

Cloning of Genes Differentially Expressed during the Initial Stage of Fruit Development in Melon (*Cucumis melo* cv. Reticulatus)

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We have cloned genes involved in the initial stage of fruit development in the melon by suppression subtractive hybridization. A cDNA library of unfertilized ovules was subtracted from that of fruit 9 days after pollination (DAP); 10 of the 40 selected cDNA clones were identified by reverse Northern analysis as genes differentially expressed in fruit at 9 DAP. Seven of the ten genes were homologous to genes of known function; two were related to genes with unknown functions, and one was novel. With the exception of cucumisin, none of the cDNAs had been previously identified in melon. According to Northern analyses, six of the genes were expressed at high levels early in fruit development. Expression of cucumisin, Cmf-25, Cmf-30, and Cmf-124 was highest at 9 DAP, implying that these genes are involved in the initial stage of fruit development. Cmf-30, a seed nucellus-specific gene, was also expressed early in seed development. The other genes were expressed at a moderate level throughout fruit development, with the highest expression occurring in fruit at 9 and 18 DAP. In conclusion, nine new genes involved in early fruit development in melon were cloned, and their temporal and spatial expression patterns indicate that they are preferentially expressed during the active growing stage of fruit.

Keywords: cDNA Clones; Differential Screening; Gene Expression; Melon.

Introduction

Fruit development has been intensively studied because of its unique aspects and its dietary importance. Molecular and genetic research on fruit ripening has provided information on the genetic control of processes associated with ripening, such as ethylene biosynthesis, ethylene responses, and cell wall metabolism (Giovannoni, 2001). Although the early stage of fruit development is as important as ripening, less is known about it perhaps because the dramatic biochemical and physiological changes that occur during ripening represent more attractive research challenges.

Fruits develop from the gynoecium and early development can be divided into three phases (Gillaspy *et al.*, 1993). The earliest is the development of the ovary and fruit set; at this stage the decision is made to either abort or proceed with cell division. In the second phase, active cell division occurs. Differences in fruit size depend in part on the number of cells in the pericarp of most fruits, and the fruit size of melon is primarily determined by the extent of cell proliferation in the pericarp during early fruit development (Higashi *et al.*, 1999). The third phase is cell expansion, which continues until the fruit reaches its final size. During this period, cell expansion and the formation of intercellular spaces generate more substantial and prolonged enlargement (Yamaguchi *et al.*, 2002).

The fertilized ovules are generally responsible for triggering the development of the ovary into a fruit; the signals involved are not known. Hormones act as growth factors during cell division and expansion (Gillaspy *et al.*, 1993 and references therein). Cell division in the pericarp results in differentiation into exo-, meso-, and endocarp;

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subsequently a set of mesocarp cells differentiate into vascular cells. Also during the early stage, cells in the ovary differentiate into placental and locular cells. It is believed that the developing seed or embryo produces signals that regulate the expansion and sink activity of the surrounding fruit cells (Gillaspy *et al.*, 1993). In the expansion step, genes related to cell wall loosening, biosynthesis, and assembly are also expressed (Gillaspy *et al.*, 1993). In contrast to the extensive studies focused on the ripening process, few molecular or genetic studies have addressed the important physiological and morphological changes that occur during early fruit development. Thus, it is imperative to identify the genes involved and to determine how they and their products are regulated in response to developmental signals.

Although the tomato has long served as the primary model for the study of climacteric fruit development, the melon has been used recently as an alternative model, especially for studies of the ripening process (Rose *et al.*, 1998). Melon is widely cultivated and has become a model Cucurbitaceae. Unlike the tomato, melon uses stachyose and raffinose, together with sucrose, as primary translocation forms of sugars (Chrost and Schmitz, 1997; Mitchell *et al.*, 1992).

We therefore decided to carry out a comprehensively study of melon genes involved in the early stage of fruiting. As a first step, we used suppression subtractive hybridization (SSH) to identify genes that are specifically expressed early on in development, and we hoped to obtain genes common to climacteric fruits as well as genes specific to the development of melon. SSH has been successfully used to isolate differentially expressed genes in plants and is now often used for differential screening (Kim *et al.*, 2001 and references therein). Here we report the isolation of genes that are differentially expressed during the early stages of fruit development in melon and their expression patterns during subsequent development.

Materials and Methods

Plant materials Charentais melon (*Cucumis melo* L cv. Reticulatus) seeds were provided by Dr. Alan Bennett of the University of California, Davis. Plants were grown under standard conditions in a greenhouse in Suwon, Korea. Female flowers were hand-pollinated, and tagged on the day of pollination. Fruits were harvested at 0, 9, 18, 27 and 36 days after pollination (DAP) corresponding to: the unfertilized ovary, actively growing stage 1, growing stage 2, mature green fruit, and mature ripened fruit, respectively (Fig. 2). The whole fruit was harvested in the case of the unfertilized ovary and 9 DAP fruit, and the inner mesocarp was sampled for the others. Tissue was sliced evenly, then immediately frozen in liquid nitrogen and stored at -80°C .

RNA extraction Total RNA was extracted with Trizol reagent

(Invitrogen) according to the manufacturer's protocol, and the polysaccharides were removed from the crude RNA by the method of Asif *et al.* (2000). Poly A⁺ RNA was isolated from the unfertilized ovary and 9 DAP fruit using a PolyATtract mRNA Kit (Promega).

Suppression subtractive hybridization SSH was performed with 2 μg of poly A⁺ RNA using the PCR-SelectTM cDNA Subtraction Kit (Clontech). Ovary mRNA was used as driver, and 9 DAP mRNA as tester. PCR-amplified DNAs differentially expressed in 9 DAP fruit were cloned into the pGEM-T Easy vector (Promega).

Reverse Northern blot analysis The subtracted cDNA clones were digested with *EcoRI* and separated on a 1% agarose gel. The gel was blotted onto duplicate Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech), and each blot was screened with [α -³²P] dCTP-labeled cDNA probes prepared from the total RNAs of ovary and 9 DAP fruit, respectively. Hybridization was carried out at 65°C in a solution containing 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 $\mu\text{l}/\text{ml}$ sheared salmon sperm DNA. The membranes were washed at high stringency (0.2× SSC at 65°C for 30 min) as described by Sambrook *et al.* (1989) and exposed to X-ray film.

Northern blot analysis Total RNA (20 μg) was electrophoresed on a 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane. Hybridization, washing, and exposure to X-ray film were carried out as described above for reverse Northern analysis.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's guidelines. The extracted RNA was treated with DNase to completely remove residual genomic DNA. First strand cDNA was synthesized from total RNA (5 μg) by reverse transcription using oligo (dT) as antisense primer. The first strand reaction product was used for subsequent PCR reactions to detect gene expression using gene-specific primers designed from the coding sequence of each gene. RT-PCR was performed for between 15 and 40 cycles at 5-cycle intervals, and the best RT-PCR result giving non-saturating levels of amplification was chosen to quantify the transcripts of each gene. Melon rRNA RT-PCR was used as internal standard. The primer sets for RT-PCR were: Cmf-5 (5'-CATGGAGTGCACGAT-3', 5'-CATCAAAGTAGCGGCGCT-3'), Cmf-11 (5'-ATTGTCGA-GTTTTGATT-3', 5'-GCTC-TCTTCATTCCAA-3'), Cmf-122 (5'-GACTTATAAGGTGCTGGAT-3', 5'-TAAACTAAGTTCTC-CAT-3'), Cmf-123 (5'-CAAGGAAGCTGCCAACT-3', 5'-GTCCTTCTTCTCCACACTCT-3'), and melon rRNA (5'-AGAT-CATTGCAAATTGTTG-3', 5'-CACCTACGGAACCTTGT-3').

DNA sequence analysis Sequences were determined using a DNA sequencer (Applied Biosystems). DNA and predicted amino acid sequences were searched against DNA and protein databases using the BLAST program at the NCBI website.

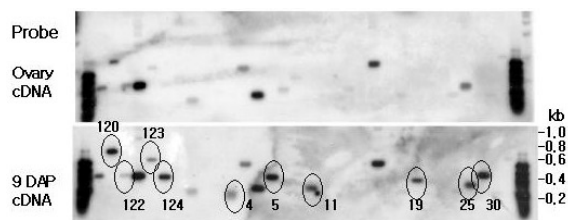


Fig. 1. Identification of differentially expressed genes by reverse Northern analysis. The subtracted cDNA clones were digested with *Eco*RI and separated on a 1% agarose gel. The gel was blotted onto duplicate nylon membranes, and each blot was screened with [α - ^{32} P] dCTP-labeled cDNA probes prepared from the total RNA of ovary and 9 DAP fruit. The circled bands indicate cDNAs differentially expressed in 9 DAP fruit. Numbers on the panels indicate clone numbers (e.g., 120 indicates clone Cmf-120).

Results and Discussion

Isolation and analysis of fruit-specific cDNA clones To clone genes differentially expressed in the early stage of melon fruit development, a subtraction cDNA library was constructed from 9 days after pollination (DAP) fruit by suppression subtractive hybridization (SSH). Approximately 40 cDNA clones were isolated and further screened by reverse Northern analysis (Fig. 1). About 20 clones exhibited differential expression in 9 DAP fruit, and their nucleotide sequences were determined by single-run sequencing. Because the isolated cDNAs were small, ranging from 200 to 600 bp (Fig. 1), single-run sequencing allowed us to determine the complete sequence of most clones. These nucleotide sequences were submitted to dbEST at NCBI (see accession numbers in Table 1), and the sequence information was searched against databases using the BLASTn and BLASTx programs. A summary of the differentially expressed cDNA clones is given in Table 1. Clones Cmf-4, Cmf-25, Cmf-120, and Cmf-124 were the most frequent among the 20 clones that resulted from the reverse Northern analysis, indicating that these genes are probably more highly expressed in 9 DAP fruit. Among the cDNA clones were three cell wall or membrane-related proteins (cucumisin, annexin, and extensin), one seed development protein, three metabolism-related proteins (KH domain, elongation factor, and geranylgeranyl reductase), and three unknown proteins.

Cmf-4 was identified as cucumisin, a serine protease that is expressed during the early stage of fruit development in melon (Yamagata *et al.*, 1994; 2002); its detection by SSH is a good indication that the differential screening was successful. Extensin, a hydroxyproline-rich cell wall protein, is also expressed during early seed development in soybean (Cassab and Varner, 1987), and Cmf-5 was found to be an extensin homologue. Cmf-19 was homologous to annexin, a calcium-dependent phospholipid-binding protein that is induced during development in bell pepper (Proust *et al.*,

Table 1. Partial cDNA clones isolated by SSH at the initial stage of fruit development.

cDNA Clone	Putative function (GenBank accession No./plant)	GenBank accession No.
Cmf-4	Cucumisin (A55800/ <i>Cucumis melo</i>)	AT007005
Cmf-5	Unknown protein (BAC79719/ <i>Oryza sativa</i>)	AT007011
Cmf-11	KH domain protein (NP194378/ <i>Arabidopsis thaliana</i>)	AT007007
Cmf-19	Annexin (AAF01250/ <i>Fragaria x ananassa</i>)	AT007012
Cmf-25	Extensin-like protein (BAA11854/ <i>Populus nigra</i>)	AT007013
Cmf-30	Seed nucellus-specific protein (AAB82329/ <i>Citrullus lanatus</i>)	AT007010
Cmf-120	Unknown protein (AAK44106/ <i>Arabidopsis thaliana</i>)	AT007009
Cmf-122	Geranylgeranyl reductase (NP177587/ <i>Arabidopsis thaliana</i>)	AT007008
Cmf-123	Elongation factor-1 alpha (AAD56020/ <i>Lilium longiflorum</i>)	AT007014
Cmf-124	Unknown	AT007006



Fig. 2. Various developmental stages of melon fruits. Numbers over panels refer to fruits harvested at the designated days after pollination (DAP).

1996). Cmf-30 showed a high degree of homology to the seed nucellus-specific protein of watermelon (GenBank accession No. AF008925), the function of which is unknown. Cmf-11 showed extensive homology to KH protein, an RNA-binding protein; its homologue in BY-2 cells is thought to play a role in the differentiation of starch-storing cells (Miyazawa *et al.*, 2002). Cmf-122 had extensive homology to the geranylgeranyl reductase of *Nicotiana tabacum*; this enzyme is involved in chlorophyll and tocopherol synthesis and is up-regulated during the development of the chloroplast from the etioplast (Tanaka *et al.*, 1999). Cmf-123 exhibited a high degree of homology to translation elongation factor 1-alpha 1 (EF-1- α 1) of *Lilium longiflorum*. The translated polypeptides of Cmf-5 and Cmf-120 were homologous to the unknown *Arabidopsis* proteins NP567596 and AF370291, respectively. No proteins related to Cmf-124 were found in the databases, suggesting that Cmf-124 is a novel gene.

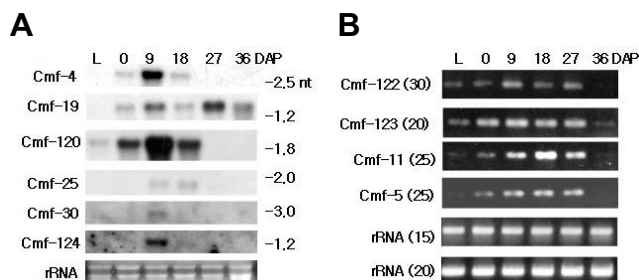


Fig. 3. The expression of differentially expressed genes during fruit development. **A.** Northern blot analyses showing the induction of transcripts at the early stage of fruit development. The DNA fragments used as probes were prepared by *Eco*RI digestion of the subtracted cDNA clones. **B.** RT-PCR showing the induced levels of transcripts during fruit development. L (leaf). The numbers over the panels represent days after pollination (DAP). Numbers in parenthesis indicate the numbers of PCR cycles producing non-saturating levels of the amplified gene. The melon rRNA RT-PCR was used as internal standard.

Expression of the early fruit development mRNAs Figure 2 illustrates the fruit development process, which is composed of five stages: the unfertilized ovary (1), actively growing stage 1, growing stage 2, mature green fruit, and mature ripened fruit. We examined the mRNA levels of ten fruit development genes at each stage of fruit development (Figs. 3A and 3B). The cucumisin (Cmf-4) transcript was weakly expressed at 0 DAP, reached its highest level at 9 DAP, and decreased dramatically after 18 DAP; Cmf-120 had a similar expression pattern and was highly specific to the early stage of development. Expression of Cmf-25 (an extensin homologue) was strong and specific to 9 and 18 DAP fruit, indicating that it is expressed at higher levels during the active growing stage. The mRNA of Cmf-19 (an annexin homologue) was detected throughout fruit development, but its level fluctuated, the highest levels being detected in 9 and 27 DAP fruits. Expression of Cmf-30 and Cmf-124 (a novel gene) was essentially confined to 9 DAP fruit. The mRNA of Cmf-30, a watermelon seed nucellus-specific protein homologue, was detected in 9 DAP fruit, suggesting that this gene is involved from the outset of seed development. Cmf-30 transcripts may have been absent from 18 to 36 DAP fruit because seeds were not included in the plant material used for the Northern blot analysis. The expression pattern of Cmf-124 was similar to that of Cmf-30, but unlike the latter, it was weakly expressed in 18 DAP fruit. Thus, its expression was restricted to the early stage of fruit development, and its expression in 18 DAP established that the absence of transcripts of Cmf-30 at 18 to 36 DAP was not due to the lack of seeds in the sampled material.

We used RT-PCR to study the expression of genes whose transcripts were not detected in the Northern blots (Fig. 3B). Cmf-5 (an unknown protein) and Cmf-11 (a KH domain

protein) displayed higher expression levels in fruit than in leaves. The levels of these mRNAs increased as development progressed until 18 DAP; they began to decrease from 27 DAP and were barely detectable in 36 DAP fruit.

Transcript levels of Cmf-122 (a geranylgeranyl reductase) and Cmf-123 (an elongation factor-1 alpha) increased slightly during fruit development and decreased at the ripening stage. The mode of regulation of Cmf-11, -122, and -123 during fruit development is not known, nor is it known whether the functions of these three genes are specifically related to fruit. We intend to examine further the functions of these differentially expressed genes in fruit development.

In conclusion, in this study we cloned one previously known gene (cucumisin) and nine new representatives of the melon genome involved in early fruit development. With the exception of one novel gene, all of the genes were identified by BLAST searches as homologues of gene families in *Arabidopsis* or other plants. We believe that each of the nine identified genes cloned by SSH belongs to a melon gene family, considering the great complexity of the melon genome compared with the *Arabidopsis* genome. Hence the expression pattern of each gene in Fig. 3 could represent not the expression of that specific gene alone but of a set of homologues. The cloning of other members of each suspected family and examination of their expression should permit us to test this proposal.

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