Preliminary study of exo-β-1,3-glucanase encoding genes in relation to the protective activity of *Pichia anomala* (strain K) against *Botrytis cinerea* on postharvest apples

C. Grevesse, M.H. Jijakli, O. Duterme, D. Colinet and P. Lepoivre
Unité de Phytopathologie, Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 passage des déportés, 5030 Gembloux, Belgium.
Corresponding author: C. Grevesse, E-mail address: c.grevesse@fsagx.ac.be

Abstract

In a previous study, the yeast *Pichia anomala* strain K was found to provide a high and reliable level of protection against *Botrytis cinerea* on wounded apples. An exo-β-1,3-glucanase (paexg2), active in reducing *B. cinerea* germinative tube growth, was purified to homogeneity from a culture filtrate of strain K. In order to find genes coding for exo-β-1,3-glucanase in strain K, a PCR approach using degenerated primers was used. Two PCR fragments (*PAEXG*1a and *PAEXG*2a) were found to share a significant similarity at the deduced amino acid level with exo-β-1,3-glucanases from other fungi. *PAEXG*1a and *PAEXG*2a have 90% identity in their homologous region at the amino acid level and 80% identity at the nucleotide level. Haploid populations have been produced from the diploid strain K by ascus dissection. *PAEXG*1a and *PAEXG*2a served as probes in Southern blots on genomic DNA extracted from strain K and its haploid descendants. Segregation of *PAEXG*1a and *PAEXG*2a was discussed in relation to the in vivo protective effect and the in vitro exo-β-1,3-glucanase activity of the yeasts.

Introduction

Botrytis cinerea Pers.:Fr. is one of the most devastating pathogens of postharvest apples. Its control is based primarily on chemical fungicides. Biological control of postharvest diseases appeared to be a realistic approach because of the defined and stable environmental conditions in storage rooms and the high value of some harvested commodities. Numerous microbial strains exhibiting an antagonistic activity against *B. cinerea* have been reported in the literature (Wilson and Wisniewski 1989). Jijakli (1996) isolated the yeast *Pichia anomala* (Hansen) Kurtzman strain K from the surface of an apple and demonstrated that it exhibits a high and reliable biocontrol activity against infection of *B. cinerea* on wounded Golden Delicious apples at 5 °C and 25 °C.

Knowledge of the mechanisms of antagonism operative in biocontrol may be helpful in enhancing the antagonistic efficiency. However, reports postulating mechanisms of antagonism used by yeasts in gray mold control on apple have been few. These include preemptive exclusion by yeasts of fungal infection sites, competition for nutrients and mycoparasitism (Mercier and Wilson 1994, Roberts 1990, Janisiewicz et al. 1994, Wisniewski et al. 1991). Elucidation of the mechanisms of action are often hampered by the complex interactions between host-pathogen-antagonist. Moreover, the mechanisms studied in vitro do not necessarily reflect in situ activity.

An exo-β-1,3-glucanase activity has been measured in culture filtrates of strain K grown in the presence of a *B. cinerea* cell wall preparation. An exo-β-1,3-glucanase (paexg2)

purified to homogeneity from strain K culture filtrate showed in vitro inhibitory effects on germinative tube growth of *B. cinerea*. An exo-β-1,3-glucanase activity was detected on strain K-treated apples and might be related to paexg2. Moreover, the addition of *B. cinerea* cell wall to suspension of *P. anomala* stimulated both in situ exo-β-1,3-glucanase activity and protective activity against the pathogen (Jijakli 1996). Overall results suggested that the exo-β-1,3-glucanase activity might be involved in the protective activity of strain K. However, experimental evidence of such a role is still missing and would require genetic studies of the yeast.

In this respect, this work aims to study exo-β-1,3-glucanase encoding genes in the genome of strain K and its haploid descendance in relation to the protective activity against *B. cinerea* on wounded apples and the in vitro exo-β-1,3-glucanase activity production of those yeasts.

Materials and Methods

Strains and growth conditions. *Botrytis cinerea* Pers.:Fr. strain V was isolated from a rotting strawberry and stored on oatmeal agar (oatmeal 4.5%, boiled for 30 min, 2% agar) at 4 °C in the dark. The pathogen was cultivated on oatmeal agar at 21 °C for 8 to 12 days. Conidial suspension was obtained as described by Jijakli (1996) and adjusted to 10^6 spores per ml. And *B. cinerea* cell wall preparation was prepared with a method derived from the method described by Jijakli (1996).

Pichia anomala (Hansen) Kurtzman strain K was isolated from an apple in its diploid form. It was induced to sporulate on 1% potassium acetate, pH 6.5, 1.5% agar at 20 °C for 8 days. Ascospores were isolated by micromanipulation on YEPD-agar after treatment with lyticase (from *Arthrobacter luteus*, Sigma Chemical Co., St Louis, MO). Ten haploid segregants from strain K (strain Kh 1 to 10) have been used in this study. All yeasts were stored on YEPD-agar at 5 °C. Cells suspensions were prepared by subculturing the yeasts three times at intervals of 24 h on YEPD-agar at 25 °C. Cells from the third culture were suspended in sterile isotonic water (0.85% NaCl). Yeast concentrations were determined spectrophotometrically at 595 nm following a standard curve and adjusted to the desired concentration.

Purification and sequencing of an exo-β-1,3-glucanase from strain K. An exo-β-1,3-glucanase (paexg2) was purified from a culture filtrate of strain K grown in yeast nitrogen broth (YNB, Difco) supplemented with 0.2% *B. cinerea* cell wall preparation as described by Jijakli (1996). The N-terminus was sequenced by Wattiez R (UMH, Mons, Belgium).

Nucleic acid preparation. Yeast genomic DNA was prepared by a glass beads disruption method described by Ausubel et al. (1991). PCR reactions were performed in 50 μl volume of 1 x PCR buffer (Boehringer Mannheim GmbH, Germany) containing 100 ng of yeast genomic DNA, 1 μM of forward and reverse primers (Pharmacia Inc., Piscataway, NJ), 200 μM of each dNTP, 1 unit of Taq DNA polymerase (Boeringer). Denaturation of the template was carried out at 94 °C for 5 min. The following thermal cycling scheme was used for 40 reaction cycles (PTC-200, MT Research, Watertown, MA): 94 °C for 1 min, 45 °C (cycles 1-5) or 50 °C (cycles 6-40) for 30 s, 72 °C for 30 s. A final 10-min elongation step was performed at the end of the cycles. PCR amplification products were size fractionated by 1% agarose gel electrophoresis in TAE buffer (Maniatis et al. 1982). Bands of expected lengths were excised and eluted with the QIAEX gel extraction kit (Qiagen Inc., Chatsworth, CA) and cloned into the pCR™II plasmid with the TA cloning kit (Invitrogen, San Diego, CA) according to

manufacturer's instructions. The nucleotide sequence of the cloned PCR fragments were obtained by the enzymatic dideoxy chain termination method with the T7 Sequencing kit (Pharmacia) according to provided instructions.

Two PCR degenerate primers [Pichia 1] and [Pichia 3as] were designed from conserved amino acid regions found in exo-β-1,3-glucanases of several yeasts (EXG from *Hansenula polymorpha* (Genbank accession code: Z46868), *Kluyveromyces lactis* (Z46869), *Schwanniomyces occidentalis* (Z46871), *Yarrowia lypolitica* (Z46872), EXG1 (M34341) and SPR1 (S52932) from *Saccharomyces cerevisiae*, XOG from *Candida albicans* (X56556) and one filamentous fungus (EXG1 of *Agaricus bisporus* (X92961)). The forward primer [Pichia 1] was 5'-GCATCCCNATHGGNTAYTGG-3' (N = A+C+G+T, H = A+T+C, Y = C+T) and coded for the amino acid sequence IPIGYW. The reverse primer [Pichia 3as] was 5'-GARTTRTCRAANCCRTTYTG-3' (R = A+G) and was complementary to the sequence coding for QNGFDN. The forward degenerate primer [Pichia 7] was 5'-GATAARTTTCGGGGGNGTN-3' and corresponded to the amino acid sequence DKFRGV from the N terminal sequence QPWRQQNDKFRGVNLG of paexg2.

Southern blots were performed as follows: approximately 1µg of DNA was digested by restriction endonucleases in 2X One Phor All (OPA) buffer (all enzymes and 10X OPA buffer were purchased from Pharmacia Biotechnologies). Digested DNA was size fractionated by electrophoresis in 0.5% agarose gel and transferred to positively charged nylon membranes (Boeringer) by vacuum blotting. Blots were hybridized to DNA probes P^{32}-labelled with a multiprime DNA labelling kit (Amersham International plc, Bucks, UK). Prehybridization was performed in 10x Denhardt's, 3 x SSC, 0.1% SDS, 0.01% herring sperm DNA, at 65 °C for 2 h. Hybridization was performed overnight at 65 °C in the same solution containing the probe. Membranes were submitted to 2 washes (15 min and 30 min) in 2 x SSC, 0.1% SDS and 2 washes (15 min and 30 min) in 0.2 x SSC, 0.1% SDS.

Enzyme assays and protein measurements. 50 ml YNB supplemented with 0.2% *B. cinerea* cell wall preparation as sole carbon source were inoculated with 2.10^7 CFU of the tested yeast. Flasks were shaken at 25 °C for 5 days at 100 rpm. Culture filtrates were collected after centrifugation at 10,000 rpm for 10 min at 4 °C and extensively dialyzed against 0.05 M potassium acetate buffer (pH 5.5) at 4 °C. For enzyme activity measurements, 250 µl of 0.05 M potassium acetate (pH 5.5) containing 1% laminarin (from *Laminaria digitata*, Sigma) were added to 250 µl culture filtrate. This reaction mixture was incubated with gentle agitation at 50 °C for 16 h. The exo-β-1,3-glucanase (EC 3.2.1.-58) activity was assayed from 100 µl reaction mixture by following the release of free glucose from laminarin with a commercial glucose oxydase kit (Sigma) using glucose as a standard according to manufacturer's instructions. One unit (U) of exo-β-1,3-glucanase activity was defined as the amount of enzyme releasing 1 µg of glucose equivalent per minute, per millilitre of enzyme solution. Specific activity was expressed in units per mg of protein (SU). The experiment was repeated once independently (two culture filtrates per yeast). Results of the two experiments were submitted to variance analysis and means were separated by the Fisher's least significant difference at $P \leq 0.01$ with the SYSTAT software (SYSTAT Inc., Evaston, IL, USA).

Biological protection assays. Apple fruits (cv. Golden Delicious) were brought in a commercial store and maintained at 4 °C until used. Fruits were surface-disinfected with 10% sodium chloride for 2 min, rinsed with sterile distilled water and wounded with a cork borer (two wounds of 6 mm diameter and 3 mm deep at the equator of each fruit). The wounds were treated with 50 µl of a suspension of each yeast (in isotonic water or in an aqueous suspension of 0.2% cell wall preparation) or with 50 µl isotonic water as a control. The fruits were left in

closed plastic boxes with a wetted filter paper at 21 °C in darkness. After 24 h, the wounds were inoculated with 50 µl of a *B. cinerea* conidial suspension. Fruits were incubated in the same conditions for 7 days before measuring diameters of decay lesions. Five apples were used per treatment. Each experiment was conducted twice independently. Results were subjected to analysis of variance and means were separated by the Fisher's least significant difference at $P \leq 0.01$ (first assay) or the Dunnet's test at $P \leq 0.05$ (second assay) with the SYSTAT software.

Results and Discussion

Isolation of *PAEXG1* and *PAEXG2* fragments. Degenerate primers [Pichia 1] (sens) and [Pichia 3as] (antisens) based on conserved regions between exo-β-1,3-glucanases from different fungi gave rise to the amplification of a 163 bp DNA fragment (*PAEXG1a*) from strain K genomic DNA, the expected length of the PCR product being about 200 pb. A 390 bp DNA fragment (*PAEXG2a*) was also amplified from strain K genomic DNA with the primers [Pichia 7] derived from the N terminal sequence of the purified paexg2 and [Pichia 3as], the expected length of that PCR product being about 400 bp. The PCR products *PAEXG1a* and *PAEXG2a* were cloned and sequenced (Fig. 1). They both showed a significant similarity, at the deduced amino acid sequence level, with exo-β-1,3-glucanases of other fungi. *PAEXG1a* shared a 76% similarity with the protein coded by XOG of *Candida albicans* and *PAEXG2a* shared 66% with the protein coded by EXG1 of *Saccharomyces cerevisiae*. *PAEXG1a* and *PAEXG2a* shared a 90% identity at the amino acid level and a 80% identity at the nucleotide level in their homologous region. These results suggest that at least 2 exo-β-1,3-glucanase encoding genes (*PAEXG1* and *PAEXG2*) are present in strain K genome. *PAEXG2a* correspond probably to the gene coding for the purified paexg2. That result corroborates the observation, with native gel detection, of two bands of exo-β-1,3-glucanase activity of different intensity in the culture filtrate of strain K grown with cell walls (Jijakli 1996). The highest activity being produced by paexg2, *PAEXG1* could then code for an exo-β-1,3-glucanase partly or entirely responsible for the less active band. *PAEXG1a* and *PAEXG2a* were used as probes in Southern blot hybridizations.

PAEXG1a: 1-atccccatcggttactgggcattttatctattagaagatgatccatacgttcaaggtcaagaaccttatttggataaggctttggaa tgggccaaacagaatgatttgaaagtttggattgacttgcatggtccaggctctcaaaacggatttgataactcaa-163

PAEXG2a: 1-gatatgtttcggggcgttaacttaggtgggtggtttgttttagaacctttatcactccttctttatttgaagcttttgaaaatcaaggc caagatgttcctgttgatgaataccattacaccaaagcccttggtaaggatttagctaaagagagattggtcaacattggagttcatggattgttgaag ctgatttccaaagcattgcaggtgctggtttaaactttgttcgtattccaatcggttattgggcattccaattgttggataatgatccatacgttcaaggtca agaatcatatttggaccaagctttagaatgggctaagaaatatgatattaaagtttggattgatttacatggtgctctcagttctcaaaacggcttt-390

PAEXG1a amino acid sequence: LQNSACIPIGYWAFYLLEDDPYVQGQEPYLDKALEWAKQ NDLKVWIDLHGPGSQNGFDNS

PAEXG2a amino acid sequence: DMFRGVNLGGWFVLEPFITPSLFEAFENQGQDVPVDEYHYT KALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPIGYWAFQLLDNDPYVQGQESYLD QALEWAKKYDIKVWIDLHGALSSQNGF

Fig. 1. Nucleotide sequences and derived amino acid sequences of the PCR products *PAEXG1a* and *PAEXG2a*.

Analysis of genomic DNA of strain K and its segregants. Genomic DNA extracted from the diploid strain K was cut with 10 restriction enzymes and subjected to Southern blots hybridizations with *PAEXG*1a and *PAEXG*2a as probes. Both probes hybridized to one or two bands depending on the restriction enzyme used (results not showed). Restriction enzymes giving two hybridization bands were chosen (EcoRI, EcoRV for *PAEXG*1a; EcoRV, BamHI for *PAEXG*2a) to treat DNA from strain K and its ten haploid segregants. For the ten segregants, *PAEXG*1a or *PAEXG*2a hybridized to a single band corresponding either to one or the other band detected on strain K genomic DNA blots. Blots with EcoR V digestions are showed in Fig. 2. Repartition of bands obtained on blots with EcoRI and BamHI digestions, probed with *PAEXG*1a or *PAEXG*2a respectively, was the same as on EcoR V blots. So, *PAEXG*1 and *PAEXG*2 turned out to be single copy genes and the two bands observed on strain K genomic blots were due to the diploid status of this yeast. Assuming that the two fragments hybridizing with each probe were representative of two alleles of each *PAEXG*1 or *PAEXG*2 gene (alleles *PAEXG*1-1 and *PAEXG*1-2, alleles *PAEXG*2-1 and *PAEXG*2-2), we may identify four genetic types among the 10 segregants (Table 1). This shows that *PAEXG*1 and *PAEXG*2 segregate independently.
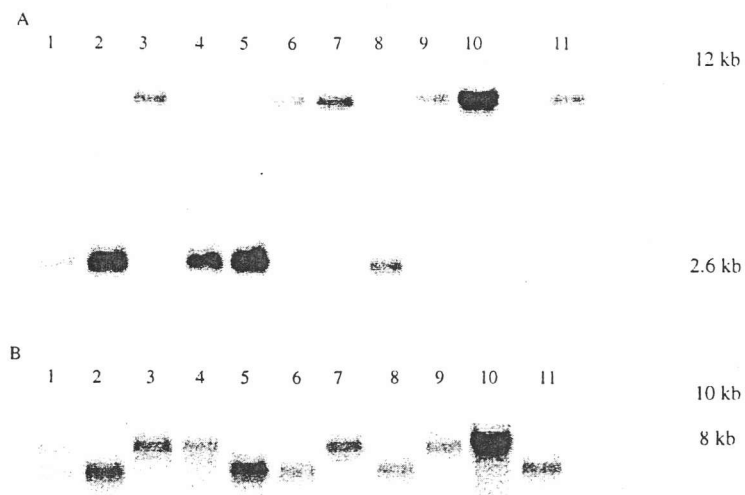

Fig. 2. Blots of total genomic DNA from strain K (lane 1) and its ten haploid segregants strain Kh 1 to 10 (lanes 2 to 11) cut with EcoRV. The blots were probed with P32-labelled PCR products *PAEXG*1a (blot A) and *PAEXG*2a (blot B).

Table 1. Segregation of alleles of the two genes *PAEXG1* and *PAEXG2* in the haploid segregants derived from strain K.

Yeast Strain	Alleles			
	PAEXG1-1	*PAEXG1-2*	*PAEXG2-1*	*PAEXG2-2*
K	●	●	●	●
Kh 1	●		●	
Kh 2		●		●
Kh 3	●			●
Kh 4	●		●	
Kh 5		●	●	
Kh 6		●		●
Kh 7	●		●	
Kh 8		●		●
Kh 9		●		●
Kh 10		●	●	

Genetic type	Yeast Strain
PAEXG1-1 PAEXG2-1	Kh 1, Kh 4, Kh 7
PAEXG1-1 PAEXG2-2	Kh 3
PAEXG1-2 PAEXG2-1	Kh 5, Kh 10
PAEXG1-2 PAEXG2-2	Kh 2, Kh 6, Kh 9

Exo-β-1,3-glucanase activity measurements. Strain K and its segregants were grown in presence of *B. cinerea* cell walls as sole carbon source. The in vitro exo-β-1,3-glucanase activity was measured in the culture filtrates. Out of the ten haploid yeasts tested, strain Kh 1 and strain Kh 7 showed a level of exo-β-1,3-glucanase specific activity significantly higher ($P \leq 0.01$) than strain K activity, the others producing the same specific activity level as strain K (Fig. 3). No relation could be found between established genetic types and the different levels of enzymatic activity produced in vitro.

Biological protection assays. The antagonistic activity against *B. cinerea* of strain K and its segregants was tested in vivo on wounded apples. A first experiment was conducted with yeasts inoculated at 10^6 CFU/ml in isotonic water. All yeasts reduced lesion diameter (protection levels between 56 and 77%) (Fig. 4). strain K protection level was consistant with the levels observed in the same conditions by Jijakli (1996). Strain Kh 2, strain Kh 3 and strain Kh 9 protected significantly less ($P \leq 0.01$) than strain K, strain Kh 1, strain Kh 6 and strain Kh 7, the other haploids showing an intermediate protective effect. A second experiment was conducted with each yeast inoculated at 10^5 CFU/ml in isotonic water or in an aqueous suspension of 0.2% cell wall preparation. Strain K, strain Kh 1, strain Kh 5 and strain Kh 6 showed an increased protective effect ($P \leq 0.05$) when inoculated with *B. cinerea* cell walls (Fig. 5). Concerning the other yeasts, the addition of cell walls add no significant effect on the antagonism. For these two experiments, no relation could be found between genetic types and either the different levels of antagonism or the different effects of the addition of *B. cinerea* cell walls on the antagonism.
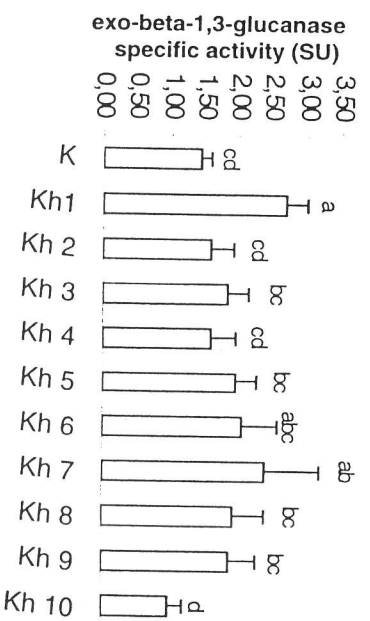
Mean diameter of decay lesion (mm)

K	a
Kh1	a
Kh2	c
Kh3	bc
Kh4	abc
Kh5	ab
Kh6	a
Kh7	a
Kh8	abc
Kh9	bc
Kh10	abc
Control	d

Fig. 4. Mean diameters of decay lesions when apple wounds were inoculated with 50 µl of yeast suspension (strains K and Kh 1 to 10) at 10⁶ cfu/ml in isotonic water and 50 µl of *B. cinerea* spore suspension at 10⁶ spores/ml at 24 h interval. Data from the two repetitions of the experiment were submitted to variance analysis and pooled. Means with the same letter are not significantly different from each other (Fisher's LSD test, $P \leq 0.01$).
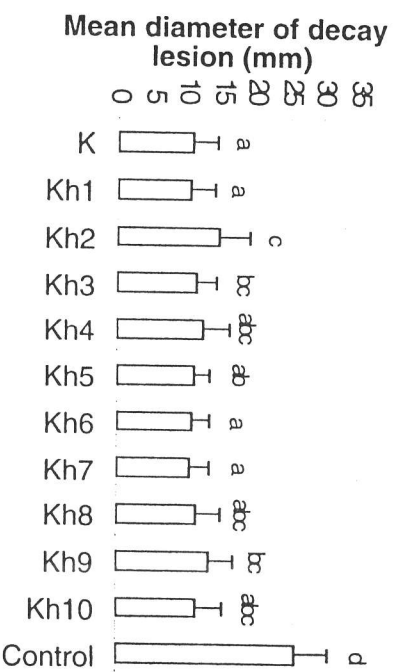
exo-beta-1,3-glucanase specific activity (SU)

K	cd
Kh1	a
Kh 2	cd
Kh 3	bc
Kh 4	cd
Kh 5	bc
Kh 6	abc
Kh 7	ab
Kh 8	bc
Kh 9	bc
Kh 10	d

Fig. 3. Mean exo-β-1,3-glucanase specific activities produced by strain K and its segregants (strains Kh 1 to 10) when grown with *B. cinerea* cell walls as sole carbon source. Data from the two repetitions of the experiment were submitted to variance analysis and pooled. Means with the same letter are not significantly different from each other (Fisher's LSD test, $P \leq 0.01$).
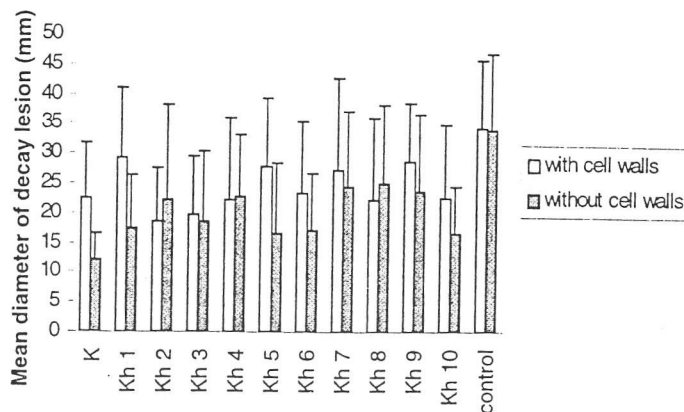
Fig. 5. Mean diameters of decay lesions when apple wounds were inoculated with 50 µl of yeast (strains K and Kh 1 to 10) at 10^5 cfu/ml in aqueous suspension of 0.2% cell wall preparation and 50 µl of *B. cinerea* spore suspension at 10^6 spores/ml at 24 h interval. Data from the two repetitions of the experiment were submitted to variance analysis and pooled.

Conclusion

None of the studied segregants derived from strain K was defective in in vitro exo-β-1,3-glucanase activity production or in protective activity against gray mold on wounded apples. The in vitro enzymatic activity of the segregants was not related to their protective activity. Moreover, segregation of the strain K genes *PAEXG1* and *PAEXG2* influence neither in vitro exo-β-1,3-glucanase activity nor in vivo protective activity of the yeasts postulating that the diploid strain K may be homozygous at those loci. Hence the variations observed between haploid segregants for the studied characteristics point out that other factors (genes or regulating elements) than the genes *PAEXG1* and *PAEXG2* are active in the in vivo exo-β-1,3-glucanase production and the protective effect.

Accordingly, the possible implication of the exo-β-1,3-glucanase activity in the protective effect remains to be elucidated. As a first step, the in situ transcription and translation of *PAEXG1* and *PAEXG2* will be investigated on apples at the site of *B. cinerea*-*P. anomala* interaction. Since some haploid segregants derived from strain K produce as much exo-β-1,3-glucanase activity as strain K (or more) and protect apples against *B. cinerea* with the same efficiency as strain K (in the experimental conditions), the implication of *PAEXG1* and *PAEXG2* in the antagonism will be further studied through their disruption by integrative transformation in the genome of the haploid material. To reach that purpose, we are currently attempting to isolate *PAEXG1* and *PAEXG2* from a strain K genomic library in the phagic vector EMBL3 to sequence them.

Literature

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-Intersciences, New York, USA.

Franclet, J. 1994. Enquête ANPP-Maladies de conservation des pommes. L'arboricul. Fruit. 473:24-26.

Janisiewicz, W.J., Peterson, D.L., and Bors, R. 1994. Control of storage decay of apples with *Sporobolomyces roseus*. Plant Dis. 78:446-470.

Jijakli, M.H. 1996. Study of antagonistic properties of two strains of yeasts against *Botrytis cinerea* Pers. on postharvest apples (thèse de doctorat, in French). Faculté Universitaire des Sciences Agronomiques de Gembloux, Belgium.

Maniatis, T., Fristch, E.F., and Sambrook, J. 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, NY.

Mercier, J., and Wilson, C.L. 1994. Colonization of apple wounds by naturally occuring microflora and introduced *Candida oleophila* and their effect on infection by *Botrytis cinerea* during storage. Biol. Cont. 4:138-144.

Roberts, R.G. 1990. Postharvest biological control of gray mold of apple by *Cryptococcus laurentii*. Phytopathology 80:526-530.

Rosenberg, D.A. 1991. Postharvest diseases (Blue mold - Gray mold). Pages 53-58 in: Compendium of Apple and Pear Diseases. A.L. Jones and H.S. Aldwinckle, eds. APS Press The American Phytopathological Society, USA.

Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C., and Chalutz, E. 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guillermondi* 1. Characterization of attachment to *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 39:245-258.