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Identification of differentially expressed genes from cherry tomato fruit (*Lycopersicon esculentum*) after application of the biological control yeast *Cryptococcus laurentii*

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ABSTRACT

Postharvest decay of fruit may be controlled by the use of a variety of diverse microorganisms acting as biocontrol agents, but the mechanisms associated with control are not fully understood. In order to gain insight into the action of antagonistic microorganisms on fruit, a forward subtractive suppression hybridization (SSH) cDNA library was constructed. SSH was performed with cDNA from cherry tomato fruit (*Lycopersicon esculentum*) inoculated with water as the "driver" and cDNA from tomato fruit inoculated by *Cryptococcus laurentii* as the "tester". A total of 150 clones in the SSH library were sequenced and found to represent 50 unigenes. BLASTX results reveal that 35 cDNAs had significant sequence homologies with known sequences in the NCBI database. The identified cDNAs encode proteins involved in cellular processes such as primary metabolism, signal transduction, defense and responses to pathogens, stress-related, cell wall assembly, and photosynthesis and transcription related sequences. Six cDNA clones were selected for temporal expression analysis using RT-PCR. The results show that a number of transcripts encoding proteins/enzymes which are known to be up-regulated under some biotic and abiotic stresses are also up-regulated after the application of biological control yeast to cherry tomato fruit. The expression of these proteins may play a role in increasing fruit resistance to postharvest pathogen infection.

1. Introduction

Microbial biocontrol agents have been shown to have potential as an alternative to synthetic fungicides, either alone or as part of an integrated pathogen management program (approach) for the control of postharvest decay of fruit and vegetables (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992). Several biocontrol agents, which include yeast and bacteria, have been investigated and shown to protect against a number of postharvest pathogens on a variety of harvested commodities. For postharvest applications on pome and citrus fruit, a yeast, *Candida oleophila* (Aspire), and two strains of the bacterium, *Pseudomonas syringae* ESC-10 and ESC-11 (Biosave-10 and Biosave-11) have been commercialized (Janisiewicz and Jeffers, 1997; Droby et al., 1998).

Antagonistic yeasts have been selected for use as postharvest biocontrol agents for three reasons: firstly, their activity does not involve the production of toxic metabolites, which could have a negative impact on the environment and animals; secondly, they are capable of growing rapidly in surface wounds; thirdly, they appear to be less sensitive than many postharvest rot fungi to chemical fungicides. Control of plant pathogenic fungi by antagonistic yeasts often involves several modes of action including competition for nutrients and space, production of hydrolytic enzymes, direct contact, and possibly induction of plant resistance (Janisiewicz and Korsten, 2002). Another possible mode of action of the yeast antagonist has also been suggested. In apple wounds infected by *Botrytis cinerea* Per.ex Fr. and *Penicillium expansum* Linkex Thom, the yeast *Cryptococcus laurentii* (Kufferath) Skinner may out-compete the wound pathogens by tolerating oxidative stress (Castoria et al., 2003).

There is also evidence indicating that antagonistic yeasts are capable of inducing resistance mechanisms in host tissue. The yeast *C. laurentii* (Kufferath) Skinner may control a postharvest disease of jujube fruit by producing β-1,3-gucanase, a cell wall degrading enzyme involved in plant host defense (Tian et al., 2007). Similarly, Fan et al. (2002) demonstrated that *Pichia membranefaciens* (Hansen) Hansen and *Candida guilliermondii* (Cast) Langeronet Guerra, two antagonistic yeasts, could produce chitinase and β-1,3-glucanase *in vitro* and induce an increase in β-1,3-glucanase and chitinase activities in the wounds of nectarine fruit, resulting

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in an effective decrease in decay caused by *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. In peach fruit infected by *P. expansum* Linkex Thom, the yeast *P. membranefaciens* Hansen induces a number of proteins related to host defense mechanisms (Chan et al., 2007). Furthermore, the yeast *P. membranefaciens* induces production of H_2O_2-metabolizing enzymes and total protein synthesis and reduces oxidative stress in harvested sweet cherry fruit (*Prunus avium*) (Chan and Tian, 2006; Xu and Tian, 2008).

Recently, we have isolated a yeast, *C. laurentii*, from the surface of pear fruit and demonstrated that it effectively controlled decay of pear, peach, and sweet cherry (Zhang et al., 2005, 2007a,b). *In vivo* studies revealed that *C. laurentii* is capable of inducing the accumulation of resistance-related enzymes (Tian et al., 2007). In order to maximize the potential use of *C. laurentii* for the control of postharvest decay of fruit, the mechanism of the action has to be characterized. Therefore the objectives of this research were to investigate the molecular responses of cherry tomato fruit after they were treated with the biocontrol yeast *C. laurentii.* To achieve this goal we used suppression subtractive hybridization for the analysis of differential gene expression.

2. Materials and methods

2.1. Plant material

Cherry tomato fruit (*Lycopersicon esculentum* Mill. cv. Miny Tomato) were collected at the mature green stage MG4 from tomato plants grown under greenhouse conditions.

2.2. Yeast inoculation

Yeast were grown on nutrient yeast dextrose agar (NYDA) medium (containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar in 1 L distilled water) at 28 °C. Liquid cultures of yeast cells were grown for 16 h at 28 °C on a rotary shaker at 200 rpm in 250 mL flasks containing 50 mL of nutrient yeast dextrose broth (NYDB). Cells were then collected by centrifugation at 7000 × g for 10 min and washed twice with sterile distilled water to remove the growth medium. Cell numbers were determined using a hemocytometer and diluted with sterile distilled water as required.

Fruit freshly collected from the greenhouse were surface sterilized by a 10 min incubation in a 1% NaClO solution and subsequently washed in distilled water three times. Fruit were air-dried and wounded (5 mm diameter and approx. 3 mm deep) using a sterile borer at one point around the equator. 5 μL of suspensions of 1×10^8 cell mL^{-1} yeast were inoculated in the wound. Control fruit were either intact (ripening control) or inoculated with 5 μL of sterilized water (wounding control). Inoculated fruit and the corresponding ripening and wounding controls were maintained at 20 °C and 85–90% RH. Pericarp tissue from around the infection site was collected 24 h after inoculation, then frozen immediately in liquid nitrogen and stored at −80 °C. Pericarp from control fruit was also collected and processed in the same way.

2.3. RNA preparation

Total RNA was isolated from both *C. laurentii*-treated tomato fruit pericarp and control tomato fruit using Trizol reagent. The RNA yield and quality were determined spectrophotometrically at 260 and 280 nm and adjusted to a final concentration of 1 μg μL^{-1}. The RNA integrity was checked on 1.5% (w/v) agarose/formaldehyde gels. Poly(A)$^+$ RNA was enriched from 5 μg total RNA using an Oligotex kit (Qiagen Oligotex mRNA mini kit).

2.4. Suppression subtractive hybridization (SSH)

SSH was performed using the PCR-select SSH kit of Clontech (TaKaRa) according to the manufacturer's protocols. In the forward-subtracted library the cDNA from the yeast-treated fruit was used as the "tester" and in the reverse-subtracted library the cDNA from sterile ones was used as the "driver".

Two rounds of SSH and PCR amplification were carried out to normalize and enrich the differentially expressed cDNAs. Products of the second-round PCR from the forward and reverse subtraction were directly inserted into p-GEMT vector (Promega, USA). The ligation mixture was then transformed into *Escherichia coli* DH5α cells and cultured at 37 °C overnight on a LB media plate containing ampicillin and X-Gal/IPTG. The white clones were selected to construct the subtracted cDNA library, each of which contained more than 1600 clones. Those white colonies were obtained and single colonies were grown overnight in 1 mL of liquid LB medium with 100 g mL^{-1} ampicillin. Bacterial colonies were stored at −80 °C as glycerol stocks for PCR amplification of the cDNA inserts.

2.5. PCR amplification of cDNA inserts

The recovered clones from glycerol stock were grown in a 96-well plate in LB medium containing ampicillin (100 μg mL^{-1}) for 2 h at 37 °C. A master mix containing the nested primers 1 and 2R and Taq DNA polymerase (TaKaRa) was added to 1 μL of bacteria culture. Thermal cycling was conducted under the following conditions: 94 °C for 2 min, then 23 cycles of 94 °C for 20 s and 68 °C for 3 min. PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and photography, and then purified with Qiaquick 96-well purification kit (Qiagen). The purified PCR products were used for further reverse northern microarray analysis.

2.6. Dot blot analysis

Approximately 400 clones from the forward and reverse subtracted libraries were blotted onto hybond N nylon membranes and screened by dot hybridization with the forward and reverse subtracted cDNA as probes. Clones hybridizating with the forward SSH probe were selected from the forward library. The opposite criteria were used for selecting clones from the reverse subtracted library. Preparation of the forward and reverse SSH probes were carried out as described by Diatchenko et al. (1996, 1999). Essentially, the adaptor sequences from the ends of the library clones were removed by restriction enzyme digestion and the purified products were labeled with [a-^{32}P]-dCTP using the random primed labeling kit (TaKaRa).

The cDNA dot blot method was based on the method described by Diatchenko et al. (1999), except that the cDNA inserts were amplified directly from colonies by PCR (primed with primers N1 and N2R in the Clontech PCR-select cDNA subtraction kit). cDNA dot blots were performed in duplicate.

2.7. Clone sequencing and analysis

Plasmid DNA was purified from clones using a DNA Gel Extraction Kit (AXYGEN BIO), and submitted to Invitrogen Co. (Shanghai) for sequence analysis. Reactions were primed with universal forward and reverse primers. Database searches of DNA sequences obtained from the SSH clones were performed using the NCBI (National Center for Biotechnology Information) BLAST servers.

2.8. RT-PCR analysis

Total RNA was isolated as described above. Total RNA used for RT-PCR verification was obtained from fruit that were treated with