Biological control of fungal and bacterial plant pathogens
IOBC/wprs Bulletin Vol. 43, 2009
pp. 327-330

cDNA-AFLP analysis of *Candida oleophila* (strain O) genes differentially expressed during the biocontrol of *Botrytis cinerea* on harvested apples

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Abstract: Genes potentially involved in the biocontrol activity of *Candida oleophila* strain O against *Botrytis cinerea* on stored apples were identified by cDNA-AFLP. Gene expression of strain O in the absence (O) and in the presence (OB) of *B. cinerea* spores was compared after 7 h of incubation at 25°C on apple wounds. A total of 1467 (O) and of 1214 (OB) cDNA fragments were visualised using 15 primer pair combinations, the average number of fragments per primer pair being respectively 105 and 87. Of these fragments, 73 were differentially expressed in O compared to OB: 61 down-regulated (4.2%) and 12 up-regulated (1%). Among them, nine sequenced fragments were subjected to real-time RT-PCR to confirm their differential expression. Confirmation was observed for three down-regulated genes (mitochondrial inner membrane transporter, NADP(+)-dependent dehydrogenase, and alcohol dehydrogenase 2) and one up-regulated gene (acid phosphatase).

Key words: biological control, post-harvest, real-time PCR

Introduction

The yeast *Candida oleophila* strain O was isolated from the surface of 'Golden Delicious' apples and selected for its greater and reliable effectiveness against *Botrytis cinerea* and *Penicillium expansum*, two worldwide serious pathogens of stored apples (Jijakli et al., 1993). Its application as soon as possible after harvest is recommended for an optimal biocontrol of post-harvest decay caused by *B. cinerea* (Bajji & Jijakli, 2007). While colonization of apple wound sites seems to be a prerequisite to control *B. cinerea* (Jijakli et al., 1993), the molecular aspects of this biocontrol activity is still unknown. Differential gene expression methods are generally used to unravel the complex molecular mechanisms underlying such a multifactorial process.

cDNA-AFLP method (Bachem et al., 1998) is a non-biased PCR technique offering high reproducibility and sensitivity; it is very appropriate for non-model species such as *C. oleophila* as it requires no prior sequence information. This method was first used to isolate genes of *C. oleophila* strain O induced by galacturonic acid *in vitro* (Massart et al., 2002). It was then developed and applied *in situ* to identify strain O genes potentially involved in the biocontrol of *B. cinerea* (Massart et al., 2004), yeast cells and *B. cinerea* spores being, however, separated by a nitrocellulose filter. In the present work, potential biocontrol genes of *C. oleophila* strain O were identified by cDNA-AFLP in conditions close to those encountered in natural post-harvest infection and their differential expression was validated by real-time PCR.

Material and methods

Microorganisms and fruit material

Before each experiment, *Candida oleophila* strain O was subcultured each day (3 times) on potato dextrose agar (PDA) at 25°C. *Botrytis cinerea* was cultivated for 10 to 15 d on PDA. Apple fruits ('Golden Delicious') were bought from a local market.

Biocontrol assays

Biocontrol assays were carried out essentially as previously described (Bajji & Jijakli, 2007). The model used consisted of apples treated with strain O alone (O), apples treated with strain O and then inoculated with *B. cinerea* (OB), apples inoculated with *B. cinerea* alone (B), and non-treated apples (A). For each assay, the wound content was recovered by pipeting and the lesion diameters were recorded after 7 h and 7 d of incubation at 25°C. Four independent assays were performed and the protection level of strain O against *B. cinerea* was evaluated for each one.

RNA extraction

Only samples recovered from assays with high levels of protection were considered for total RNA extraction. This was performed using the RiboPure™-Yeast Kit (Ambion), including DNase treatment. RNA concentration was quantified and its purity and quality were checked.

cDNA-AFLP

Double-stranded cDNAs were synthesized using the BD SMART™ PCR cDNA Synthesis Kit (Clontech). They were then digested, ligated, pre-amplified and selectively amplified using the AFLP® Analysis System for Microorganisms and the AFLP Microorganism Primer Kit (Invitrogen). Fifteen primer pair combinations (*Eco*RI+1/*Mse*I+2) were tested. *Eco*RI+1 primers were labelled with [γ^{33}P] dATP. Amplified products were separated on a 5% denaturing polyacrylamide gel. Gels were dried before autoradiography.

Characterization of cDNA fragments

Bands of interest were cut from the gel and re-amplified with the appropriate (selective) primers. Re-amplified fragments were visualized on agarose gel, purified (QIAEX II Agarose Gel Extraction Kit, QIAGEN), cloned using the GenJET PCR Cloning Kit (Fermentas) and then sent for sequencing (GATC Biotech). Database searches were performed using the Blastx program (NCBI). The origin of the fragments was determined by PCR using specific primers and DNA extracted from strain O cells, a culture of *B. cinerea*, and from apple leaves.

Real-time RT-PCR

Two independent RNA extracts (including the one used for cDNA-AFLP) were considered for each sample (O, OB, B and A). Specific primers were designed for each sequenced fragment (Primer3). First strand cDNA synthesis, amplification and real-time quantification (SYBR Green), PCR efficiency determination (LinRegPCR program), relative expression evaluation ($\Delta\Delta$Ct method), and statistical analysis (ANOVA) were performed as previously reported (Massart & Jijakli, 2006). Actin was used for data normalization.

Results and discussion

In the present work, we used cDNA-AFLP method to identify *C. oleophila* strain O genes modulated during the biocontrol of *B. cinerea* on apple wounds. A key point in the success of such a method is the selection of a relevant comparison model. The first possible model may be the comparison of gene expression of contrasting strains of the same species regarding their biocontrol activity. In our case, we did not identify any reliable non-antagonistic strain against *B. cinerea* on stored apple at 25°C to be compared with strain O (our unpublished

data, Massart et al., 2004). The other possible model is the comparison of gene expression of the studied biocontrol agent in contrasting conditions. Here, we compared strain O in the absence and in the presence of *B. cinerea* conidia on apple wounds. Such an *in situ* model in which the triple interaction antagonist-pathogen-host is considered reflects more adequately the "natural" situation.

cDNA-AFLP was applied on RNA extracted from the assay which displayed a compromise between the protection level and RNA quantity and quality. Sampling of the apple wound content was performed after 7 h of incubation, which corresponds to mid-exponential phase of strain O growth in apple wounds. Band patterns of strain O in the absence (O) and in the presence (OB) of *B. cinerea* conidia were compared considering those obtained for the two controls: *B. cinerea* alone (B) and untreated apples (A).

Using 15 primer pair combinations, approximately 105 (O) and 87 (OB) bands were visualized per primer pair, resulting in a total number of inspected bands of 1467 and 1214, respectively. Of these bands, 73 were differentially expressed in OB compared to O: 61 down-regulated (4.2%) and 12 up-regulated (1%). The down-regulated bands were excised from O lanes and the up-regulated ones from OB lanes. Once the elution and re-amplification steps done, 45 out of 73 fragments of interest were retained for cloning and sequencing, giving rise to 32 useable sequence data.

Sequenced cDNA fragments could originate either from strain O cells, *B. cinerea* and/or apple tissues. The determination of fragment origin by PCR revealed that 17 out of 32 were of strain O origin. Among them, nine fragments (six down-regulated and three up-regulated) were subjected to real-time RT-PCR to check whether or not cDNA-AFLP data are confirmed by another independent method. Down-regulation was confirmed for three of six fragments while up-regulation was validated for one of the three tested fragments. Sequence analysis of the four fragments for which the differential expression was confirmed are likely to encode for the following proteins: mitochondrial inner membrane transporter, NADP(+)-dependent dehydrogenase, acid phosphatase, and alcohol dehydrogenase 2 (Table 1).

Table 1. Sequence analysis of *Candida oleophila* strain O fragments identified by cDNA-AFLP and validated by RT-PCR.

Size (bp)	Homology[a]	Species	Accession	E-value	Expression[b]
238	Mitochondrial inner membrane transporter	*Candida albicans*	Q6BX67	$1x10^{-17}$	Down
381	NADP(+)-dependent dehydrogenase	*Saccharomyces cerevisiae*	NP_013953	$6x10^{-40}$	Down
160	Acid phosphatase	*Candida albicans*	XP_722656	$8x10^{-8}$	Up
398	Alcohol dehydrogenase 2	*Candida albicans*	XP_717649	$2x10^{-56}$	Down

[a]Based on Blast homology comparison.
[b]Expression in the presence of *Botrytis cinerea* after 7 h of incubation on apple wounds.

In conclusion, cDNA-AFLP was used to search for *C. oleophila* strain O genes of biocontrol relevance against *B. cinerea* on apples. Four fragments for which the differential expression was confirmed by real-time RT-PCR were identified. Based on their homology, these fragments have primary metabolism functions and could be involved in strain O colonisation of apple wounds, a prerequisite to control *B. cinerea* (Jijakli et al., 1993). The

identification of chemicals able to modulate their expression could improve the efficacy of strain O against *B. cinerea* on post-harvest apple fruits.

Acknowledgements

This work was financially supported by BioNext sprl.

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