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Application of RAP-PCR and cDNA-AFLP to isolate genes of *Candida oleophila* (strain O) induced by the presence of galacturonic acid

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Abstract: The yeast *Candida oleophila* (strain O) possesses an antagonistic activity against *Botrytis cinerea* (gray mold) on postharvest apples. This activity has been stimulated by the addition of galacturonic acid (GA) at 0.01 and 0.001 % (w/v) while GA didn't show any protective activity alone. RNA arbitrarily primed polymerase chain reaction (RAP-PCR) and cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) were used to identify strain O genes induced by the presence of GA. For the RAP-PCR, 82 sets of primers were used, resulting in a total number of 1438 or 1439 bands observed from mRNA of strain O incubated in presence or absence of GA. The average number of bands per primer set was 17.5. Eight bands were selected for their strongest differential expression in presence of GA but were no more differentially observed during repetitions of the RAP-PCR (on the same and/or other batches of mRNA). cDNA-AFLP application with 10 primer sets resulted in the detection of 77 bands in average per primer set and 100 % of reproducibility using the same cDNA batch. One band was differentially expressed in presence of GA. This result remains to be confirmed.

Keywords: *Candida oleophila*, galacturonic acid, RAP-PCR, cDNA-AFLP, biological control, postharvest disease, apple

Introduction

The yeast *Candida oleophila* (strain O) has been isolated from apple (cv. Golden delicious) and selected for its high and reliable antagonistic properties against *Botrytis cinerea* one of the most devastating pathogens of postharvest apples (Jijakli et al., 1999). The addition of galacturonic acid (GA), at 0.01 and 0.001 % (w/v), enhanced the protective activity of strain O (10^5 cfu/ml) to the protective level obtained by strain O applied alone at 10^7 cfu/ml (around 90 %). Furthermore, the GA didn't show any protective activity by itself (Dickburt et al., 2001). In order to isolate strain O genes up-regulated by the presence of this molecule, two molecular techniques were applied to compare mRNA populations extracted from strain O cultivated in YNB + glucose (G) (0.5%) and YNB + G (0.4%) + GA (0.1%). The first technique, the RNA Arbitrarily Primed Polymerase Chain Reaction or RAP-PCR (Sokolov & Prockop, 1994), is based on the retrotranscription and the amplification by PCR of mRNA previously extracted. The amplified fragments are displayed and compared on agarose gel. After mRNA retrotranscription, the second technique, the cDNA Amplified Fragment Length Polymorphism or cDNA-AFLP (Bachem et al., 1996), relies on the digestion of cDNAs by restriction enzymes followed by their ligation with adaptators which serve as priming site during amplification. It allows performing a specific amplification at high stringency, making this technique theoretically more reproducible.

Material and methods

Yeast strain and media

The strain O was cultivated on Potato Dextrose Agar (PDA, Duchefa) and conserved at 4°C during 6 month. Before experiment, it was subcultured on PDA at 25°C. One millilitre of a suspension of strain O (4.10^8 cfu/ml) was inoculated in 50 ml of YNB +G [containing 6.7 g/l of Yeast Nitrogen Base (Gibco) and 5 g/l of G (Merck)]. Six hours later, when the strain O was in exponential phase, 8.10^6 ufc were transferred in 250 ml of differential media (YNB + G or YNB + G+ GA) [containing 6.7 g/l of YNB, 4 g/l of G and 1 g/l of GA (Fluka)]. Flasks were always incubated at 20 °C on a rotary shaker at 120 rpm.

RNA extraction

The yeast cells (10^9 cells per sample) were harvested in exponential phase after 14 hours of incubation in the two differential media by filtration on a 0.45 µm filter (Gelman Laboratories) and resuspended in 1 ml of RNA buffer (0.5 M NaCl; 0.2 M TrisCl pH 7.4; 10 mM EDTA). One millilitre of buffered phenol–chloroform–isoamylic alcohol (PCAi; 25:24:1) and glass beads (425-600 µm, Sigma) were added. After vortexing 10 x 1 min. (with 30 seconds on ice between each vortexing), 3 ml of RNA buffer and 3 ml of PCAi were added. The samples were centrifuged at 5860 g during 10 min. (Rotor GSA, Sorvall RC-5B). Two other extractions with PCAi were carried out on the recuperated aqueous phase. The extract was ethanol precipitated. Samples were DNase treated with 20 U of DNase RNase free (Boehringer Mannheim) in presence of 40 U of RNase inhibitor (Roche).

RAP-PCR

The mRNAs were purified using the batch system of Oligotex mRNA midi kit (Qiagen) and single stranded cDNAs were synthetised following the instructions of the kit "Superscript™ pre-amplification system for the first strand cDNA synthesis" (Invitrogen) using random hexamer primers. The subsequent amplification step was carried out on a UNO II thermocycler (Biometra) as follow: 95°C-15 min., 45 X (94.5°C – 1 min., 34°C – 1min., 72°C – 1min. 30 s), 72°C 10 min. The 50 µl reaction contained 2 µl of cDNA solution (20 ng), 1X PCR buffer and 5 U of Hotgoldstar (Eurogentec), 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 1 mM of each arbitrary decamer primer. The PCR products were analysed by electrophoresis on agarose gel (1,8 %, Difco) in TBE 1X stained by ethidium bromide (100 ppm).

cDNA-AFLP

First strand synthesis of cDNA from total RNA was primed with oligo dT primers and followed directly by second strand replacement synthesis using RNaseH and Taq polymerase I (Superscript™ double stranded cDNA synthesis kit, Invitrogen). The double stranded cDNA was digested with EcoRI and MseI, ligated with EcoRI and MseI adaptators and pre-amplified on a UNO II thermocycler (Biometra) following instructions of the kit "AFLP® analysis system for microorganisms" (Invitrogen). After a 50 fold dilution, the specific amplification was carried out on the same thermocycler with selective Eco and Mse primers, corresponding to the sequence of the adaptators and containing one (Eco+1 and Mse+1) or two (Eco+2 and Mse+2) additional nucleotides. The Eco primer was labelled with [γ^{33}P] ATP. The specific amplification was carried out following instruction of the kit "AFLP® analysis system for microorganisms" (Invitrogen). Two enzymes were tested: the Taq Polymerase (Roche) and the Expand High Fidelity System (Roche). Amplified products were separated by electrophoresis at 100 W during 1.5 h on a denaturing polyacrylamide gel (6 %) containing 7 M urea. Gels were dried on whatman 3MM paper before autoradiography.

Results

RAP-PCR

Eighty-two primer sets were used. The total number of bands observed from mRNA of strain O incubated in YNB + G or YNB + G + GA was 1439 or 1438 respectively. The average number of bands per primer set was 17.5 ranging from 5 to 31 bands per lane. Thirteen bands were only or more intensively expressed in presence of GA. Among these, eight were selected for their strongest differential expression and the corresponding primer combinations were used for a second screening using the same batch of mRNA. Only one band (1000 bp) was still differentially expressed during this second screening. This selected band wasn't more differentially detected in the patterns observed for three different batches of mRNA. By counting the number and the size of the bands for each repetition, the reproducibility was estimated at 65 % when using the same batch of mRNA and 50 % for different batches.

cDNA-AFLP

Three primer combinations, Eco+1/Mse+1, Eco+2/Mse+1 and Eco+1/Mse+2, were assessed. The highest average number of bands per primer combination (77), were obtained with the Eco+1/Mse+2 primer combination. The Expand High Fidelity system was preferred to the Taq polymerase because the observed bands were sharper and of higher intensity. Based on 6 primer sets, revealing a total of 222 bands, a reproducibility of 100 % for the digestion, the ligation and the pre-amplification steps was observed by processing the same batch of ds cDNA at three different times (Figure 1a). Ten primer sets have been tested resulting in 775 and 767 bands for YNB + G and YNB + G + GA respectively. Among those, one band has shown a stronger expression in presence of GA (arrow, Figure 1b).

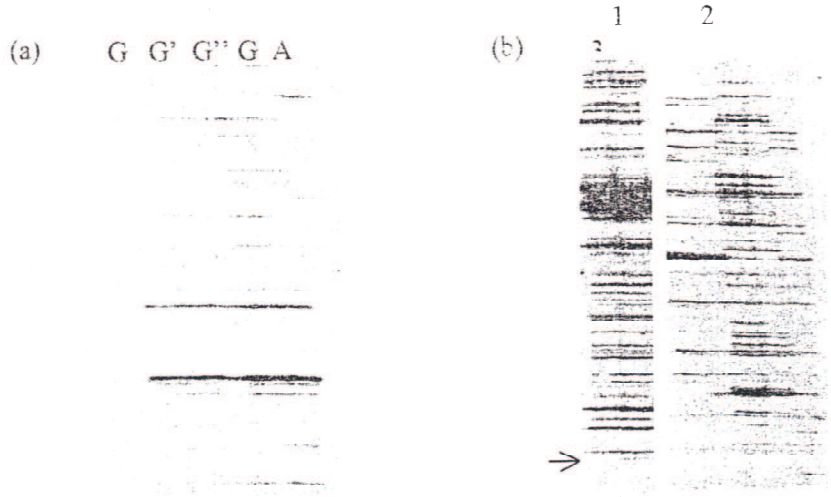

Figure 1. DNA sequencing gel autoradiography of cDNA-AFLP fingerprints from mRNA of strain O cultured in YNB + G (G) and YNB + G + GA (A). (a) Example of results obtained with one primer set by processing the digestion, the ligation and the pre-amplification steps at three different times from the same cDNA sample. (b) Example of fingerprint obtained with 3 primer sets (1, 2, 3). Positive control (C): DNA from *E. coli*. → : band specifically observed in presence of GA in the culture media.

Discussion

The RAP-PCR is quick and easy to perform. Nevertheless, its lack of reproducibility (only 50 % for different batches of mRNA) represents its major drawback. Depending of the model, the reproducibility of differential display has already been evaluated at 60 % (Bauer et al., 1994) and 45 % (Kuhn, 2001). The RAP-PCR was used to compare mRNA populations expressed by the strain O in YNB + G or YNB + G + GA. Among 8 differentially expressed bands from our first screening, no one has shown a reproducible differential expression after further repetitions. The cDNA-AFLP was also applied on the same model after some optimization (primer combinations and polymerase selection). Our preliminary results indicate a better reproducibility for the cDNA-AFLP as already described by Jones & Harrower (1998) and Gellatly *et al* (2001). After testing 10 primer pairs, one band of interest has been observed. The screening will be completed and the experiment will be repeated for the bands of interest. Bands reproducibly observed will be characterized. Furthermore, thanks to the weak amount of total RNA needed, the cDNA-AFLP could be applied *in vivo* on apple.

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