IN SITU DEVELOPMENT AND APPLICATION OF CDNA-AFLP TO ISOLATE GENES OF *CANDIDA OLEOPHILA* (STRAIN O) POTENTIALLY INVOLVED IN ANTAGONISTIC PROPERTIES AGAINST *BOTRYTIS CINEREA*

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ABSTRACT

The yeast *Candida oleophila* (strain O) presents a high level of protective activity against *Botrytis cinerea* (gray mold) on postharvest apples. The cDNA-AFLP technique allows the comparison of mRNA populations extracted from cells grown in different conditions. In order to isolate yeast genes potentially involved in biological control properties, that technique was applied on strain O cells growing on apple wounds. The biological control properties of 8 *C. oleophila* strains and strain O were assessed in order to compare the gene expression of a non antagonistic strain against gene expression of strain O. In the absence of a non-antagonistic strain, an other comparison model was designed. It was based on the growth of strain O in different *in situ* conditions: strain O applied on apple wounds (O), strain O applied on apple wounds in presence of *B. cinerea* (B) and *B. cinerea* alone on apple wounds (F). A recovering technique, based on the washing of cells in the wound and a RNA extraction method followed by a DNase treatment were optimised before cDNA-AFLP application. Thirteen primer pairs were used. Their application resulted in an average of 54 and 55 bands for O and B respectively whereas no bands were observed for F. Among these bands, 8 were expressed more intensely in presence of the pathogen (1.1% of the fragments).

Keywords: *Candida oleophila, Botrytis cinerea,* cDNA-AFLP, biological control, postharvest disease, apple.

INTRODUCTION

The yeast *Candida oleophila* (strain O) has been isolated from apple (cv. Golden delicious) and selected fot its high and reliable antagonistic properties against *Botrytis cinerea*, one of the most devastating pathogens of postharvest apples (Jijakli & Lepoivre, 1993).

Among the multiple steps involved in the development of a biocontrol agent (BCA), the understanding of the mode of action is a key point to develop rational procedure, for the selection of more efficient strains, to develop appropriate production and formulation methods and to fulfil requirements of registration procedure.

Numerous techniques have been used to study the mode of action of the biocontrol agents (e.g. microscopy, population dynamic, *in vitro* dual culture, biochemical studies, molecular biology tools,...). During recent years, molecular biology tools have been developed and used to study the mode of action of biocontrol agents (Grevesse *et al.*, 2003; Yehuda *et al.*, 2003). Often, they are focused on a unique gene whereas biological control properties depend on numerous factors. Techniques displaying differential gene expres-

sion can potentially solve this problem as they are able to grasp the complexity of gene expression and interaction.
The cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP; Bachem *et al.*, 1996), a technique of differential gene expression analysis, relies on the digestion of cDNAs by restriction enzymes followed by their ligation with adaptators which serve as priming site during amplifications. It allows to perform a specific amplification at high stringency, making this technique theoretically very reproducible. In this study, we describe the adaptation and the application of the cDNA-AFLP in order to isolate strain O genes potentially involved in the antagonistic activity against *B. cinerea*.

MATERIAL AND METHODS

Yeast strain and media. The strain O was isolated from an apple. It was cultivated on Potato Dextrose Agar (PDA, Duchefa) and conserved at 4°C during 6 months. Strains CBS 2219, CBS 2220, CBS 4371, CBS 7419, CBS 8269, ATCC 60367 and ATCC 201074 were purchased at the CentraalBureau voor Schimmelcultures (CBS) or at the American Type Culture Collection (ATCC) respectively. Before experiments, they were subcultured on PDA at 25°C. *Botrytis cinerea* was isolated from a rotting strawberry and stored on PDA at 5°C in the dark. Before experiments, the pathogen was cultivated on PDA at 25°C. Spores were scraped from fungal colonies and suspensions were filtered through nylon sterile gaze.

Assay of biocontrol properties. The biocontrol properties of *C. oleophila* strains were evaluated on apple wounds as previously described (Jijakli & Lepoivre, 1993) with two independent repetitions. This protocol was slightly adapted during development and application of cDNA-AFLP. The diametre of the wounds was 21 mm and yeast cells (2.10^7 cells by wound) were separated from *B. cinerea* spores (1.5 10^5 spores by wound) by a nitrocellulose filter (0.45 µm of porosity).

Cell recovering for cDNA-AFLP. Strain O was applied 1 hour after wounding. *B. cinerea* spores were further inoculated one hour after BCA treatment. Strain O cells were recovered 7h30 after their application on the wound. After removal of the filter, one millilitre of PKBT (potassium phosphate 0.05M pH 6.5; 0.05% Tween 80) was pipetted inside of the wound. The buffer was further filtrated (20 µm) to eliminate contaminating apple tissue.

RNA extraction. The yeast cells were pelleted by centrifugation 1 min. at 16000g and 4°C (Eppendorf centrifuge 5415R) and resuspended in 400 µl of RNA buffer (0.5 M NaCl; 0.2 M TrisCl pH 7.4; 10 mM EDTA). RNA extraction and DNase treatment were carried out as previously described (Massart *et al.*, 2002).

cDNA-AFLP. For first strand synthesis, total RNA were primed with oligo dT primers using the SMART™PCR cDNA Synthesis Kit (BD Clontech). The ds cDNA were digested, ligated, pre-amplified and specifically amplified as previously described (Massart *et al.*, 2002).

Band elution, re-amplication and cloning. Bands were eluted from the gel as previously described (Campalans *et al.* 2001). They were further submitted to a re-amplification step using the corresponding PCR conditions of the cDNA-AFLP. Re-amplified bands were cloned into the PCR 2.1 vector using the TA cloning kit (Invitrogen) according to manufacturer instructions. Insertion of the re-amplified band was checked by colony PCR.

RESULTS AND DISCUSSION.

Design a comparison model. In order to find genes potentially involved in biological control properties, the first considered model was the comparison of gene expression between strain O and a non antagonistic strain of *C. oleophila*. The antagonistic properties of 8 *C. oleophila* strains, which correspond to the majority of *C. oleophila* strains available worldwide in the public collections, were compared to strain O. Statistical analysis (Fisher's test, $p \leq 0.01$) did not reveal any significant difference between biocontrol properties of the nine tested strains.
In the absence of a non antagonist strain, an other comparison model, based on the comparison of strain O gene expression in different growing conditions, was developed. In order to isolate strain O genes induced by the presence of *B. cinerea* spores on apple wounds, we compared the gene expression of strain O in presence or in absence of *B. cinerea* spores using the following model: strain O+*B. cinerea* spores on fresh wounds (B); strain O alone on fresh wounds (O) and *B. cinerea* spores alone on fresh wounds (F).

Development of cDNA-AFLP. The recovering method was optimised following two parameters: maximising the yeast cell recovery and minimising the presence of apple tissue in the extract. Several methods were tested and compared in triplicate (unpresented results). The "pipeting" method was selected because it allowed the recovering of $1.8\ 10^7$ cells, which correspond to 62 % of the total yeast population growing on apple wounds, and no discernable apple tissue was observed before RNA extraction. After checking the RNA quality of the extracts by electrophoresis on agarose gel, the cDNA-AFLP method was applied to the selected model.

Application of cDNA-AFLP. Thirteen primers pairs were tested, resulting in the observation of 721 bands for B and 708 bands for O. The average number of bands was 55 and 54 for B and O respectively. No bands was observed for F. By comparing the patterns of B and O, 8 bands were selected. They corresponded to bands specifically or more intensely expressed in the presence of the pathogen. These bands were eluted from the gel, re-amplified and cloned.

CONCLUSION AND FUTURE PROSPECTS

In this paper, we described the adaptations to *in situ* conditions of a RNA extraction method and the subsequent application of cDNA-AFLP, both previously optimised *in vitro*.
The design of a comparison model has a crucial importance in the application of cDNA-AFLP. The comparison models may be divided in two different

strategies: comparison of gene expression of different strains in the same media or comparison of gene expression of a unique strain in different media. The feasability of the first approach was assessed through the evaluation of biocontrol properties of 8 *C. oleophila* strains to find a non antagonsitic strain. As all tested strains showed an antagonisitic activity, this strategy was abandoned. Nevertheless, the comparison of gene expression between a biocontrol agent and a non antagonistic strain can provide a relevant comparison model for other BCA species where a non antagonisitic strain exists. The second strategy was therefore applied. The comparison model comparing gene expression of strain O in presence or in absence of *B. cinerea* spores reflects very closely the interactions existing on apple wounds between the BCA and *B. cinerea*. It is therefore well suited to isolate genes potentially involved in biocontrol properties of the BCA during early phase of growth of booth micro-organisms.

Comparing to *in vitro* experiments, *in situ* studies of BCA's mode of action are often much more complex to set up due to various factors (complex media; limited growth of the antagonist; numerous interactions between the biocontrol agent, the apple, the pathogen and the microclimate,...). Nevertheless, the succesful transfer of techniques from *in vitro* to *in situ* conditions is very important because *in vitro* studies are often hampered by a *biais* masking or increasing the role of the different phenomenons. For these reasons, we transferred *in situ* the application of a cDNA-AFLP method, including the RNA extraction and the cell recovering steps, previously optimised *in vitro* (Massart *et al.*, 2002).

In this experiment, the application of RNA extraction method from cells growing on apple wounds resulted in an average yield of 7.4 µg by sample ($1.8\ 10^7$ cells) before DNase treatment. This yield corresponded approximatively to those previously obtained during RNA extraction from cells growing *in vitro* (Massart *et al.*, 2002). The RNA was also of good quality for cDNA-AFLP application.

After application of the cDNA-AFLP, 8 bands were selected for their strongest differential expression between O and B. These bands corresponded potentially to genes over-expressed in presence of the *B. cinerea* spores during early growth of strain O on apple wound. These bands are now cloned and they will be sequenced. Their sequences will be compared to databases of published fungal genes in order to find their putative function. The obtained sequences will also allow the design of primers for real-time experiments in order to validate the differential expression of the genes.

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