Identification of Differentially Expressed Genes by cDNA-Amplified Fragment Length Polymorphism in the Biocontrol Agent *Pichia anomala* (Strain Kh5)

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ABSTRACT

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cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was used to identify genes potentially involved in biological control, by strain Kh5 (*Pichia anomala*), of *Botrytis cinerea*, an important postharvest pathogen on apples. Strain Kh5 was grown in yeast nitrogen base (YNB) plus glucose (G medium) or YNB plus cell walls of *B. cinerea* (B medium). Thirty-five primer pairs were used in AFLP amplifications, resulting in a total of more than 2,450 bands derived from the mRNA of strain Kh5 grown in B medium. Eighty-six bands (3.5%) corresponded to genes upregulated in B medium compared with G medium. Of these 86 bands, 28 were selected, cloned, sequenced, and subjected to real-time reverse transcription-polymerase chain reaction (RT-PCR) to confirm their differential expression. An appropriate housekeeping gene, G2, was selected and used to normalize the results of RT-PCR. Eleven genes presented an increased gene expression in the presence of *B. cinerea* cell walls (expression ratio > 1). Statistical analysis showed a significant increase for 5 of these 11 genes. The overexpressed genes show homologies to yeast genes with various functions, including β-glucosidase, transmembrane transport, citrate synthase, and external amino acid sensing and transport. Some of these functions could be related to cell wall metabolism and potentially involved in mycoparasitic properties.

Since the early 1970s, fruit losses due to postharvest diseases have reached up to 25% (23). Chemical treatments are still widely used to control diseases caused by postharvest pathogens, but the appearance of fungicide-resistant strains, the deregistration of fungicides, and public concerns about health and environmental impact may limit the future application of fungicides and increase the need to develop alternative protection methods such as biological control. In this context, the yeast *Pichia anomala* (E.C. Hansen) Kurtzman (strain K) has been isolated from the surface of apples (cv. Golden Delicious) and selected for its strong and reliable ability to antagonize *Botrytis cinerea* Pers.Fr., one of the most devastating pathogens of harvested apples (26). The antagonistic activity of *P. anomala* strain K was maintained during pre- and postharvest practical applications of industrial fermented cells (24).

Understanding the mechanisms of action of a biocontrol agent is a prerequisite to its practical application, because this allows a more rational mode of production and a formulation enhancing its action. Various mechanisms, based mainly on competition for space and nutrients, mycoparasitism, antibiosis, and elicitation of plant defenses, are reported to contribute simultaneously or sequentially to the biocontrol properties of microorganisms (23). Jijakli et al. (27) suggested that competition for space and nutrient is involved in the biological control of *B. cinerea* by *P. anomala* strain K. In contrast, *P. anomala* strain K did not induce defense responses of the apple and did not produce detectable antibiotic or killer toxin (M. H. Jijakli, *unpublished data*). Using biochemical methods, Jijakli and Lepoivre (25) isolated an exo-β-1,3-glucanase from culture filtrate from *P. anomala* strain K growing on cell wall preparations of *B. cinerea*. This enzyme showed in vitro a strong inhibitory effect on germ tubes of *B. cinerea*. Moreover, addition of cell wall preparation to a suspension of strain K stimulated both in situ exo-β-1,3-glucanase activity and protective activity against the pathogen. Based on these results, the hypothesis of an involvement of mycoparasitism in biocontrol properties of *P. anomala* strain K against *B. cinerea* was formulated.

Genetic studies have also been undertaken to help build knowledge on the antagonistic mechanisms of *P. anomala* strain K and its segregants (21). The gene coding for the exo-β-1,3-glucanase previously studied (25) was isolated, sequenced, and disrupted. The disruption abolished all extracellular exo-β-1,3-glucanase activity in vitro and in situ, and recent results suggest an effect on the biocontrol properties of the strain (D. Friel, *personal communication*). These results strengthen the hypothesis of an involvement of mycoparasitism in the biological control of *B. cinerea* by *P. anomala* strain K. However, this strategy is focused on one or two genes, whereas biocontrol properties, e.g., mycoparasitism, often depend on numerous genes, with their respective regulatory pathways and mutual interactions. For example, at least 18 genes are thought to be involved in mycoparasitic properties of the biocontrol agent *Trichoderma virens* (41). In this context, a deeper investigation is required to identify other genes involved in *B. cinerea* cell wall degradation by *P. anomala* strain K and putatively related to mycoparasitism.

Techniques displaying differential gene expression can address this drawback, as they shed light on the complexity of gene expression and gene interactions. Techniques, such as differential display, RNA arbitrarily primed (RAP)-polymerase chain reaction (PCR), or suppressive subtractive hybridization (reviewed in literature citation 20) have been developed for this purpose. These approaches have become highly popular and have led to the identification of new genes in various agriculture-related domains, including plant development (47), plant defense responses (7), plant

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pathogen germination and development (11), and plant–pathogen interactions (36). Differential display is now being applied in the biological control field. It recently allowed the identification of an aspartyl protease from *Trichoderma asperellum* (45) and genes differentially expressed during nonself fungal–fungal interactions of the decomposing basidiomycete *Physisporinus sanguinoletus* (22). Compared with differential display, cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (5), a nonbiased technique based on PCR amplification, offers the advantage of higher reproducibility (16). Additionally, it is more sensitive than hybridization-based techniques and is also highly specific (10). However, differential expression of genes corresponding to cloned cDNA-AFLP fragments has to be confirmed by an independent technique. For this purpose, real-time PCR offers several advantages over other commonly used techniques such as northern blotting or reverse northern blotting: it has a higher sensitivity, necessitates less starting material, and is a less time-consuming procedure.

The aim of this study was to identify genes of a biocontrol agent, *P. anomala* strain Kh5, overexpressed in the presence of *B. cinerea* cell walls and potentially involved in its biocontrol properties. cDNA-AFLP was used to compare gene expression in strain Kh5 using either *B. cinerea* cell walls or glucose as the sole carbon source. Overexpression of the isolated cDNA fragments was confirmed by reverse transcription (RT)-PCR and the corresponding deduced amino acid sequences were run against the Universal Protein Resource (Uniprot) database (Uniprot database is available online) to obtain preliminary information about the functions of these genes.

MATERIALS AND METHODS

Strains and media. Strain Kh5 is a segregant of *P. anomala* strain K (21). It displays the same biocontrol properties (*unpublished data*) and the same chromosomal pattern as strain K (14). Strain Kh5 was grown at 25°C in an Erlenmeyer flask containing 50 ml of a medium consisting of yeast nitrogen base ([YNB] 0.65%, wt/vol) (Becton Dickinson & Co., Sparks, MD) and glucose (0.2%, wt/vol) (Merck, Darmstadt, Germany). Cells were harvested after 8 h, washed with sterile water, and plated on petri dishes containing YNB supplemented with either 1% glucose or 1% *B. cinerea* cell walls. *B. cinerea* cell walls were prepared by five successive rounds of crushing and washing followed by ultrasonication, washing, and lyophilization (details provided in literature citation 25). Cells were harvested with a platinum wire in sterile isotonic solution (NaCl, 0.85%, wt/vol) after 16 h and total RNA was extracted.

RNA extraction. The total RNA extraction protocol described previously by Ausubel et al. (3) was applied, with slight modifications during cell lysis. The cells (10^8 cells by sample) were resuspended in 400 μl of RNA buffer. An equal volume of phenol/chloroform/isoamylic alcohol (25:24:1) and glass beads was added. Cells were lysed by vortexing the mix during 20 × 1 min (with 30 s on ice between each vortexing). RNA extracts were DNase-treated for 30 min at 37°C with 20 units of RNase-free DNase (Roche, Basel, Switzerland) in the presence of 40 units of RNase inhibitor (Roche).

cDNA-AFLP. Double-stranded cDNA was synthesized from 15 μg of total RNA according to the instructions for the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The double-stranded cDNA was digested with *Eco*RI and *Mse*I, ligated with *Eco*RI and *Mse*I adaptors, and pre-amplified with Eco (5′-GACTGCGTACCAATTC-3′) and Mse (5′-GATGAGTCCTGAGTAA-3′) primers following the instructions of the AFLP analysis system kit for microorganisms (Invitrogen). After 10-fold dilution of the PCR fragments, specific amplifications were carried out with combinations of an Eco primer and an Mse primer containing, respectively, one and two additional bases at the 3′ end. The Eco primers were labeled with [γ^{33}P] dATP. Amplification products were separated by electrophoresis at 100 W on a vertical denaturing polyacrylamide gel (6%) containing 7 M urea. Gels were dried on Whatman 3MM paper before autoradiography.

Isolation of cDNA fragments. cDNA-AFLP fragments of interest were recovered as described previously (6) and amplified by two rounds of PCR with the selective primers used to generate the corresponding cDNA-AFLP profile.

Cloning and sequencing. After visualization on a 1.5% agarose gel stained with ethidium bromide, the reamplified cDNA fragments were purified with QIAEX (Qiagen, Venlo, the Netherlands) and cloned into *Escherichia coli* TOP10F′ cells according to the instructions of the TA cloning kit (Invitrogen). Big Dye terminator technology (Applied Biosystems, Foster City, CA) was used to sequence the fragments with an automated sequencer. Sequence analysis was performed with the help of the Belgian EMBnet Node (available online) and the putative protein sequences were used in BlastX (2) queries against the UniProt database (available online).

Northern blotting. Total RNA samples (20 μg per sample) were denatured and electrophoresed in 1.2% agarose-formaldehyde gels. After capillary transfer to Hybond N+ filters (Amersham Biosciences (GE healthcare), Buchs, UK), they were hybridized with a random-labeled DNA probe for 48 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's reagent, 0.1% sodium dodecyl sulfate (SDS) (wt/vol), and 1% salmon sperm (vol/vol). Filters were further washed for 4 × 5 min in 2× SSC and for 30 min in 2× SSC, 0.5% SDS (wt/vol). Northern blotting was repeated independently twice. Each repetition was done with different RNA samples. Band intensity was analyzed with the Photocapt 99.01 Software (Vilber Lourmat, Marne-la-Vallée, France).

RT-PCR. Specific primers were designed for each sequenced fragment using eprimer3 software from the wEmboss platform (Belgian EMBnet Node). For each set of growth conditions (glucose or cell walls as the carbon source), two RNA samples, extracted independently (2.5 μg each), were reverse-transcribed with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). One non-reverse-transcribed control was also processed for each batch. The GeneAmp 5700 (Applied Biosystems) was used for amplification and real-time quantification. RT-PCR was performed in 20-μl volumes containing 1× SYBR Green PCR Master Mix (Applied Biosystems), 300 μM of each primer, and 1 ng of cDNA. The thermal cycle consisted of a first step of 2 min at 50°C followed by one denaturation step at 95°C for 10 min and 40 cycles at 95°C for 30 s and 60°C for 30 s. Each sample was amplified in triplicate.

Individual PCR efficiencies were determined according to Ramakers et al. (35). This approach is based on the basic formula for exponential PCR: $N_c = N_o \times E^C$, where N_o is the initial number of target DNA molecules in the sample, N_c is the number of amplicons at the end of cycle number C, and E is the efficiency of the reaction. This formula is linearized as follows: $\log(N_c) = \log(N_o) + \log(E) \times C$. The log-linear part of the PCR data can be determined for each sample by selecting a lower and an upper limit of a "windows-of-linearity". Linear regression analysis is then used to calculate the slope. The individual PCR efficiencies are further obtained by $E = 10^{\text{slope}}$. A computer program to perform this calculation is available on request (35). PCR efficiencies of each primer pair (12 values) were subjected to the analysis of variance (ANOVA) procedure of the Minitab 13.20 software (Minitab Inc., State College, PA). Statistical significance was tested at the $P < 0.05$ level.

Individual threshold cycle (Ct) values corresponded to the cycle to which the fluorescence curve crosses an arbitrary threshold. Individual ΔCt values were calculated for each replicate as follows: (individual Ct value of the studied gene) – (average Ct value of

the housekeeping gene of the corresponding sample). Individual ΔCt values were further subjected to the ANOVA procedure of the Minitab 13.20 software (Minitab Inc.). Statistical significance was judged at the $P < 0.05$ level. The relative expression ratio (R) of the target gene between cells grown on glucose or cell walls was evaluated by the ΔΔCt method described by Applied Biosystems ($R = 2^{\Delta\Delta Ct}$).

RESULTS

Genes overexpressed in the presence of *B. cinerea* cell walls. A total of 35 primer combinations were used in cDNA-AFLP screening and more than 2,450 bands were amplified from the material derived from cells grown on each carbon source. An example of the patterns is presented in Figure 1. Forty-five bands were downregulated in the presence of *B. cinerea* cell walls. Eighty-six bands were either specific to cells grown in the presence of *B. cinerea* cell walls or more intense when obtained from these cells. In this study, a deliberate choice was made to select only bands specific to cells grown in the presence of *B. cinerea* cell walls or more intense when obtained from these cells. This selection does not mean that repressed bands are not involved in biological control properties. Among the upregulated bands, 28 were selected on the basis of their strong differential expression. They were further cloned and sequenced. The reproducibility of this differential expression was evaluated by independent cDNA-AFLP analysis performed on other mRNA samples with three primer pairs. Highly similar band patterns were observed in the two independent experiments, with only slight variations in band intensity (*unpublished data*).

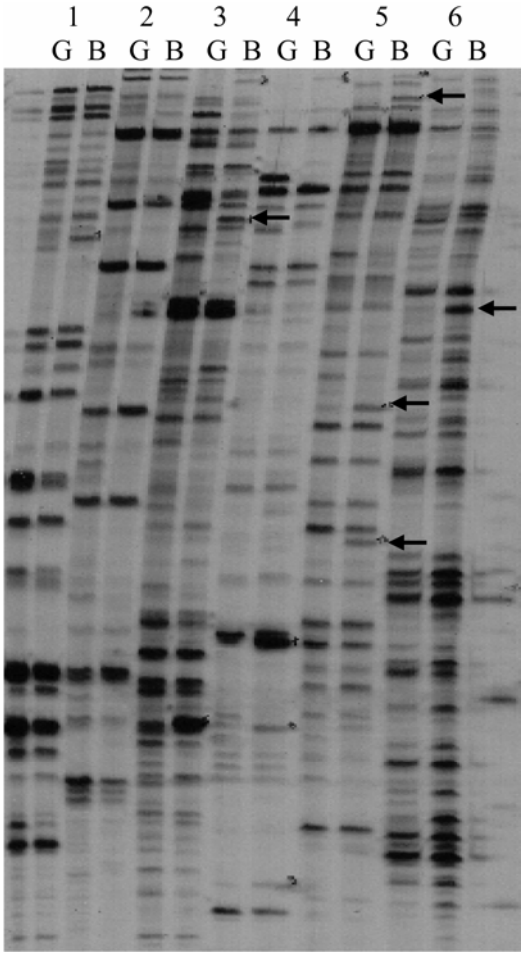

Fig. 1. cDNA-amplified fragment length polymorphism autoradiography showing patterns for strain Kh5 grown in yeast nitrogen base (YNB) medium plus *Botrytis cinerea* cell walls (B) or YNB plus glucose (G). Lanes 1 to 6 are the primer pairs used. Black arrows indicate fragments more intensively or exclusively expressed in the presence of *B. cinerea* cell walls.

Selection of a gene with constant expression for real-time RT-PCR. To normalize the results of RT-PCR, a gene with constant expression in both media had to be identified. Several authors warn that the expression of common housekeeping genes can vary depending on the cell state and environmental conditions (38). Therefore, we chose the G2 fragment, displaying constant band intensity in our cDNA-AFLP analysis, and subjected it to northern blotting analysis (*unpublished data*). In this experiment, the difference in band intensity observed after growth on glucose or cell walls was only about 5%, confirming the stable expression of this gene under the growth conditions studied.

Confirmation of gene overexpression. Twenty eight of the overexpressed fragments were subjected to RT-PCR. For each single run, PCR efficiency with the studied gene fragments was never statistically different from the PCR efficiency with the G2 gene fragment. An example of fluorescence emission and ΔΔCt calculations during the amplification cycles is presented in Figure 2. For some primers, contamination by genomic DNA was observed in the no-RT control. This contamination was considered negligible, as its relative abundance with respect to cDNA never exceeded 1:65,000. Eleven cDNA fragments were more abundant in the case of cells grown on *B. cinerea* cell walls than when the initial mRNA extract was obtained from glucose-grown cells (Table 1). The increase was statistically significant ($P < 0.05$) for the cDNA fragments 7, 12, 28, 42a, and 59.

Similarities observed with the overexpressed genes. Translations of the overexpressed cDNA fragments were run against the Uniprot database. The obtained similarities are detailed in Table 1. Homology was found between the amino acid sequence corresponding to cDNA fragment 7, showing the highest increase in gene expression, and a citrate synthase of *Saccharomyces cerevisiae.* This amino acid sequence contained a perfect match with the consensus motif of the citrate synthase family, described by the Swiss Institute of Bioinformatics {G-[FYA]-[GA]-H-x-[IV]-x(1,2)-[RKT]-x(1,2)-D-[PS]-R}. Citrate synthase catalyses the synthesis of citrate from oxaloacetate and acetyl-CoA.

The amino acid sequence corresponding to fragment 12 was similar to that of an Opt1 protein produced by the yeast *Yarrowia lipolytica*. This protein is involved in an extracellular protease synthesis pathway that seems to be pH independent. It may act as an oligopeptide transporter involved in extracellular amino acid sensing for protease induction (19).

The amino acid sequence corresponding to fragment 28 was homologous with various proteins having hydrolase activity. The best match was with the SPBC1683.04 protein of *Schizosaccharomyces pombe* (46). This protein, involved in carbohydrate metabolism, has β-glucosidase activity and catalyses the hydrolysis of *O*-glycosyl compounds. *O*-Glycosyl links are though to be involved in the attachment of the highly *O*-glycosylated Pir proteins to the 1,3-β-glucane network, which confers rigidity to the yeast cell wall (40).

The amino acid sequence corresponding to fragment 42a shared similarities with various sugar transporters. The lowest e-score was obtained with a high-affinity glucose transporter from *Candida albicans* (43). This transporter is an integral membrane protein belonging to the major facilitator superfamily; it may be linked to drug resistance in *C. albicans*. Yet the conserved signature of this family (IDKVGRRPLLIGG) was not found in the amino acid sequence deduced from our fragment.

The deduced amino acid sequence of fragment 59 showed similarities with various oligopeptide transporters, mainly Opt tetra/pentapeptide transporters, and the best match was with an unnamed protein of *Kluyveromyces lactis* (13).

The increase in gene expression was not significant for the six other fragments. Among these, fragments 33 and 46 did not yield

any database hits. The amino acid sequence corresponding to fragment 34 showed similarity with the KNQ1 protein identified in *K. lactis* (42). This is a drug-efflux permease possibly responsible for decreasing concentrations of several toxic compounds in the cytoplasm of yeast cells. Its expression is induced by oxidative stress caused by hydrogen peroxide. Similarity was found between the amino acid sequence encoded by fragment 42b and an aspartyl protease of *Saccharomyces cerevisiae* encoded by the *MKC7* gene. Within the amino acids sequence of fragment 42b appeared the {D[T/S]G} sequence containing the catalytic Asp residues of the aspartyl protease family. Overexpression of *MKC7* results in production of a membrane-associated proteolytic activity (29). The sequence encoded by fragment 1 showed homology with a *Saccharomyces cerevisiae* nicotinamidase (NAM) catalyzing the deamidation of nicotinamide. Expression of the corresponding *Saccharomyces cerevisiae* gene has been found to increase in cells grown under stress conditions (18).

DISCUSSION

A key point in the successful application of techniques displaying differential gene expression is the selection of a relevant comparison strategy. Cell wall preparations of plant pathogens have been widely used to isolate enzymes (25,44) or genes (9) related to mycoparasitic properties. The expression of numerous enzymes related to mycoparasitic properties, such as exo-glucanase (1), chitinase (28), and proteinase (17), is triggered by the presence of cell wall preparations, which are often used to simulate mycoparasitic conditions. These observations and previous experiments on *P. anomala* strain K (25) prompted us to select a comparison strategy based on the growth of its segregant strain Kh5 on media containing glucose or cell wall preparation of *B. cinerea* as the sole carbon source in order to identify genes participating in *B. cinerea* cell wall recognition and degradation. In comparison with the use of intact hyphae or germinating conidia of *B. cinerea*, this model also reduced the complexity of the experimental set up and of the cDNA-AFLP pattern analysis.

Comparing the gene expression profiles obtained when a yeast strain is grown under different culture conditions is a good way to identify differentially expressed genes (8). Here we have used this strategy to identify genes potentially involved in antagonism exerted by the *P. anomala* strain Kh5. Given the scant genetic and physiological information available, any technique used for this purpose should require no genetic information and should be applicable whatever the complexity of the transcriptome of the studied species. Heterologous hybridization with commercially available DNA microarrays can fulfill these requirements. Use of a *Saccharomyces cerevisiae* microarray has provided deep insights into transcriptional regulation in *Pichia pastoris* (37). Yet, as observed by Schoondermark-Stolk et al. (39), a high degree of gene sequence similarity between different yeast species cannot be assumed a priori. For this reason, we have preferred a gene

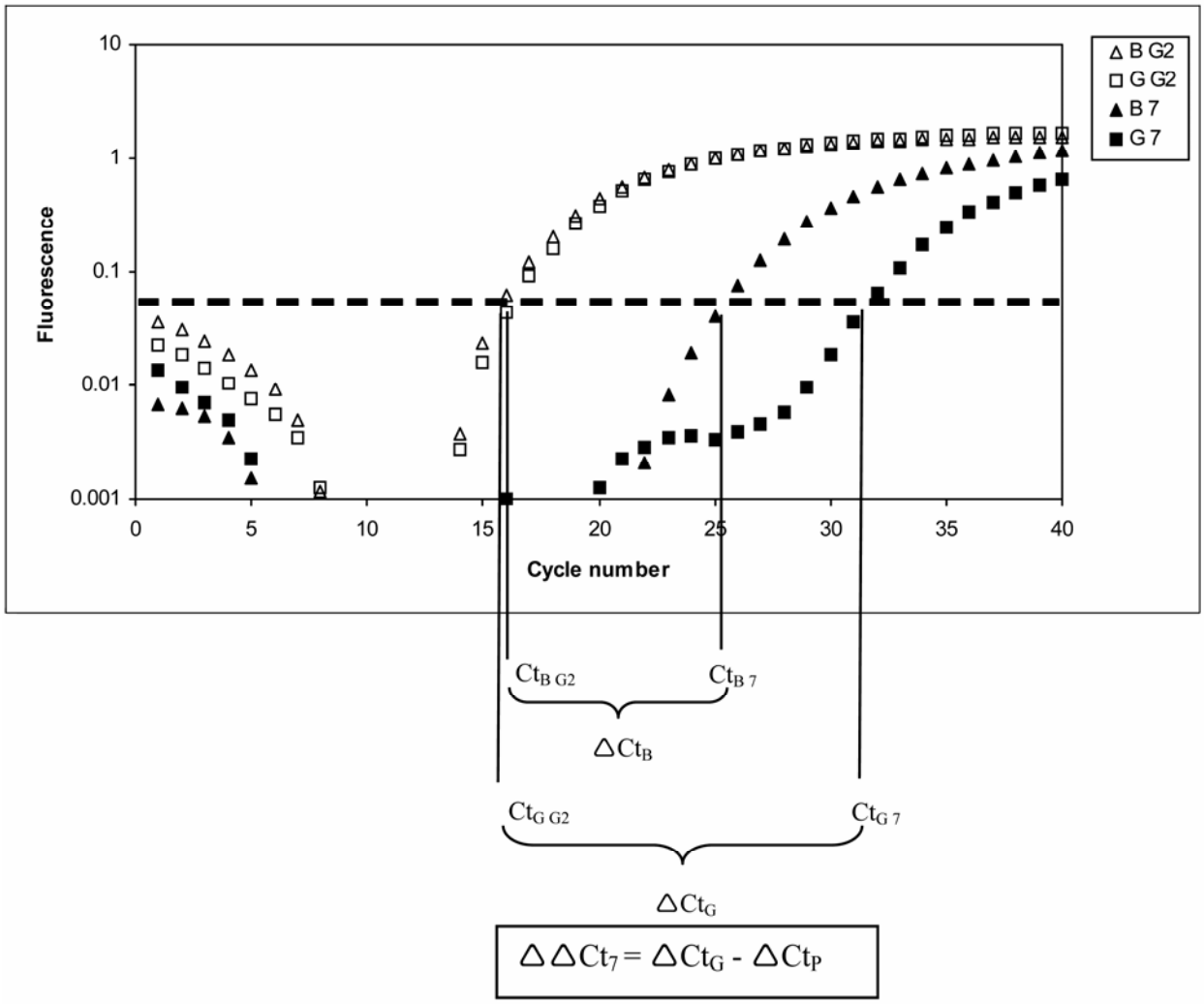

Fig. 2. Real-time measurement of emitted SYBR green fluorescence during amplification cycles for the housekeeping gene G2 and for the differentially expressed cDNA-amplified fragment length polymorphism fragment 7. Amplification of cDNA from strain Kh5 grown in yeast nitrogen base (YNB) plus *Botrytis cinerea* cell walls (B) or YNB plus glucose (G). Horizontal dashed line indicates selected threshold.

expression profiling technique based on PCR amplification. We chose the cDNA-AFLP technique both for its known reproducibility and because it allows screening of hundreds of genes simultaneously and the isolation of uncharacterized genes. It is thus well suited for the study of gene expression in microorganisms envisaged as biocontrol agents.

Because artefactual cloning is a problem inherent in DNA isolation from denaturing polyacrylamide gels (4), it is necessary to confirm differential expression of fragments identified by cDNA-AFLP analysis by an independent technique like RT-PCR or northern blotting. In the literature, the confirmation rate provided by these techniques ranges from 30% (6,15) to 100% of the selected genes (12). Cooper (7) has observed discrepancies between cDNA-AFLP and RT-PCR results. Calculation of individual ΔCt values and relative expression ratios assumes an equal efficiency between the target and the reference gene. Determination of PCR efficiency by the analysis of the same data set used to determine the Ct (35) has several advantages over the standard curve method (34). The standard curve method assumes that the PCR efficiency is constant for every sample, which is not always true (31,35). Furthermore, it necessitates more data points to determine efficiency. In the present study, PCR efficiencies were determined by the method of Ramakers et al. (35). For each run, no statistical difference was detected between PCR efficiencies of the target genes and the G2 gene. Relative expression ratios were further calculated and confirmed upregulation of 40% of the selected genes. To refine our analysis, we subjected the RT-PCR results to a statistical analysis, which revealed a significant difference in gene expression for five fragments, corresponding to 18% of the selected genes. Statistical analysis has scarcely been used in confirmation experiments, although it constitutes a valuable tool in gene selection prior to downstream characterization.

The selection of a gene with constant expression under the studied conditions is a problematic step. In this study, we selected a cDNA-AFLP fragment exhibiting similar expression in both media on polyacrylamide gels. Its stable expression was confirmed by northern blotting. This fragment presented similarities with an unnamed protein product from *K. lactis* (Table 1). Further characterization of this fragment, including full-length sequencing and larger transcription analysis, will be required to evaluate its suitability as a common housekeeping gene for *P. anomala*. In our study, a statistically significant effect of the run on PCR efficiencies and Ct values of G2 was detected. Similar results were obtained by Marino et al. (31). The origin of this effect is not known but it can be the result of minute changes in the preparation of the reaction mix. For this reason and as recommended (31), the gene displaying constant expression was always amplified during the same run than the studied gene.

The isolated fragments 28 and 42b were related to genes already studied in relation to the biocontrol properties of microorganisms (17,30,45). The other matches (various transporters, a citrate synthase, a drug-efflux permease, and a nicotinamidase) have never been linked to biocontrol properties. To some (fragments 33 and 46), no function has ever been assigned. Our results, thus, are in agreement with previous results obtained with other biocontrol agents, but also open new lines of investigation likely to shed light on the molecular mechanisms underlying and/or regulating antagonism and environmental sensing.

Based upon their homologies to known genes, the eight fragments can be divided into four different groups having distinct putative roles in the mycoparasitic interaction. These groups might represent the first outline of a global response model to the presence of *B. cinerea* cell walls in the immediate vicinity of *P. anomala* strain Kh5 cells. Fragments 28 (β-glucosidase) and 42b (protease) code potentially for secreted enzymes having a direct effect on *B. cinerea* viability and pathogenicity. These enzymes have been thoroughly studied in other biocontrol agents. Fragments 12 and 59 are related to the oligopeptide transporters Opt-family. Disruption of an Opt-like gene affects protease induction (19). These genes may be therefore involved in sensing or transmission of environmental signals required for protease induction. Similarly, the G-protein and mitogen activated kinase signaling pathways have already been identified in biocontrol agents and their role in mycoparasitism was suggested (32,33). To our knowledge, this is the first identification of genes belonging to the Opt-protein family hypothesized to play a role in biological control. These genes may correspond to a new pathway transmitting environmental signals for mycoparasitic response through protease induction.

Other fragments may be linked to stress response due to oxidation (fragments 1 and 34) or to other toxic compounds (fragment 42a). Secondary metabolism can be activated in the absence of a directly available carbon source or during interaction with another fungal species. This switch is often accompanied by the production of reactive oxygen species that can damage biological macromolecules (22). For example, overexpression of a nicotimamidase gene (fragment 1) enhances the concentration of NAD+ in the cell (18), which acts as a coenzyme for oxydoreductase. Several transport systems (fragments 34 and 42a) also catalyze the extrusion of a large number of structurally and functionally unrelated compounds out of the cells. These stress-response genes may be involved in the detoxification of strain Kh5 cells in the presence of *B. cinerea* cell walls. Response to cellular stress has

TABLE 1. Homologies of translated sequences of cDNA-amplified fragment length polymorphism (AFLP) fragments to Uniprot sequence database calculated by the BlastX software and expression ratio of the cDNA-AFLP fragments when growing strain Kh5 on *Botrytis cinerea* cell wall in comparison with glucose

	BlastX results				
Number	Homology	Accession no.[x]	Organism	e-score[y]	Ratio[z]
1	Putative nicotamidase	Sw:P53184	*Saccharomyces cerevisiae*	4×10^{-11}	1.3
7	Citrate synthase CIT1	Spt:AAO32482	*Saccharomyces castellii*	5×10^{-3}	66.9*
11	Hypothetical protein	Spt:Q8NJM	*Neurospora crassa*	1×10^{-10}	1.2
12	Oligopeptide transporter	Spt:Q8WZL3	*Yarrowia lipolytica*	2×10^{-6}	3.2*
28	Beta-glucosidase	Spt:Q9P6J6	*Schyzosaccharomyces pombe*	6×10^{-17}	4.6*
33	No homology	…	…	…	2.2
34	Permease KNQ1	Spt:Q874B4	*Kluyveromyces lactis*	9×10^{-7}	1.8
42a	Glucose transporter	Sw:O74713	*Candida albicans*	2×10^{-25}	14.2*
42b	MKC7 proteinase	Sw:Q12303	*Saccharomyces cerevisiae*	1×10^{-7}	1.4
46	No homology	…	…	…	1.1
59	Unnamed protein product	Spt:Q6CQP6	*K. lactis*	2×10^{-28}	6.3*
G2	Unnamed protein product	Spt:Q6CQE3	*K. lactis*	1×10^{-16}	…

[x] Primary accession number of the homologous protein in the Uniprot sequence database.

[y] The blast e-score scores local ungapped alignments. It is normalized by the length of the query and database. The lowest is the e-score, the best is the alignment.

[z] Ratio was calculated by the ΔΔCt method applied on real-time reverse transcription-polymerase chain reactions. The analysis of variance was applied on individual ΔCt values, and statistical significance (*) was judged at the $P < 0.05$ level.

been scarcely studied in the biological control domain, but Iakovlev et al. (22) suggested that fungal interactions between a biocontrol agent and a plant pathogen may be accompanied by stress-induced reaction. Finally, fragment 7 is part of the tri-carboxylic acid cycle in the mitochondrial matrix. Its overexpression might be the consequence of a higher demand of energy from the cells growing on *B. cinerea* cell walls or of the use of amino acids in the tri-carboxylic acid circle to provide energy to the cell.

The isolation of these genes is a first step leading to a larger study of their further characterization and an enhanced understanding of their role in biological control. In further work, these putative functions should be confirmed by functional complementation, and their role in biocontrol properties investigated by simple or multiple disruption approaches.

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