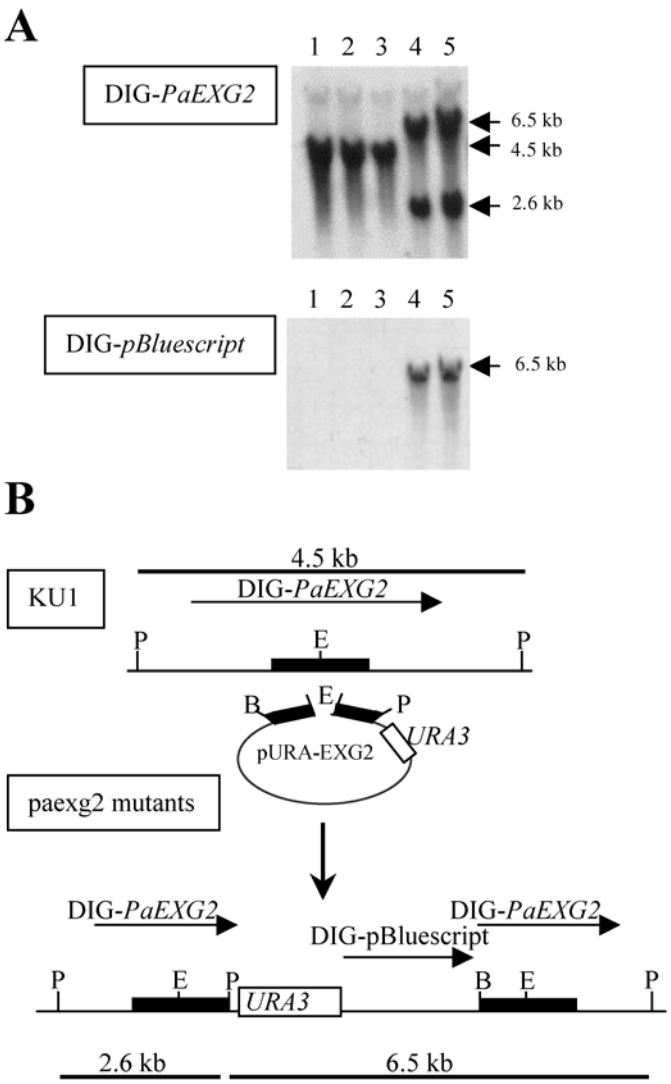

Fig. 3. A, Southern blot analysis of *Pst*I-digested genomic DNA of *Pichia anomala* probed with digoxigenin-labeled *PaEXG2* (DIG-*PaEXG2*) and digoxigenin-labeled pBluescript (DIG-pBluescript). Lanes 1, 2, and 3 are DNA from the parent strains K (diploid), KH6 (descendant of K), and KU1 (uracil auxotrophic derivative of KH6), respectively. Lanes 4 and 5 are DNA from the *paexg2* mutants B1-1 and B1-2, respectively. **B,** Integration pattern of the plasmid pURA-EXG2 by homologous recombination with the *PaEXG2* locus. The solid box indicates the targeting fragment EXG2. E = *Eco*RI; B = *Bam*HI; P = *Pst*I; S = *Ssp*I.

expressed as the number of generations during a 24-h cultivation (r = 0.999). When compared to the activity of the prototrophic strain KH6, the total amount of exo-β-1,3-glucanase activity in the supernatants was lower for KU1, which displays reduced growth, and double for the diploid strain K (Table 2).

Colonization of apple wounds. Inoculated at the same concentrations (5×10^4 CFU per wound), all the prototrophic strains (K, KH6, and B1-2) gave rise to about five generations at the wound site during the first 24 h of growth, whereas the uracil auxotroph KU1 produced only slightly more than two generations (Fig. 4). After 72 h, all yeast strains reached their saturation level, and cellular densities of prototrophic strains (around 10^7 CFU per wound) were about four times higher than KU1 density.

Exo-β-1,3-glucanase activity in situ. As shown in Figure 5, no exo-β-1,3-glucanase activity was detected in apple wounds inoculated with the *paexg2* mutant B1-2 alone or in the presence of CWP. When spores of *B. cinerea* were applied to wounds not treated with yeast or treated 24 h before with the *paexg2* mutant, a similar limited activity was extracellularly produced.

TABLE 2. Exo-β-1,3-glucanase activity and growth rate of *Pichia anomala* strains K (diploid prototroph), KH6 (strain K segregant, prototroph), KU1 (uracil auxotroph derived from KH6), and B1-2 (*paexg2* mutant derived from KU1, prototroph) grown with *Botrytis cinerea* cell wall preparation as the sole source of carbon

	Exo-β-1,3-glucanase activity[x]		
Strain[w]	U/(10^6 CFU)	U/ml	G_{24h}[y]
H_2O	NA[z]	0.00 f	NA[z]
K	0.69 b	7.93 c	3.1 g
KH6	0.87 b	4.12 d	1.3 h
KU1	0.83 b	2.87 e	0.7 i
B1-2	0.00 a	0.00 f	1.6 h

[w] Yeast was replaced by H_2O in the control. Yeasts (at an inoculum concentration of 2×10^6 CFU per ml) were grown at 25°C for 24 h with 0.2% cell wall preparation.

[x] Activities were measured by the release of free glucose (glucose-hexokinase method) from laminarin at 37°C, pH 5.5, during 24 h. One unit (U) of exo-β-1,3-glucanase activity is the amount of enzyme releasing 1 µg of glucose equivalent per hour. Activities are means of three independent trials, expressed in units of 1×10^6 CFU or 1 ml of supernatant. In each column, means followed by the same letter are not significantly different (Fisher's test at $P \leq 0.01$).

[y] Growth rates under the same conditions, expressed as the number of generations (G_{24h}) produced by each strain during a 24-h cultivation. Means followed by the same letter are not significantly different (Fisher's test at $P \leq 0.01$).

[z] Not applicable.

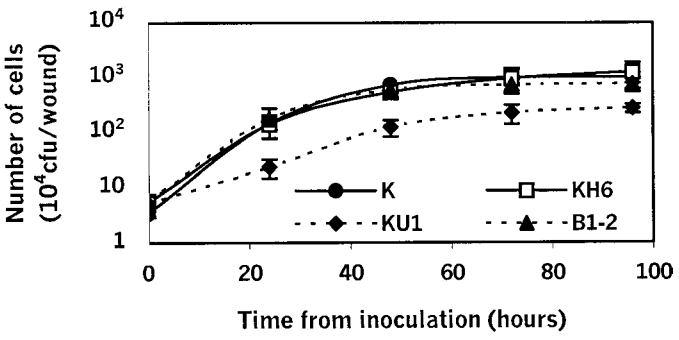

Fig. 4. Colonization of apple wounds by yeast strains. *Pichia anomala* strain K is a diploid prototroph; KH6 is a prototroph descended from strain K; KU1 is a uracil auxotrophic mutant of KH6; and B1-2, a prototroph derived from KU1, is disrupted in *PaEXG2*. About 5×10^4 CFU of each yeast was deposited in each wounded site (three wounds per strain). Populations were determined at 24-h intervals over 4 days at 21°C. The assay was repeated three times independently. The vertical bars represent standard errors.

Activities detected in wounds treated with strains K, KH6, and KU1 were higher than in the control wounds and equivalent between strains in the same treatment (yeast alone, yeast + CWP, and yeast + pathogen spores). Increased activity was observed for all these strains when inoculation was followed 24 h later by treatment of the wound with CWP or spores of *B. cinerea* (except for inoculation with KH6 in the presence of spores).

Biocontrol activity. Whatever the concentration of yeast strains applied at the wound sites, the disruption of *PaEXG2* had no detectable effect on the biocontrol of *B. cinerea* under the experimental conditions, either when the yeast treatment preceded inoculation with the pathogen by 24 h or when wounds were coinoculated with the yeast and the pathogen (Table 3). In contrast, in inoculation at 5×10^4 CFU per wound 24 h before challenge with *B. cinerea*, KU1 showed no protective activity (no significant difference from that of the H_2O control).

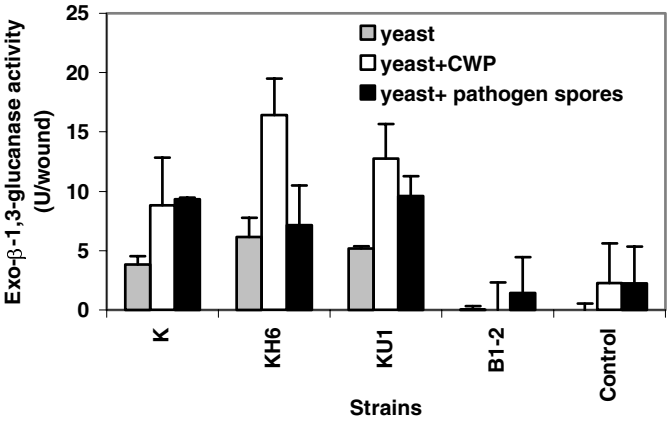

Fig. 5. Exo-β-1,3-glucanase activity, in units (U) per wound, in apple wounds treated with (i) yeast alone, (ii) yeast followed 24 h later by *Botrytis cinerea* cell wall preparation (CWP), or (iii) yeast followed 24 h later by *B. cinerea* spores. *Pichia anomala* strain K is a diploid prototroph; KH6 is a prototroph descended from strain K; KU1 is a uracil auxotrophic mutant of KH6; and B1-2, a prototroph derived from KU1, is disrupted in *PaEXG2*. Controls were included in which wounds were inoculated with water instead of yeast for each treatment. Enzymatic activity was detected in water used to rinse the the wounds 48 h after treatment with yeast or water. Exo-β-1,3-glucanase activity was detected through the release of free glucose from laminarin at 37°C and pH 5.5 over 24 h by the glucose-hexokinase method. One unit (U) of exo-β-1,3-glucanase activity was defined as the amount of enzyme releasing 1 µg of glucose equivalent per hour. The narrow vertical bars represent standard errors.

TABLE 3. Protection of wounded apples against gray mold by wild-type *Pichia anomala* strains (strain K and its descendant KH6) and a mutant strain (B1-2) disrupted in *PaEXG2*

	Percentage decrease in lesion diameter (compared to a control without yeast) at yeast inoculum concentration (CFU per wound) of[y]			
Strain	5×10^3 (24 h)	5×10^4 (24 h)	5×10^5 (24 h)	5×10^5 (coinoculation)
H_2O[z]	0.0 a	0.0 a	0.0 a	0.0 a
K	61.5 c	86.1 b	95.3 c	72.7 c
KH6	27.0 b	84.5 b	83.2 bc	50.3 bc
B1-2	34.2 b	86.6 b	94.6 c	62.1 bc

[y] Apples were inoculated with 5×10^3, 5×10^4, or 5×10^5 yeast CFU per wound, 24 h before inoculation with *Botrytis cinerea* (5×10^4 spores per wound), or with 5×10^5 yeast CFU per wound in coinoculation with *B. cinerea*. One yeast concentration was tested per trial. Five apples (a total of 10 wounds) were tested for each yeast. Each yeast concentration was tested three times independently, except the concentration of 5×10^5 CFU per wound in both types of inoculation, which was tested once. In each column, mean percentages followed by the same letter are not significantly different (Fisher's test, $P \leq 0.01$).

[z] Yeast was replaced by H_2O in the control.

DISCUSSION

PaExg2, the strain K exo-β-1,3-glucanase coded by the gene *PaEXG2,* showed consistency with the overall conservation of exo-β-1,3-glucanases among yeasts. It is an acidic protein, as are its counterparts previously extracted from cell-free extracts, cell walls, or culture filtrates of many yeasts. It belongs to the family 5 glycosyl hydrolases, as do all molecularly characterized yeast exo-β-1,3-glucanases (7,10,11,23,24,33). The glucanolytic system of *P. anomala* has features in common with the well-studied system of *S. cerevisiae.* The *S. cerevisiae EXG1* gene codes for a polypeptide of which differential glycosylation accounts for the two main extracellular exo-β-1,3-glucanases (ExgIa and ExgIb) with different K_m values and substrate specificities detected in culture supernatants of exponentially growing cells (24). The extracellular secretion of PaExg2 has been experimentally observed. Jijakli and Lepoivre (16) found two exo-β-1,3-glucanases with different electrophoretic mobilities in *P. anomala* strain K culture filtrates when grown with CWP as the sole source of carbon. *PaEXG2* could then code for two enzymatic forms with different glycosylation patterns on the unique potential site of PaExg2. The abolishment of exo-β-1,3-glucanase activity in vitro after disruption of the *PaEXG2* gene under the conditions in which two exo-β-1,3-glucanases were observed by Jijakli and Lepoivre (16) gives further support to this hypothesis.

The absence of any detectable exo-β-1,3-glucanase activity in vivo, in apple wounds treated with the *paexg2* mutant only, strongly suggests that the exo-β-1,3-glucanase activities detected for wild-type strains in vitro and in vivo have the same origin, the *PaEXG2* gene. It also strongly suggests that the major part of the activity detected in wounds treated with strains with an active *PaEXG2* gene can be attributed to PaExg2, while only a residual activity seems to be produced by apple or by the pathogenic spores, as observed in wounds treated with *B. cinerea* spores (free of yeast or treated with the *paexg2* mutant). The possibility that apple tissue challenged with *B. cinerea* spores produces this limited activity is strengthened by the observation of a similar level of activity in wounds treated with CWP, although pathogenesis-related plant glucanases are mostly endo-acting. Why the activity observed in wounds treated solely with CWP is not observed in wounds treated with CWP and the *paexg2* mutant is still unknown.

Biochemical studies (16) have provided presumptions of the role of exo-β-1,3-glucanases in the protective activity of *P. anomala* strain K against *B. cinerea* on stored apples. Under our experimental conditions by a strategy using *PaEXG2*-disrupted mutants, the extracellular production of that activity has been shown to be dispensable for the biocontrol effect. To our knowledge, this is the first report on the use of the disruption strategy on *P. anomala.* However, inactivation of all exo-β-1,3-glucanase-encoding genes would be required to conclude that enzymes with that substrate specificity are not implicated in the biocontrol.

It has been suggested that exo-β-1,3-glucanases play a role in yeast morphogenesis (apical growth, branching, budding, mating, and the formation and release of ascospores), the mobilization of glucan for use as a fuel, and the hydrolysis of exogenous material for uptake as a nutrient (22). Disruption of *PaEXG2* expression did not affect the growth of the yeast either in vitro or in situ. The *PaEXG2* gene is therefore dispensable for growth when CWP is the carbon source in vitro or in apple wounds.

P. anomala strain K has been shown to efficiently protect pears and citrus against *B. cinerea* and *Penicillium* (Jijakli, *personal communication*). The implication of *PaEXG2* in the inhibition of those pathogens on those hosts should not be excluded. Illustrating this in preharvest conditions, *Trichoderma harzianum* strain P1 provided equivalent biocontrol of *Pythium ultimum* on bean seed, better biocontrol of *Rhizoctonia solani* on bean seed, and reduced biocontrol of *B. cinerea* on bean leaves after disruption of the endochitinase-encoding *ech42* gene, in comparison with the wild-type strain (34).

The use of mutant KU1 confirmed that hampered wound colonization can be related to lower biocontrol activity, as has already been shown (16,17). The direct effect or negative effects of (i) the inactivation of the *URA3* housekeeping gene in particular and (ii) reduced wound colonization in general on the production of substances possibly involved in biocontrol remains to be established. However, this argues for the hypothesis that *P. anomala* strain K inhibits *B. cinerea* by competition for nutrients or physical preemptive exclusion. Wound colonization and biocontrol activity were restored when a functional *URA3* gene was reintroduced in the strain (*paexg2* mutants), showing that the *URA3* disruption procedure had no side effects on the observed characters.

Gene targeting by homologous recombination is a powerful tool, which can be used to generate precise mutations in the genome of an organism of interest. While the technique is used with high efficiency in yeasts such as *S. cerevisiae* (29) and *S. pombe* (19), it is more difficult to achieve with a number of other yeasts. Ogata et al. (27) showed that *P. anomala* strain HU-1 was able to undergo gene targeting by homologous recombination, as was observed for strain K descendants in our work. However, strain K derivatives exhibited a great tendency to integrate transforming DNA in an ectopic manner, rendering targeted disruption drudgery. This phenomenon had not previously been reported for *P. anomala* and could then be specific to strain K descendants, although a locus effect, as observed by Krawchuk and Wahls (20) in *S. pombe,* should not be ruled out. Very recently, Bundock et al. (4) overcame such a problem by using T-DNA from *Agrobacterium tumefaciens* as an efficient tool for gene targeting in *K. lactis* (92% of homologous recombination using a T-DNA construct, against 18% by electroporation with a plasmid DNA). This procedure would certainly be of interest for strain K transformation.

The construction of a mutant defective in exo-β-1,3-glucanase production has been very informative in the investigation of the *P. anomala* strain K glucanolytic system and its implications for biocontrol. But this study also emphasizes the difficulty of raising relevant hypotheses concerning the mode of action of biocontrol agents, the importance of an uncontroversial demonstration of the mode of action in situ, and the power of genetic engineering to reach that goal. However, residual levels of exoglucanase activity in wounds treated with *paexg2* mutants in the presence of *B. cinerea* spores could deserve further investigation as long as their origin is not elucidated. It could be due to possible compensation effects, stemming from gene disruption. This could explain the conservation of biocontrol activity, although one could wonder if these enzyme levels are not too low, especially if the "aggressive" nature of glucanase as it is the case of a mycoparasite behavior is considered. The same levels of activity were detected when only spores of *B. cinerea* were present in wounds. This shows that apple or pathogenic spores produced exoglucanase activity that could quantitatively suffice to explain the observed residual activity. This prompts us to reconsider our disruption strategy after genomic or proteomic studies of the host in the pathogen–biocontrol agent system.

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LITERATURE CITED

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, L. G., Smith, J. A., and Strill, K. 1991. Current Protocols in Molecular Biology. Green Publishing Associates and Wiley-Interscience, New York.
2. Bennetzen, J. L., and Hall, B. D. 1982. The primary structure of the *Saccharomyces cerevisiae* genes for alcohol dehydrogenase. J. Biol. Chem. 257:3026-3031.

3. Boeke, J. D., Lacroute, F., and Finx, G. R. 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-Fluoro-orotic acid resistance. Mol. Gen. Genet. 197:345-346.
4. Bundock, P., Mróczek, K., Winkler, A. A., Steensma, H. Y., and Hooykaas, P. J. J. 1999. T-DNA from *Agrobacterium tumefaciens* as an efficient tool for gene targeting in *Kluyveromyces lactis.* Mol. Gen. Genet. 261:115-121.
5. Bussey, H. 1988. Proteases and the processing of precursors to secreted proteins in yeast. Yeast 4:17-26.
6. Castoria, R., De Curtis, F., Lima, G., and De Cicco, V. 1997. β-1,3-Glucanase activity of two saprophytic yeast's and possible mode of action as biocontrol agents against postharvest diseases. Postharvest Biol. Technol. 12:293-300.
7. Chambers, R. S., Broughton, M. J., Cannon, R. D., Carne, A., Emerson, G. W., and Sullivan, P. A. 1993. An exo-β-(1,3)-glucanase of *Candida albicans*: Purification of the enzyme and molecular cloning of the gene. J. Gen. Microbiol. 139:325-334.
8. Chet, I., Benhamou, N., and Haran, S. 1998. Mycoparasitism and lytic enzymes. Pages 137-160 in: *Trichoderma* and *Gliocladium*: Enzymes, Biological Control and Commercial Applications. Vol. 2. G. E. Harman and C. P. Kubicek, eds. Taylor and Francis, London.
9. Droby, S., Robin, D., Chalutz, E., and Chet, I. 1993. Possible role of glucanase and extracellular polymers in the mode of action of yeast antagonists of postharvest diseases. (Abstr.) Phytoparasitica 21:167.
10. Esteban, P. F., Casarégola, S., Vazquez de Aldana, C. R., and Del Rey, F. 1999. Cloning and characterization of the *EXG1* gene from the yeast *Yarrowia lipolytica.* Yeast 15:1631-1644.
11. Esteban, P. F., Vasquez de Aldana, C. R., and Del Rey, F. 1999. Cloning and characterization of 1,3-β-glucanase-encoding genes from nonconventional yeasts. Yeast 15:91-109.
12. Hahn, S., Hoar, E. T., and Guarente, L. 1985. Each of three 'TATA elements' specifies a subset of the transcription initiation sites at the *CYC-1* promoter of *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA 82: 8562-8566.
13. Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280:309-316.
14. Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P., and Davies, G. 1995. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc. Natl. Acad. Sci. USA 92:7090-7094.
15. Hubbard, S. C., and Ivatt, R. J. 1981. Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 50:555-583.
16. Jijakli, M. H., and Lepoivre, P. 1998. Characterization of an exo-β-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88:335-343.
17. Jijakli, M. H., Lepoivre, P., and Grevesse, C. 1999. Yeast species for biocontrol of apple postharvest diseases: An encouraging case of study for practical use. Pages 31-49 in: Biotechnological Approaches in Biocontrol of Plant Pathogens. K. G. Mukerji, B. P. Chamola, and R. K. Upadhyay, eds. Kluwer Academic, New York.
18. Jijakli, M. H., Lepoivre, P., Tossut, P., and Thonard, P. 1993. Biological control of *Botrytis cinerea* and *Penicillium* on postharvest apples by two antagonistic yeasts. Meded. Fac. Landbouwwet. Rijksuniv. Gent 58: 1349-1358.
19. Keeney, J. B., and Boeke, J. D. 1994. Efficient targeted integration at leu1-32 and ura4-294 in *Schizosaccharomyces pombe.* Genetics 136: 849-856.
20. Krawchuk, M. D., and Wahls, W. P. 1999. High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. Yeast 15:1419-1427.
21. Kyte, J., and Doolittle, R. F. 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. 157:105-132.
22. Larriba, G., Basco, R. D., Andaluz, E., and Luna-Arias, J. P. 1993. Yeast exoglucanases: Where redundancy implies necessity. Arch. Med. Res. 24:293-299.
23. Muthukumar, G., Suhng, S.-H., Magee, P. T., Jewell, R. D., and Primerano, D. A. 1993. The *Saccharomyces cerevisiae SPR1* gene encodes a sporulation-specific exo-1,3-β-glucanase which contributes to ascospore thermoresistance. J. Bacteriol. 175:386-394.
24. Nebreda, A. R., Villa, T. G., Villanueva, J. R., and del Rey, F. 1986. Cloning of genes related to exo-β-glucanase production in *Saccharomyces cerevisiae:* Characterization of an exo-β-glucanase structural gene. Gene 47:245-259.
25. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10:1-6.
26. Ogata, T., Iimura, Y., Obata, T., and Tamura, G. 1992. Isolation and nucleotide sequence of *Hansenula anomala URA3* gene encoding orotidine-5′-phosphate decarboxylase. J. Ferment. Bioeng. 74:352-357.
27. Ogata, T., Okumura, Y., Iimura, Y., and Obata, T. 1995. Development of an integrative transformation system for the yeast *Hansenula anomala.* J. Ferment. Bioeng. 79:1-5.
28. Ponticelli, A. S., and Struhl, K. 1990. Analysis of *Saccharomyces cerevisiae his3* transcription in vitro: Biochemical support for multiple mechanisms of transcription. Mol. Cell. Biol. 10:2832-2839.
29. Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast. Methods Enzymol. 194:281-301.
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
31. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA-sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
32. Thompson, J. R., Register, E., Curotto, J., Kurtz, M., and Kelly, R. 1998. An improved protocol for the preparation of yeast cells for transformation by electroporation. Yeast 14:565-571.
33. Vasquez de Aldana, C. R., Correa, J., San Segundo, P., Bueno, A., Nebrada, A. R., Mendez, E., and Del Rey, F. 1991. Nucleotide sequence of the exo-1,3-glucanase-encoding gene, *EXG1*, of the yeast *Saccharomyces cerevisiae.* Gene 97:173-182.
34. Woo, S. L., Donzelli, B., Scala, F., Mach, R., Harman, G. E., Kubicek, C. P., Del Sorbo, G., and Lorito, M. 1999. Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol. Plant-Microbe Interact. 12:419-429.
35. Zaret, K. S., and Sherman, F. 1982. DNA sequence required for efficient transcription termination in yeast. Cell 28:563-573.