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Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display

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Abstract

Citrus unshiu is freeze tolerant to −10 °C when fully acclimated after exposure to cold, nonfreezing temperatures. To gain an understanding of its cold tolerance mechanism, mRNA differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) and quantitative relative RT-PCR were used to study gene expression under a gradual cold-acclimation temperature regime. Six up-regulated and two down regulated genes were identified based on their amino acid sequences. The identified proteins encoded by the up-regulated genes were: 14-3-3 protein, 40S ribosomal protein S23, putative 60S ribosomal protein L15, nucleoside diphosphate kinase III protein, regulator of chromosome condensation-like protein, and amino acid permease 6. The proteins encoded by the two down-regulated genes were: miraculin-like protein and beta-galactosidase. Their individual function has been briefly reviewed based on published information. In addition to the findings in this study, we compared the function of cold responsive genes of *Poncirus trifoliata*, a very cold hardy relative of *Citrus* species that is freeze tolerant to −30 °C when fully acclimated, to the function of genes in the current study.

Keywords: Gene expression; Cold acclimation; 14-3-3 protein; Nucleoside diphosphate kinase; Amino acid permease

1. Introduction

Crop quality and productivity are negatively affected by biotic and abiotic stresses. Low temperature is one of the most common environmental stresses and can potentially cause severe losses to major economically important plants. Disruption of cell membranes is the primary injury associated with freeze-induced dehydration (Thomashow, 1998). Many plants increase their freezing tolerance in response to low, nonfreezing temperatures, a phenomenon known as cold acclimation. Changes in gene expression were suggested to be associated with this process, and many genes related to cold acclimation have been cloned from several plants, including *Arabidopsis* (Gilmour et al., 1992), *Brassica napus* (Orr et al., 1992), and *Poncirus trifoliata* (Zhang et al., 2005a). A number of C repeat binding proteins (CBF) from *Arabidopsis* have been characterized. Overexpression of the gene encoding the protein was shown to increase tolerance of transformed plants to environmental stresses (Jaglo-Ottosen et al., 1998).

Although efforts have been taken to elucidate the cold adaptation mechanism of herbaceous plants, very limited information is available for woody plants under low temperatures. *Citrus* sp. are some of the most important fruit crops throughout the world. Yet, some of the most valued citrus crops are grown in relatively high-risk freeze areas (Yelenosky, 1985). Research has been conducted in some *Citrus* varieties and relatives, and a few genes have been cloned (Cai et al., 1995; Hara et al., 1999; Sanchez-Ballesta et al., 2003; Zhang et al., 2005a). *Citrus unshiu* is

Abbreviations: CBF, C repeat-binding proteins; cDNA, DNA complementary to RNA; AFLP, amplified fragment length polymorphism; DDRT-PCR, differential display reverse transcriptase polymerase chain reaction; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; M-MuLV, moloney murine leukemia virus; Oligo(dT), oligodeoxyribonucleotide thymidine; RNase, ribonuclease; RT, reverse transcriptase; SAGE, serial analysis of gene expression.

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considered one of the most cold hardy commercial *Citrus* species (Yelenosky, 1985), but the changes in gene expression exposed to a gradually declined temperature regime, which mimics the natural temperature changes in the southeastern-U.S., have not been studied. Transcriptome profiling of plants to environmental stresses can be studied using different techniques, which include differential display reverse transcription PCR (DDRT-PCR), serial analysis of gene expression (SAGE), subtractive hybridization, DNA-chip, and cDNA microarray. mRNA differential display has been widely used to identify genes whose expression levels have been altered under different environmental conditions because of its technical simplicity and lack of requirement for previous genomic information of the species of interest (Liang and Pardee, 1992; Carginale et al., 2004). DDRT-PCR was used to clone genes in *C. unshiu* following a gradual cold acclimation regime. The identified genes will provide insights into the adaptation of important fruit crops to low temperature and may lead to strategies to develop transgenic citrus with enhanced levels of cold tolerance.

2. Materials and methods

2.1. Plant growth conditions

One year old *Citrus unshiu* plants were grown for 5 weeks in a growth chamber with a 12 h light period at 400 μmol $m^{-2}s^{-1}$ intensity. The regimen for temperature decline was as follows: 32 °C day/21 °C night for 14 days; 27 °C day/16 °C night for 7 days; 24 °C day/13 °C night for 7 days and 18 °C day/7 °C night for 7 days. Plants were uniformly watered every day.

2.2. RNA extraction and mRNA differential display

Fully expanded leaves at the end of the second and fifth weeks were collected, immediately immersed in liquid nitrogen and stored at −80 °C for later use. RNA was extracted from leaves according to the RiboPure kit protocol (Ambion, Austin, TX). Extracted RNA was mixed with 1/9 volume of 10X DNase buffer and 4 μl DNase I (2U/μl) and incubated for 30 min at 37 °C to digest the remaining genomic DNA. Digested RNA was treated with DNase inactivation reagent (20% volume) for 2 min, followed by centrifugation for 1 min at 14 000 *g* and transferred to a new tube. The concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). The quality of RNA was checked using formaldehyde-agarose gel electrophoresis. RNA from the end of second week was used as an unacclimated control and RNA from the end of fifth week as treatment. mRNA differential display was performed using RNAimage kits and 64 primer pairs according to the protocol supplied with this kit (GenHunter, TN). 0.2 μg of RNA was reverse transcribed in a 20 μl reaction mixture at 42 °C for 60 min with M-MuLV reverse transcriptase (GenHunter). Amplification of cDNA fragments was performed in a 20 μl reaction mixture containing 2 μl of the reverse transcribed cDNA, 0.2 μM arbitrary primer (GenHunter), 0.2 μM anchored oligo (dT)-primers (H-T11M, where M = A, G, C), 2 μM of each dNTP, 10 mM Tris-Cl, pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 μl α-[S^{35}] dATP and 1 U *Taq* DNA polymerase (Qiagen, CA). The PCR program consisted of 40 cycles: 30 s at 94 °C, 2 min at 40 °C, 1 min at 72 °C; and a final 5 min elongation step at 72 °C. Amplified PCR products were separated in a 6% denaturing PAGE gel. The gel was transferred to filter paper (Whatman, England) and dried at 80 °C for 1 h in a gel dryer (BioRad, CA). PCR products on filter paper were exposed to BioMax Kodak film covered with two intensifying screens for 24 to 72 h in a −80 °C freezer. The film was developed and the differentially expressed bands between control and treatment were excised from the filter paper according to the pattern on the film. The PCR products were extracted according to the GenHunter protocol, and reamplified using the original primer pair.

2.3. cDNA-AFLP analysis

cDNA was synthesized using RETROscript™ (Ambion, TX) according to the manufacturer's instructions, and digested using the *Mse*I/*Eco*RI enzyme combination. AFLP analysis was conducted according to the protocol of AFLP kit from Li-COR (Li-COR Biosciences, NE). Sequences of the adapters and primers used for cDNA AFLP analysis were: 5′-GACGATGAGTCCTGAG-3′ (*Mse*I adapter 1); 5′TACTCAGGACT CAT-3′ (*Mse*I adapter 2); 5′-CTCGTAGACTGCGTACC-3′ (*Eco*RI adapter 1); 5′-AATTGGTACGCAGTCTAC-3′ (*Eco*RI adapter 2); 5′-GATGAGTCCTGAGTAAC-3′ (non-selective primer for *Mse*I); 5′-GACTGCGTACCAATTCA-3′ (non-selective primer for *Eco*RI); 5′-GATGAGTCCTGAGTAACNN-3′ (selective primer for *Mse*I, and NN represents 2bp extension); 5′-GACTGCGTACCAATTCANN-3′ (selective primer for *Eco*RI, and NN represents 2bp extension). Preamplification was performed in a 25 μl reaction solution, containing 0.2 mM dNTPs, 0.2 μM non-selective primers, 1.2mM $MgCl_2$, 0.5U *Tag* polymerase (Invotrogen, CA) and 10 μl ligated cDNA fragments. The PCR program consists 20cycles: 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. Selective amplification was performed in a 10 μl reaction solution, containing 0.1 mM dNTPs, 0.15 μM *Eco*RI primer and 0.3 μM *Mse*I primer, 1.2 mM $MgCl_2$, 0.5 U *Tag* polymerase (Invitrogen) and 2.5 μl pre-amplification solution (diluted at 1:40). The PCR program was carried out with the following procedure: one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; 13 cycles with annealing temperature decreasing 0.7 °C per cycle followed by 23 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. Selective reaction products were run on 6% polyacrylamide