Journal of Tropical Plant Physiology 1(1) (2006): 100-107

ISOLATION OF RIPENING-RELATED GENES OF *CAPSICUM ANNUUM* VIA A DIFFERENTIAL DISPLAY TECHNIQUE

Zamri, Z.[1*], Roslinda, S.[2] and Ismanizan, I.[1]

[1*]School of Biosciences and Biotechnology, Faculty of Science and Technology Universiti Kebangsaan Malaysia, 43000, Bangi, Selangor Darul Ehsan, and [2]Biotechnology and Strategic Research Unit, Malaysian Rubber Board Sungai Buloh, Malaysia.

ABSTRACT

A mRNA differential display was established to allow the isolation and identification of genes induced during chili fruit ripening grow under normal conditioned. Twenty eight PCR- fragments from the differential display were identified using twenty four primer combinations. Only four cDNA fragments were successfully cloned and sequenced. Database searches revealed that CUKMD1 encodes ζ-carotene desaturase and CUKMD2 was identified as encoding O-methyltransferases. The fragment CUKMD3 shares 100% homology at amino acid level to catalase isolated from *Capsicum annuum*. While CUKMD4 showed considerable homology with an amino acid transporter. These studies showed that mRNA differential display is a powerful tool to facilitate the isolation and identification of genes induced during chili fruit ripening.

Key words: *Capsicum annuum*, differential display, fruit ripening, catalase, amino acid transporter

INTRODUCTION

Fruit ripening has received considerable scientific scrutiny because of the uniqueness of the processes and also due to the importance of fruits as a significant component of human diet, mainly as a source of energy, vitamins, mineral and antioxidants (Giovannoni 2003). Ripening is a multi-faceted developmental program, which involves changes in fruit texture, color and flavor. During fruit development and maturation, both physical and morphological changes are often a result of changes in protein concentration and activities, which may reflect shifts in overall mRNA abundance. Those changes are regulated at gene level and control led by specific genes. The application of molecular techniques in order to understand fruit ripening has enabled the isolation and characterization of a large range of cDNA clones which show increased expression during ripening. The genes that cause such changes can be cloned and further studied. One of the useful approaches which can be employed to investigate such changes is by isolating mRNA transcripts encoding proteins associated with the ripening process via differential display techniques. This approach has been successfully applied to analyses plant gene expression in various physiological events including fruit ripening, signal transduction, stress response and secondary metabolism (Yamazaki

& Saito 2002). Current understanding of fruit ripening mostly based on tomato as a model crop, in part because of available mutants, excellent genetics and routine transformation. However, processes that contribute to the overall phenomenon of fruit ripening remain elusive for many fruit species.

Chili (*Capsicum annuum* L.), is the most popular and also important commercial fruit vegetable grown in Malaysia. The area cultivated with chili is approximately 4,000 hectares with a production of about 40,000 tones annually. However, Malaysia continues to import more than RM40 million worth of chili to accommodate its domestic demand. *Capsicum* is popular with consumers both as fresh fruit and in the processed form. Despite the obvious commercial potential, there are several constrains delimiting its extensive commercialization. These constrains are partly due to rapid post-harvest deterioration of its fruits and susceptibility of the chili plant towards virus infection. The aim of this research is to identify genes which are correlated with ripening in *C. annumm* by differential display. Previously, this technology has been proven reliable and efficient for identifying and cloning differentially expressed genes (Liang & Pardee 1992).

We report here the isolation of several ripening-related genes that are differentially expressed during chili fruit ripening by differential display technique. Once the function of a gene was identified it will possible to improve chili fruit quality through direct gene transfer technology.

MATERIALS and METHODS

Plant material and methods

Chili fruits of variety `MC11 were obtained from MARDI Jln. Kebun, Kelang. Only mature and good quality fruits were chosen for experimental work. All fruits were collected, grouped into five different stages of ripening (0% mature green, 25% breaker, 50% red, 75% red and 100% red ripening) according to coloration. The fruits were cleaned, weighed and immediately frozen in liquid nitrogen followed by storage at -70 °C prior to RNA extraction.

RNA extraction of chilli fruit

Total RNA from five different stages of fruit ripening was extracted according to Lopez-Gomez (1992) with slight modification made to the extraction buffer (4M thiocyanate was included). RNA was precipitated with 4M lithium chloride and the pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA concentration and purity was determined spectrophotometrically. Integrity of RNA was evaluated by fractionation on 1.2% agarose gel. The total RNA samples were stored at –80°C prior to differential display experiment.

mRNA differential display

Differential display was carried out using total RNA samples that were isolated and pooled from four stages of ripening. These RNA samples were subsequently used as templates in three reverse transcription reactions employing $HT_{11}M$ anchored primers (where M is degenerate A, C and G). Eight 13 mers "arbitrary" primers (HAP9 to HAP16) were used in combination with respective $HT_{11}M$ primers according to the manufacturer's recommendation (GenHunter). Total RNA samples were DNAse I treated using RNAse free DNAse I . DNAse free total RNA samples (5ug) were used for first strand cDNA synthesis. Amplified cDNAs were electrophoresed through a 6% denaturing polyacrylamide gel containing 7M urea. Electrophoresis was done at constant 60W for 3-4 h followed by drying the gels on Whatman 3MM without fixing.

Isolation and cloning of cDNA bands

Autoradiograms and gels were aligned and appropriate bands identified on the autoradiographs were excised. Differentially displayed cDNAs were recovered by eluting into 50µl sterile water and heating at 95°C for 5 min. cDNAs were then reamplified using the same PCR conditions set using primers as before. PCR products were then analysed on a 1.5% agarose gels. cDNAs were purified from agarose slices and subsequently cloned into pGEMT Easy vector (Promega) system according to the instruction of the manufacturer (Promega)

.

DNA sequence and sequence analysis

The cDNA inserts were sequenced on the ABI 310 DNA sequencer (Applied Biosystems, USA) using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Applied Biosystems).DNA sequence data were analyzed using DNA STAR software. DNA homology searches against the GenBank database were performed with the BLAST network services at National Centre for Biotechnology Information (Altschul et al. 1990).

RESULTS AND DISCUSSION

The changes in gene expression during chili fruit ripening at the transcriptional level was investigated using the mRNA differential display technique. Differential display involves random amplification of cDNA transcripts to identify particular mRNA species that are expressed under defined conditions. To identify genes that are specifically expressed during chili fruit ripening, we compared the mRNA expression profiles by mRNA differential display using two different mRNA populations extracted from different maturation stages, ripe and unripe (entirely based on colouration) fruits. RT-PCR was conducted on the extracted RNA with several primer combinations and the PCR products were then analyzed on a 6% polyacrylamide gel and visualised by autoradiography as shown in Figure 1. A total of 24 primer combinations were tested. Each primer combination resulted in a

highly reproducible banding pattern. Several cDNA bands were present in the ripe RNA sample and but not detected in the unripe RNA sample were selected for further evaluation. This allowed the identification of 28 differential cDNA transcripts that were specifically expressed during chili fruit ripening. These bands were excised and eluted from the polyacrylamide gel. The eluted DNA which potentially correspond to differential displayed products were reamplified by PCR and the resulting amplicons were cloned into pGEM-T easy and subsequently sequenced. Out of 28 excised bands, only 16 cDNA bands were successfully reamplified (Table 1), four of which were isolated following cloning into pGEMT Easy vector. Initially, the sequences obtained from this study were designated CUKMD1 to CUKMD4.

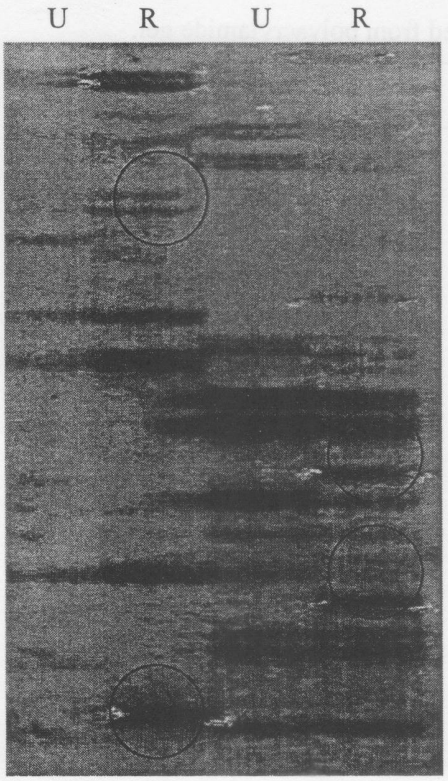

Figure 1. Autoradiography of a typical differential display gel. Total RNA (U- unripe and R- ripe) was reversed transcribed with the anchored and arbitrary primers . Circled are cDNA bands which were excised and used for PCR reamplification followed by cloning into pGEMT vector.

Comparison of the deduced amino acid sequence of fragment CDUKM1 with the GenBank database demonstrated that fragment CUKMD1 shows highly significant homology with ζ-carotene desaturase of *C. annuum* (Table 2). Zeta carotene desaturase plays a pivotal role in conversion of zeta-carotene to lycopene

in carotenoid biosynthetic pathway. Carotenoids are essential components of all photosynthetic organisms due to their chemical properties that protects against chlorophyll bleaching. Another important roles of carotenoids in plants is to furnish flowers and fruits with distinct colours that are designed to attract animals. Many genes involve in carotenoid biosynthetic pathway have been isolated and characterized from a wide range of organisms such as bacteria, algae, fungal and recently from higher plants (Hirschberg, 2001). Expression analysis of *zds* revealed that the level was lowest in immature green and increased 4-6 folds at breaker, this observation in parallel with the formation of carotenoid (Josse et al., 2000).

Table 1. List of cDNA fragments excised from polyacrylamide gel.

cDNA Fragments	Size (nt)	Primers
G9	500	G + HAP9
G11(2)	450	G + HAP11
G12	350	G + HAP12
G14(d2)	500	G + HAP14
G16(2)	400	G + HAP16
G16(3)	400	G + HAP16
G16(4)	300	G + HAP16
A10(2)	300	A + HAP10
A11(3)	300	A + HAP11
A11(4)	300	A + HAP11
A14(d1)	450	A + HAP14
A14(2)	150	A + HAP14
A14(5)	300	A + HAP14
A14(6)	200	A + HAP14
A13(3)	400	A + HAP13
C9(1)	100	C + HAP1

A second differential display product (CDUKM2) when compared with nucleotide sequences in the GenBank database revealed significant similarity with *O*-methyltransferase (Table 2). Plant *O*-methyltransferases are involve in the transfer of the methyl groups of S–adenosyl-L-methionines (AdoMet) to the oxygen, nitrogen, or carbon atoms of various acceptor molecules via the formation of the methylated derivative and S-adenosyl-L-homocystein. Molecular cloning and functional expression of many genes encoding *O*-methyltransferases have been carried out with the aim to study their function in plant metabolism (REfere). Recently, the 3-D structure of alfalfa COMT has been determined using X-ray

crystallography. The three dimensional crystal structure of this COMT revealed the structure of the complex of enzyme and their substrates, as well as the structures of their binding sites. It was observed that the alfalfa COMT showed an unusual spacious catalytic site. This finding leads to a better explanation for the broad specificity of the enzyme which could act on numerous compounds (Zubieta et al. 2002).

Table 2. Homology searches for fragments isolated from differential display.

Clone	Size (bp)	Related Gene	Related Sequence	Homology
CUKMD1	180	ξ-carotene desaturase	*Capsicum annuum*	100
CUKMD2	250	O-methyl transferase	*Prunus dulcis*	57
CUKMD3	250	Catalase	*Zea mays*	100
CUKMD4	200	Amino acid transporter	*Arabidopsis thaliana*	53

The third clone known as CUKMD3 shows 100% homology at the nucleotide level with *CaCat1* which encodes catalase from *C. annuum* L. (Kwon & An 2001). Catalase is a tetrameric, hence containing enzyme which exists in all aerobic organisms (Guan et al. 1995). In plants, catalase is involved in scavenging H_2O_2 into water and oxygen to protect the cells from oxidative stress. Oxidative stress is an intrinsic phenomenon of fruit ripening whereby it promotes the process of oxidative deterioration that contributes to a general deterioration of cellular metabolism (Thompson et al. 2001). It has been reported that oxidative stress plays an important role in the induction of chromoplast carotenoid biosynthesis and is involved in the transformation of chloroplast to chromoplast (Bouvier et al. 1998). In plants, catalase comprises of a small gene family. Three catalase genes were reported in *Zea mays* (Guan & Scandalios 1995) and pumpkin (Esaka et al. 1997). These genes are regulated differentially in response to changes in either developmental phases or environmental conditions (Redingaugh et al. 1990). The expression of *CaCat1* transcripts were detected in the early stage of fruit development (Kwon & An 2001). Since CUKMD3 and *CaCat1* have high degree of identical nucleotide sequences, it is suggested that both genes having similar function during chili fruit ripening.

The cDNA fragment designated as CUKMD4 that was isolated using this technique has shown considerable similarity with an amino acid transporter (Table 2). Amino acid transporters are involved in transporting amino acids across the plant plasma membrane. Long distance transport of amino acids is necessary to ensure not only for coordinated plant growth and development, but also for efficient

storage of proteins in the fruits. Amino acid transporters have shown a wide specificity and energized by co-transport with protons and cations (Wyse & Komor 1984). Studies carried out on purified plasma membrane vesicles from sugar beet leaves revealed the presence of at least 4 transport systems that differ in their substrate specificity (Bush 1993). Similar studies on *Ricinus communis* roots by Weston et al. (1995) also demonstrated the multiplicity of amino acid transport systems in this organ. Data obtained from the study of *Arabidopsis* have shown that plants possess a wide range of different amino acid transporters. These transporters differ in their specificity, spatial and temporal expression, as well as their responses to various stresses. Based on the data obtained and our observation, we proposed that the gene corresponding to CUKMD4 was expressed during fruit ripening in order to transport and thus provide amino acids to the tissues of the fruits.

CONCLUSION

Differential display is a powerful and suitable technique used for isolating various cDNA clones corresponding to mRNA expressed during fruit ripening. We have reported on the isolation of various ripening related cDNA clones using this technique. Homology searches against GenBank database revealed that the cDNAs have shown very significant homology with genes from the database. Further characterization of these cDNA clones will enable us to understand the possible functions of these genes during chili fruit ripening and can be in crop improvement program.

ACKNOWLEDGEMENTS

This work was supported by IRPA grant 01-02-02-0002EA035 (awarded to Dr. Zamri Zainal) from the Ministry of Science, Technology and Innovation, Malaysia. Ms. Roslinda Sajari was supported by NSF studentship.

REFERENCES

Altschul SF, Gish W, Miller W, Myer EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Bouvier F, Backhaus RA, Camara B. 1998. Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *The Journal of Biological Chemistry* **273**: 30651-30659.

Bush DR.1993. Proton coupled sugar and amino acid transporters in plant. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 513-542.

Esaka M, Yamada Y, Kitabayashi M, Setoguchi Y, Tsugeki R, Kondo M, Nishimura M. 1997. cDNA cloning and differential gene expression of three catalases in pumpkin. *Plant Molecular Biology* **33**: 141-155.

Guan L, Scandalios JG. 1995. Developmentally related responses of maize catalase gene to salicylic acid. *Proceedings National Academy Science, USA* **92**: 5930-5934.

Hirschberg J. 2001. Carotenoid biosynthesis in flowering plants. *Current Opinion of Plant Biology* **4**: 210-218.

Josse EM, Simkin AJ, Gaffe J, Laboure AM, Kuntz M, Carol P. 2000. A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. *Plant Physiology* **123**: 1427-1436.

Kwon SI, An CS. 2001. Molecular cloning, characterization and expression analysis of a catalase cDNA from hot pepper (*Capsicum annuum* L.) *Plant Science* **160**: 961-969.

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

Lopez-Gomez R, Gomez-Lim MA. 1992. A method for extracting intact RNA from fruit rich in polysaccharides using ripe mango mesocarp. *Hortscience* **27**: 440–442.

Redingaugh MG, Sabre M, Scandalios JG. 1990. Expression of the maize *Cat3* catalase gene under the influence of of a circadian rhythm. *Proceedings National Academy Science, USA*. **87**: 6853-6857.

Thompson JE, Ledge RL, Barber RF. 1987. The role of free radicals in senescence and wounding. *New Phytologist* **105**: 317-344.

Weston K, Hall JL, Williams LE. 1995. Characterization of amino-acid transport in *Ricinus communis* roots using isolated membrane vesicles. *Planta* **196**: 166-173.

Wyse RE, Komor E. 1984. Mechanism of amino acid uptake by sugarcane suspension cells. *Plant Physiology* **76**: 865-870.

Yamazaki M, Saito K 2002. Differential display analysis of gene expression in plants. *Cell Molecular Life Sciences* **59**: 1246-1255.

Zubieta C, Kota P, Ferree JL, Dixon RA, Noel JP. 2002. Structural basis for the modulation of lignin monomer methylation by caffeic acids/5-hydroxyferulic acid 3/5- O-methyltransferase. *Plant Cell* **32**: 1265-1277.