Isolation and characterization of mRNAs differentially expressed during ripening of mango fruits

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Mango, an important climacteric fruit crop with low shelf life, requires technologies to increase shelf life to reduce post harvest losses. During ripening of mango, the authors isolated five ripening related cDNAs from two mango varieties, Alphonso and Totapuri, using RT-PCR technique. The predicted polypeptides of five of these clones exhibit similarity to database protein sequences of PRL-1 protein, transcription initiation factor, CCR-4 protein, 18S ribosomal RNA gene and 23S ribosomal RNA gene. None of these proteins appear to be directly related to events generally associated with ripening such as cell wall metabolism or the accumulation of sugars and pigments or ethylene biosynthetic pathway. They are the regulatory elements/signals known to be involved during fruit ripening and may, therefore, be involved in regulating the expression of other genes directly associated with fruit ripening. The probable role of these proteins in mango fruit ripening needs to be elucidated further.

Keywords: CCR-4, PRL-1, transcription factor, rRNA, RT-PCR

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Introduction

Mango, an economically important export crop of many tropical countries including India, faces several problems on marketing of fruit, which softens very quickly and extensively. Ripening of fleshy fruit is a differentiation process involving biochemical and biophysical changes that lead to an attractive and consumable plant product. The changes are the accumulation of sugars, synthesis of aromatic compounds and changes in tissue texture and colour. In climacteric fruits, the onset of ripening is associated with autocatalytic ethylene synthesis and a surge in respiration that trigger an array of physiological processes comprising the ripening process. The ripening process has been best characterized in climacteric fruits, such as tomato, where more than 40 genes encoding functions that are specifically correlated with ripening have been described. The ripening related genes isolated in mango are alternative oxidases, uncoupling proteins, peroxisomal thiolase, protein/casein kinases, ACC oxidase, rab11-like gene and endopolygalacturonase. However, no regulatory sequences, which control the expression of ripening related genes, have been reported in mango.

Fruit development and ripening is a complex phenomenon, where several metabolic pathways are active, resulting in expression of several genes, which might be inter-related with one another or have an independent role in ripening. It has also been reported that there are several other regulatory elements or signals, which control the expression of ripening related genes. This study reports the isolation and characterization of five cDNA clones known to be involved during fruit ripening in mango.

Materials and Methods

Plant Material

Mango varieties, Alphonso and Totapuri, were chosen for the study since Alphonso is an early ripening variety (8 days) while Totapuri is a late ripening variety (13 days). The fruits, harvested from the experimental farm of IIHR, Bangalore, were allowed to ripen at ambient temperature. Samples were taken at 0, 4 and 8 days after harvest in Alphonso and at 0, 4, 8 and 13 days after harvest in Totapuri. Skin and seed of mango fruits were removed and the mesocarp was diced. These fruit pieces were frozen in liquid nitrogen and stored at –80°C for further use.

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*Abbreviation: CCR-4, Carbon Catabolite Repressor-4; PRL-1, Pleiotropic regulatory locus 1
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RNA Preparation

Total RNA was extracted from mango mesocarp. The frozen mesocarp (30 g) was homogenized in a lysis buffer (1:2 ratio of tissue and lysis buffer; 60 ml; pH 7.5), which contained: SDS, 2; β-mercaptoethanol, 1%; EDTA, 50; and Tris-borate, 150 mM. The homogenate was quickly mixed with 0.25 vols of absolute ethanol and 0.11 vol of 5 M potassium acetate. Mixing was continued for 1 min followed by extraction with equal vol of chloroform:isoamyl alcohol (49:1) and centrifugation at 14,000 rpm for 20 min. The recovered aqueous phase was extracted once with equal vol of phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (49:1). RNA was precipitated with 10 M LiCl (3 M final conc.) at −20°C overnight. RNA was pelleted by centrifugation at 14,000 rpm for 90 min at 4°C and washed once with 3 M LiCl. RNA was resuspended in 1 ml sterile DEPC water and re-precipitated with 5 M potassium acetate (0.3 M final conc.) and 2.5 vols of absolute alcohol by incubating at -20°C overnight. RNA was recovered by centrifuging at 14,000 rpm for 40 min and then washed once with 70% alcohol. The pellet was dried and resuspended in 200 µl sterile DEPC treated water. The integrity of RNA was checked by running on formaldehyde-agarose (1.2%) denaturing gels. DNase treatment of the RNA samples was done using RNase free DNase of Qiagen as per manufacturer’s instructions. The RNA samples were diluted to 0.5 µg per µl and stored at −80°C for further use.

First Strand cDNA Synthesis

RT-PCR was done using the Access RT-PCR Introductory System™ of Promega as per manufacturer's instructions in one-step method and also by two-step method by synthesizing first strand cDNA followed by PCR in the second step. However, both gave similar results. The template for the first strand reaction was 5 µg of total RNA from different stages of ripening mango fruit from both Alphonso and Totapuri. The reaction was carried out for 1 hr in 20 µl of 5X cDNA synthesis buffer, 0.1 M DTT, RNase out 1 unit, 10 mM dNTPs and Thermoscript reverse transcriptase (Gibco BRL) primed by 100 picomoles of oligo dT(20).

PCR Amplification

Two non-specific primers, forward primer: 5'GCCATTCTCACCGGATTCAGTCGTC3' and reverse primer: 5'AGCCGCGCTCCCGTCAAGTCAG3', were used in initial experiments to check the integrity of RNA for RT-PCR and have shown amplification of differential bands over different stages of ripening of mango. Hence, the same were used to study the differential amplification at different stages of fruit ripening in Alphonso and Totapuri. PCR reaction was carried out in a vol of 50 µl with 10x High Fidelity PCR buffer (Gibco BRL), 50 mM MgSO4, 10 mM dNTPs, 50 picomoles of primer (non-specific), 2 µl of the cDNA synthesis reaction and 2.5 units of Platinum Taq polymerase of High Fidelity (Gibco BRL). Reaction was cycled once at 94°C for 2 min and 40 times at 94°C for 30 sec, 60°C for 1 min, 68°C for 2 min and one extended elongation at 68°C for 7 min.

Isolation and Cloning of Differentially Expressed Bands

PCR products were analysed by gel electrophoresis on 1.8% agarose gels. The differential bands expressed in the ripe stages were eluted using the Qiaquick gel-extraction kit of Qiagen as per manufacturers instructions and these differentially expressed bands were cloned into pUC57ZR using Ins T/A cloning kit of MBI Fermantas as per manufacturer’s instructions.

Sequencing and Characterization of the cDNA Clones

Sequencing of the differentially expressed cloned cDNA fragments was done using ABI Prism Automated DNA Sequencer Model 377 at the National DNA Sequencing Facility, Indian Institute of Science, Bangalore. Both M13 forward and reverse primers were used for sequencing. Characterization of the cDNA clones was done through homology search with BLAST (x) program (Nucleotide with protein search) of NCBI12, to identify the sequences from other plant species and yeasts from gene bank, most closely related to the differentially expressed clones and thereby determine possible functions.

Results and Discussion

Gel-fractionation of the RT-PCR products, obtained from the reaction using non-specific primers, showed a series of bands (914-180 bp) in Alphonso (Fig.1a) and Totapuri (Fig. 1b). Database searches revealed that cloned mango cDNAs were homologous to reported sequences of various genes, many of which encode proteins of known function and also 23S and 18S ribosomal RNA genes. Sequence identity levels between the mango genes and those in database ranged from 55 to 98% (Table 1).
Because the increases in transcript levels observed during ripening are likely to reflect parallel increases in the corresponding enzyme activities, the possible functions of the predicted protein products were found to be involved in diverse metabolic processes. The predicted proteins included PRL-1 interacting factor, a nuclear WD protein, which was involved in pleiotropic control of glucose and hormone responses\textsuperscript{13}, a transcription factor, which was involved in transcription initiation\textsuperscript{14}, and a CCR-4 protein, which played an important general transcriptional role in diverse cellular events\textsuperscript{15}. Two ribosomal RNA genes (18S and 23S) were also found to be differentially expressed.

The first clone encoded a protein that was highly homologous with PRL-1 protein (Pleiotropic Regulatory Locus 1) encoding a conserved nuclear WD protein that functioned as a pleiotropic regulator of glucose and hormone responses in Arabidopsis. This clone showed differential expression during 4 and 8 DAH (days after harvest) in Alphonso (75\% ripe) and 8 and 13 DAH in Totapuri (100\% ripe). It was highly expressed during later stages of ripening indicating its involvement in glucose and sucrose metabolism and its increasing sensitivity to ripening hormone, ethylene\textsuperscript{13} in Arabidopsis.

PRL-1 mutation\textsuperscript{13} resulted in transcription depression of glucose responsive gene defining a novel suppressor function in glucose signaling. The PRL-1 mutation also augmented the sensitivity of plants to growth hormones (cytokinin, ethylene, abscisic acid and auxin) and stimulated the accumulation of sugars and starch in leaves. PRL-1 protein also encodes a regulatory WD protein that interacts with many other proteins/receptors involved in several other regulatory functions and signaling pathways. PRL-1 affects the transcription of many genes. Hence its expression, as shown in the present study during the ripening stages of mango fruit supports earlier findings\textsuperscript{13} of its regulatory role in sugar and starch metabolism and its increasing sensitivity to ripening hormone ethylene which might, in turn, control the ripening of mango fruit.

The deduced amino acid sequence of the second clone was homologous to transcription factor of Arabidopsis thaliana. Transcription factors were the accessory protein factors, which assists eukaryotic RNA polymerases for the recognition of the promoter for the transcription of the several genes involved in various metabolic processes\textsuperscript{14}. Eukaryotic RNA polymerases require general transcription factors for promoter recognition and assembly of initiation complex\textsuperscript{16}. They need constitutive regulatory factors for the assembly process to be efficient and stable enough to sponsor transcription, even on strong consensus promoters. This reflects the general negative regulatory strategy in eukaryotic genomes – there are lots of genes and most are inactive most of the time, so gene expression is usually mediated by selective activation\textsuperscript{17}. Hence, the present transcription factor which was differentially expressed at 4 and 8 DAH in Alphonso and 13 DAH in Totapuri might act as transcriptional enhancer or regulatory factor for transcription initiation which might help in genes specifically expressed during ripening process of mango fruit by selective activation. However, its definitive role in regulating the expression of ripening related genes in mango needs to be elucidated.

The third clone encoded a protein that was highly homologous with a putative CCR-4 (carbon catabolite
repressor-4) protein associated with transcriptional process. CCR-4 transcription factor regulates the expression of a variety of genes and processes like unidentified genes involved in cell wall integrity, in UV sensitivity and in methionine biosynthesis. Moreover, CCR-4 is required by different transactivators to function maximally. CCR-4 protein is complexed with additional proteins, including CAF-1 (CCR-4 associated factor 1) and the cell cycle regulated protein kinase yDBF2 as well as several other polypeptides. The CCR4-NOT complex appears to act at the crossroads of intermingling signaling pathways towards TBP (TATA binding protein)/TFIID (Transcription factor II D) functions and is suggested to be one of the several factors that contribute to global gene regulation by modulation of TBP activity. The CCR-4 complex is required for optimal and proper expression of many genes. The evolutionary conservation of CAF1 and CCR4 across eukaryotes further suggests that this complex plays an important role in eukaryotic gene control. CCR4-NOT complex represses transcription by inhibiting factors more specifically required for promoters lacking a TATA sequence. NOT proteins are physically and functionally part of the CCR-4 complex, which forms a unique and novel complex that affects transcription. A putative target of the CCR4-NOT complex is TFIID (or some of its subunits) because of its probable implication in core promoter recognition.

In addition to the genes involved in several regulatory processes like glucose and hormone responses and transcription process, some ribosomal RNA genes (18S and 23S) have also been differentially expressed and isolated. There was higher expression of 18S ribosomal RNA gene in Alphonso at 4 DAH and 23S rRNA gene at 8 DAH. However, very low expression of these rRNA genes was observed in Totapuri. Ribosomal RNAs are the major structural components of ribosomes, required for protein synthesis. 18S rRNA is the component of small subunit (40S) of 80S ribosomes of eukaryotes. The large (60S) subunit contains three rRNAs of 28S, 5.8S and 5S. The 5.8S rRNA is homologous to the 5' portion of the bacterial 23S rRNA and forms complementary pairs with the equivalent eukaryotic 28S rRNA. These ribosomal RNA genes also help in regulation of expression of the several other genes like feedback control especially in translation regulation.

The differentially expressed cDNA clones isolated in this study would be useful in studying functional aspects of fruit ripening. The availability of these differentially expressed genes should also be valuable for delineating the regulatory network that controls the expression of ripening regulated genes in fruits. In addition to these, the transcription initiation factor isolated can be used for the isolation of fruit specific promoter, by isolating the upstream sequences at the site of binding of transcription initiation factor.
role of these transcription factors in regulating the expression of several ripening related enzymes of ethylene related pathway or cell wall metabolism has also to be verified experimentally.

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