Tree and Forestry Science and Biotechnology ©2009 Global Science Books

The Application of Differential Display as a Gene Profiling Tool in *Citrus*

Viviana De Luca[1] • Serena Rizza[2] • Giuliana Catara[1] • Clemente Capasso[1] • Antonio Capasso[1] • Vincenzo Carginale[1*]

[1] CNR Institute of Protein Biochemistry, Via P. Castellino, 111, I-80131, Naples, Italy
[2] Department of Phytosanitary Sciences and Technologies, University of Catania, Via S. Sofia, 100, I-95123, Catania, Italy

Corresponding author: * **v.carginale@ibp.cnr.it**

ABSTRACT

Citrus (*Citrus* spp.) is one of the most important and widespread fruit crops, with great economic and health value. However, it is among the most difficult plants to improve through traditional breeding approaches. In this context, the recent advent of genomic technology and of powerful new tools, such as differential display (DD-PCR), serial analysis of gene expression (SAGE), and cDNA microarray, have greatly increased the study of biological processes relevant to citriculture, ranging from developmental biology, to biotic and abiotic stresses, and post-harvest processes. This mini-review deals with the application of the DD-PCR technique to investigate gene expression in *Citrus*. The findings of various research groups on the isolation and profiling of genes expressed in *Citrus* during physiological events, and biotic and abiotic stress conditions with this methodology are described. Even genes expressed at very low levels, such as transcriptional factors, membrane proteins, and rare enzymes have been successfully isolated using this approach. These results suggest that DD-PCR is a very suitable method for investigating rare genes involved in life cycle of plants, especially when genomic sequence information of the species is not available.

Keywords: genomic technologies, plant gene, RNA silencing, stress response

CONTENTS

INTRODUCTION

Citrus fruits are ranked the first fruit tree crop in international trade in terms of economic value, with an annual production estimated at about 100 million tons in the period of 2004-2005 (see www.fao.org). Citrus fruits are typically produced in 140 countries located in tropical and subtropical areas, where the winter temperatures are adequate for tree survival and where there is sufficient water and suitable soils to support tree growth and fruit production (Li *et al*. 2007; Talon *et al*. 2008).

Citrus species are susceptible to different pathogens and pests that are responsible for severe losses worldwide. The survival of the citrus industry is critically dependent on genetically superior cultivars. However, citrus improvement through traditional techniques is unfortunately very difficult due to the unusual combination of biological characteristics of *Citrus* species (apomixis, sexual incompatibility, extended juvenility), their low genetic diversity, and the long-term and expensive nature of tree breeding (Spiegel-Roy *et al*. 1996; Forment *et al*. 2005).

To improve and achieve faster results in citrus breeding, new approaches are being explored that will allow for better and faster acquisition of knowledge on the genetics of this group of species, as well as on the relationship with their pathogens. Genomic technology allows for the determination of when and where a gene is to be turned on or off in a cell as it divides, differentiates, and ages.

The first steps towards the identification and characterization of the genes that are differentially expressed in developmental and environmentally regulated processes is large-scale gene analysis of the transcriptome. Although are now available other gene expression analysis techniques, such as SAGE and cDNA microarray, PubMed (www.ncbi.nlm.nih.gov) continues to log hundred of papers per year in which DD-PCR is utilized in primary research. The technique was invented in 1992 by Liang and Pardee to allow for rapid, accurate, and sensitive detection of changes in gene expression in diverse biological systems. The power of differential display lies in its simplicity. It is based on three of the most commonly used molecular biology techniques: RT-PCR, polyacrylammide-gel electrophoresis and cDNA cloning. The expected advantages are numerous: it has a good sensitivity to low-abundance mRNA transcripts; both induced and repressed genes can be simultaneously detected; more than two samples can be compared; unlike other genomic approaches, such as DNA microarray, it detects changes in mRNA profiles among multiple samples being compared without the need of any prior knowledge of genomic sequence information of the species being studied (Liang *et al*. 1992, 2007).

In the last few years, DD-PCR has produced a massive amount of literature detailing the isolation of genes from plant species that are involved in physiological events, sig-

Received: 24 July, 2008. Accepted: 21 November, 2008.

Invited Mini-Review

nal transduction, stress response, and secondary metabolism (Yamazaki and Saito 2002; Carginale *et al*. 2004; Basile *et al*. 2005; Zhang *et al*. 2008). In this review, we focus our attention to the application of this technique in studying citrus gene expression.

GENE EXPRESSION IN PHYSIOLOGICAL EVENTS

Analyses of citrus gene expression performed with DD-PCR methodology in various physiological events are summarized in **Table 1**. Jia *et al*. (2005) isolated cDNA coding for a putative terpene synthase (Grtps) from mature fruits of "Rio Red" grapefruit. This gene appears to be a sesquiterpene synthase and it is not expressed in immature fruits, roots, or leaves, but only in mature fruits. This suggests that Grtps is developmentally regulated and occurs only at the final stage of fruit maturation.

A cDNA named CsHPt1 (for *Citrus sinensis* histidine phosphotransmitter protein 1) was isolated from globular embryos of 'Valencia' sweet orange by Maul *et al*. (2006). CsHPt1 is part of two-component signal transduction systems found in numerous prokaryotic and eukaryotic organisms (Marchler-Bauer *et al*. 2003). These systems consist of a histidine protein kinase, localized in the cell membrane that senses a signal input, and a response regulator that mediates the output. The authors reported that CsHPt1 is induced in embryogenic calli, in globular-stage embryos, and in tissues with embryogenic potential at higher levels than in non-embryogenic calli or in tissues without embryogenic potential. This suggests a role for CsHPt1 as a phosphotransmitter intermediate during very early embryogenesis in *Citrus*.

GENES INVOLVED IN ABIOTIC STRESSES

Application of DD-PCR to the abiotic stress response in *Citrus* is summarized in **Table 1**. Lers *et al*. (1998) isolated an mRNA that is accumulated in grapefruit peel upon UV irradiation. Sequence analysis revealed that this cDNA represents a gene encoding for an isoflavone reductase-like protein highly homologous to isoflavone reductases characterized in legumes and was termed IRL (isoflavone reductase-like). The authors showed that this gene, whose function is not yet clear, is induced also by wounding and pathogen infection.

Porat *et al*. (2002) showed that a short hot water treatment increased chilling tolerance in grapefruit. On performing DD-PCR analysis on heat-treated grapefruits, they isolated cNHX1, a citrus Na^+/H^+ antiport gene that catalyzes the exchange of Na^+ for H^+ across the tonoplast regulating intracellular pH and sodium levels (Kagami and Suzuki 2005). The heat-induced increase in cNHX1 mRNA levels was, however, temporary when the fruits were held at 20°C, and was detected only at the specific time point of 24 h after heat treatment. Unlike the temporary effect that resulted from the heat treatment alone, the increase in cNHX1 mRNA levels induced by the combination of heat and subsequent cold storage was higher and long lasting, and was observed 2 weeks after the heat treatment and until the end of a 6 week cold-storage period.

Lang *et al*. (2005) applied DD-PCR to study gene expression under cold acclimation in *Citrus unshiu*. They identified six up-regulated (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) and two down-regulated (miraculin-like protein and β-galactosidase) genes. The identified genes were mainly related to signal transduction (14-3-3), protein synthesis (S23 and L15), amino acid transport (AAP6), arrangement of chromosome structure (regulator of chromosome condensation-like protein), plant defence (miraculin), and cell wall metabolism (β-galactosidase). A similar approach was adopted by Zhang *et al*. (2005a, 2005b) with *P. trifoliata*, a very cold hardy relative of *Citrus* species. They identified eight genes that are different from those previously identified in *Citrus*. These genes are principally involved in osmotic modulation, photo-oxidative protection, and photosynthesis adjustment.

The molecular basis for the adaptation of fruit tissue to low oxygen treatments remains largely unknown. DD-PCR was employed to isolate anoxic and/or hypoxic genes whose expression responded to short, low-oxygen levels. Porat *et al*. (2004) isolated a dehydrin gene from orange that was down-regulated by exposure to low-oxygen levels. Dehydrins are a family of plant proteins that are induced in response to various environmental stresses (Kosová *et al*. 2007).

Pasentsis *et al*. (2007) were able to isolate 25 transcripts from *Citrus* flavedo tissues subjected to low oxygen regimes. Hybridization experiments revealed that 11 genes were induced under hypoxia and/or anoxia, 11 exhibited constitutive expression, and three transcripts were suppressed by low oxygen levels. Among the up-regulated genes, five of them have no known function, while the remaining six genes are involved in C-compound and carbohydrate utilization, amino acid metabolism, and biosynthesis of brasinosteroids. The down-regulated genes included a cysteine peptidase, a dehydrin (also found to be down-regu-

Table 1 Application of differential display technique in isolating genes involved in various events in Citrus.

Event	Species	Isolated gene or encoded protein	Reference
UV irradiation	*C. paradisi*	isoflavone reductase-like protein	Lers *et al.* 1998
Heat treatment	*C. paradisi*	NHX1	Porat *et al.* 2002
Fruit ripening	*C. sinensis*	putative terpene synthase	Jia *et al.* 2005
Embryogenesis	*C. sinensis*	putative histidine-containing phosphotransmitter protein	Maul *et al.* 2006
Cold	*C. unshiu*	14-3-3 protein, 40S ribosomal protein S23, putative 60S ribosomal protein L15, nucleoside diphosphate kinase III protein, regulator of chromosome condensation-like protein, amino acid permease 6, miraculin-like protein, beta-galactosidase.	Lang *et al.* 2005
Anoxia/hypoxia	*C. sinensis*	dehydrin	Porat *et al.* 2004
	C. flavedo	auxin-induced protein-like, putative phosphatase, putative serine esterase, limonoid UDP-glucosyltransferase, pyruvate decarboxylase, hypoxia- responsive family protein, xylose isomerase family protein, glutamate decarboxylase 4a (GAD), SPX (SYG1/ Pho81/XPR1) domain-containing protein, unknown protein, cytochrome P450, cysteine peptidase, dehydrin, granule-bound starch synthase	Pasentsis *et al.* 2007
Viroid infection	*C. medica*	hypothetical protein, β-galactosidase, extensine-like protein, NADPH-dehydrogenase, metallothionein, NHX1, alcool-dehydrogenase, ERE-binding protein, regulator of gene silencing, OSJNBa0033G05.21 protein, flavonol synthase, prefoldin, Hedgehog interacting protein-like 1, RecQ DNA helicase, aminoacid permease 6, peroxidase, CONSTANS-like protein, hypothetical protein	Tessitori *et al.* 2007
Rind injury	*C. unshiu*	o-methyltransferase, ADP-ribosylation factor-like protein, unknown protein, putative auxin-induced protein, auxin-induced proteinase-inhibitor, mutator-like transposase, putative enongation factor, ribosomal protein L8, rRNA 26S, malate dehydrogenase	Fujisawa *et al.* 2003

lated by Porat *et al*. (2004)), and a granule-bound starch synthase.

Fujisawa *et al*. (2003) applied DD-PCR to detect differentially expressed mRNAs relating to citrus rind injury under various storage conditions. The genes identified shared high similarity with those detected in other plants under various stress conditions. Some auxin-inducible genes, such as a proteinase inhibitor, were also identified.

GENES INVOLVED IN BIOTIC STRESSES

In investigating the molecular mechanisms by which plants respond to pathogen attack, Tessitori *et al*. (2007) utilized DD-PCR and identified genes whose transcription was significantly altered in leaves of Etrog citron infected by *Citrus viroid III* (CVd-III), recently named *Citrus dwarfing viroid* (CDVd). Eighteen genes were identified, thirteen of which were up-regulated by viroid infection (extensin-like protein, metallothionein (MT), alcohol-dehydrogenase (ADH), ethylene-responsive element binding protein (EREBP), regulator of gene silencing (RGS), OSJNBa0033G05.21 protein, flavonol synthase (FLS), prefoldin subunit 2, hedgehog interacting protein-like 1 (HIPL1), recQ DNA helicase, peroxidase, CONSTANS-like protein (COL), and a protein of unknown function), while five were down-regulated (β-galactosidase, NADPH-dehydrogenase, NHX1, amino acid permease 6 (AAP6), and a protein of unknown function). Except for two genes that encode proteins of unknown function, the remaining genes are mainly involved in plant defence/stress responses (MT, ADH, FLS, recQ DNA helicase, peroxidase) signal transduction (HIPL1), amino acid transport (AAP6), protein metabolism (prefoldin), cell wall structure (extensin, β-galactosidase), and other functions (NHX1 and NADPH-dehydrogenase). Two of the up-regulated genes (EREBP and COL) are transcription factors that coordinate downstream gene expression in stress signal transduction pathways. Of particular interest was the finding that the expression level of a cellular suppressor of RNA silencing (RGS) gene is enhanced by CDVd infection. RNA silencing is a key plant defence mechanism against invasive RNA molecules (Bass 2000; Wang *et al*. 2004). RGS induction triggered by CDVd infection supports the hypothesis that viroids can evade host gene silencing by activating endogenous mechanisms that negatively regulate RNA silencing activity (Tessitori *et al*. 2007).

Changes in gene expression of *Poncirus trifoliata*, an interfertile *Citrus* relative, in response to infection with *Citrus tristeza virus* (CTV) were analyzed by Hernandez-Jasso *et al*. (2004) using DD-PCR. Several genes that may be indirectly involved in the global response of the plant to the virus infection, such as glutaredoxin, superoxide dismutase (SOD), and GTP pyrophosphokinase were identified.

CONCLUSIONS

As an alternative to classical genetic breeding, the genes identified with DD-PCR can be used in genetic transformation studies in efforts to improve citrus plants, production, and protection from diseases. Differential display is a powerful technique for isolating genes that are specifically induced or repressed in particular types of cells under diverse stress conditions. The results obtained indicate that plant responses to various stresses involve changes in numerous metabolic and signal transduction pathways. Even genes expressed at very low levels, such as transcriptional factors and rare enzymes, can be successfully isolated using this approach. The genes identified with differential display are, often, different from the genes identified with other genomic tools, thus indicating that all the available techniques can complement each other.

REFERENCES

Basile A, di Nuzzo RA, Capasso C, Sorbo S, Capasso A, Carginale V (2005) Effect of cadmium on gene expression in the liverwort *Lunularia cruciata*. *Gene* **356**, 153-159

Bass BL (2000) Double-stranded RNA as a template for gene silencing. *Cell* **101**, 235-238

Carginale V, Maria G, Capasso C, Ionata E, La Cara F, Pastore M, Bertaccini A, Capasso A (2004) Identification of genes expressed in response to phytoplasma infection in leaves of *Prunus armeniaca* by messenger RNA differential display. *Gene* **332**, 29-34

Forment J, Gadea J, Huerta L, Abizanda L, Agusti J, Alamar S, Alos E, Andres F, Arribas R, Beltran JP, Berbel A, Blazquez MA, Brumos J, Canas LA, Cercos M, Colmenero-Flores JM, Conesa A, Estables B, Gandia M, Garcia-Martinez JL, Gimeno J, Gisbert A, Gomez G, Gonzalez-Candelas L, Granell A, Guerri J, Lafuente MT, Madueno F, Marcos JF, Marques MC, Martinez F, Martinez-Godoy MA, Miralles S, Moreno P, Navarro L, Pallas V, Perez-Amador MA, Perez-Valle J, Pons C, Rodrigo I, Rodriguez PL, Royo C, Serrano R, Soler G, Tadeo F, Talon M, Terol J, Trenor M, Vaello L, Vicente O, Vidal C, Zacarias L, Conejero V (2005) Development of a citrus genome-wide EST collection and cDNA microarray as resources for genomic studies. *Plant Molecular Biology* **57**, 375-391

Fujisawa H, Minami M, Ogata T, Takahara T (2003) Isolation and analysis of mRNAs relating to citrus rind injury under various storage conditions**.** *Journal of the Japanese Society for Horticultural Science* **72**, 7-12

Hernández-Jasso JC, da Graça JV, Louzada ES (2004) Identification of differentially expressed genes from *Poncirus trifoliata* triggered by *Citrus tristeza virus* inoculation. *Subtropical Plant Science* **56**, 26-32

Jia Y, Xia D, Louzada ES (2005) Molecular cloning and expression analysis of a putative terpene synthase gene from citrus. *Journal of the American Society for Horticultural Science* **130**, 454-458

Kagami T, Suzuki M (2005) Molecular and functional analysis of a vacuolar Na+/H+ antiporter gene of *Rosa hybrida*. *Genes and Genetic Systems* **80**, 121-128

Kosová K, Vítámvás P, Prášil IT (2007) The role of dehydrins in plant response to cold. *Biologia Plantarum* **51**, 601-617

Lang P, Zhang CK, Ebel RC, Dane F, Dozier WA (2005) Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. *Gene* **359**, 111-118

Lers A, Burd S, Lomaniec E, Droby S, Chalutztz E (1998) The expression of a grapefruit gene encoding an isoflavone reductase-like protein is induced in response to UV irradiation. *Plant Molecular Biology* **36**, 847-856

Li H, Futch SH, Stuart RJ, Syvertsen JP, McCoy CW (2007) Associations of soil iron with citrus tree decline and variability of sand, soil water, pH, magnesium and *Diaprepes abbreviatus* root weevil: Two-site study. *Environmental and Experimental Botany* **59**, 321-333

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971

Liang P, Meade JD, Pardee AB (2007) A protocol for differential display of mRNA expression using either fluorescent or radioactive labeling. *Nature Protocols* **2**, 457-470

Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, Bryant SH (2003) CDD: a curated Entrez database of conserved domain alignments. *Nucleic Acids Research* **31**, 383-387

Maul P, Bauscher M, McCollum G, Mozoruk J, Niedz R (2006) CsHPt1, a putative histidine-containing phosphotransmitter protein induced during early somatic embryogenesis in Valencia sweet orange. *Plant Science* **170**, 44-53

Pasentsis K, Falara V, Pateraki I, Gerasopoulos D, Kanellis AK (2007) Identification and expression profiling of low oxygen regulated genes from citrus flavedo tissues using RT-PCR differential display. *Journal of Experimental Botany* **8**, 2203-2216

Porat R, Pavoncello D, Ben-Hayyim G, Lurie S (2002) A heat treatment induced the expression of a Na^+/H^+ antiport gene (cNHX1) in citrus fruit. *Plant Science* **162**, 957-963

Porat R, Pasentsis K, Rozentzvieg D, Gerasopoulos D, Falara V, Samach A, Lurie S, Kanellis AK (2004) Isolation of a dehydrin cDNA from orange and grapefruit citrus fruit that is specifically induced by the combination of heat followed by chilling temperatures. *Physiologia Plantarum* **120**, 256-264

Spiegel-Roy P, Goldschmidt EE (1996) *Biology of Citrus*, Cambridge University Press, 240 pp

Talon M, Gmitter FG (2008) Citrus genomics. *International Journal of Plant Genomics* **2008**, 528361

Tessitori M, Maria G, Capasso C, Catara G, Rizza S, De Luca V, Catara A, Capasso A, Carginale V (2007) Differential Display analysis of gene expression in Etrog citron leaves infected by Citrus viroid III. *Biochimica et Biophysica Acta* **1769**, 228-235

Wang MB, Bian XY, Wu LM, Liu LX, Smith NA (2004) On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proceedings of the National Academy of Sciences USA* **101**, 3275-3280

Yamazaki M, Saito K (2002) Differential display analysis of gene expression in plants. *Cellular and Molecular Life Science* **59**, 1246-1255

Zhang C, Lang P, Dane F, Ebel RC, Singh NK, Locy RD, Dozier WA (2005a) Cold acclimation induced genes of trifoliate orange (*Poncirus trifoliata*). *Plant Cell Reports* **23**, 764-769

Zhang C, Lang P, Ebel RC, Dane F, Singh NK, Locy RD, Dozier WA (2005b) Down-regulated gene expression of cold acclimated *Poncirus trifoliata*. *Canadian Journal of Plant Science* **85**, 417-424

Zhang J, Turley RB, Stewart JM (2008) Comparative analysis of gene expression between CMS-D8 restored plants and normal non-restoring fertile plants in cotton by differential display. *Plant Cell Reports* **27**, 553-561