Expressed Sequence Tags of *Citrus* Fruit during Rapid Cell Development Phase

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ABSTRACT. A cDNA library was constructed from satsuma mandarin (*Citrus unshiu* Marc.) fruit tissues during the rapid cell enlargement phase. A total of 950 individual cDNA clones was partially sequenced and compared with GenBank databases for characterizing the gene repertoire expressed during this developmental phase. Among these, 426 cDNA clones (44.8%) showed sequence identity with previously characterized genes with optimized (OPT) scores of ≥ 200 , while 524 clones (55.2%) resulted in low OPT scores (< 200) and did not show any significant sequence identity with previously published genes. Based on nucleotide sequence, most clones with OPT scores of ≥ 200 were assumed to be transcription-, translation-, cell-wall-metabolism-, and stress-response-related genes. Other clones showed homology with published sequences related to housekeeping and stress-response-related genes, including metallothionein-like proteins, late-embryogenesis-abundant (LEA) proteins, and heat-shock proteins. These results suggested that *Citrus* fruit during rapid cell enlargement were metabolically active and expanding in response to biotic and abiotic stress. For clones with low nucleotide sequence homology, the recurrence was evaluated by aligning nucleotide sequences of each clone and generating contig maps. Expressed sequence tags (ESTs) of 162 clones with OPT scores < 200 have not been reported for any other organism. Collectively, randomly sequenced cDNA clones described in this study provided information on types of genes expressed during the rapid cell enlargement phase in *Citrus* fruit. These genes should be used as candidates for *Citrus* genome mapping projects.

The economic importance and peculiar physiology of fruit have led to many studies dealing with fruit growth and development (reviewed by Coombe, 1976). In some fruit, growth is characterized by a single sigmoidal curve, with an initial stage of slow growth generated by cell division, followed by a rapid growth due mainly to cell expansion until the onset of ripening (Coombe, 1976). In *Citrus*, Bain (1958) divided fruit growth in 'Valencia' orange (*Citrus sinensis* Osbeck) into three stages: stage I is a period of cell division, which comprises flowering and formation of various tissues in young fruit; stage II is characterized by rapid cell enlargement, followed by changes in peel color from green to orange; and stage III is regarded as the maturation period and is characterized by a reduced enlargement rate and compositional changes associated with maturation. This definition may also be applied to satsuma mandarin (*C. unshiu* Marc.). In Japan, stage II occurs during the summer, when fruit suffer from adverse environmental conditions such as water stress; this strongly influences compositional changes in stage III and, consequently, affects fruit.

In addition to such physiological studies, recent developments in molecular biology, such as genetic transformation and map-based selection, are expected to contribute to breeding programs of *Citrus* and other fruit trees. As rapid methods for determining nucleotide and deduced amino acid sequences have been developed, the number of registered sequences in databases has grown exponentially over the past few years. Expressed sequence tags (ESTs), obtained through rapid nucleotide sequencing, will help identify gene function quickly and help in the understanding of the

complexity of gene expression. The first extensive analysis of a population of expressed genes has been reported for human brain tissue (Adams et al., 1991, 1992). Large-scale partial sequencing of cDNA clones has been also achieved in some plants, and many ESTs have already been registered: almost 29,166 in *Arabidopsis* (Cooke et al., 1996; Höfte et al., 1993; Newman et al., 1994), 1427 in *Brassica napus* (Park et al., 1993), 12,672 in *Oryza sativa* (Sasaki et al., 1994; Uchimiya et al., 1992), 1757 in *Zea mays* (Keith et al., 1993; Shen et al., 1994), 750 in castor seeds (van de Loo et al., 1995), and 965 in Chinese cabbage (Lim et al., 1996). These numbers will undoubtedly increase by 10-fold during the next several years. However, except for one report of random sequencing of a cDNA library constructed from young *Citrus* seed (Hisada et al., 1996), there are no reports on ESTs in fruit trees.

Identifying genes that are differentially expressed in a fruit (or juice sacs) tissue during a particular developmental stage is an effective approach to defining physiological events on a molecular basis. Large-scale sequencing of randomly selected cDNA clones derived from *Citrus* fruit during the rapid cell enlargement phase will provide fundamental knowledge of cell expansion and fruit enlargement. We report results of partial DNA sequence analyses of clones from a *Citrus* fruit cDNA library and discuss the possible roles of putatively identified genes in fruit enlargement.

Materials and Methods

PLANT MATERIAL AND CONSTRUCTION OF THE cDNA LIBRARY. Fruit (5.0 cm in diameter) of 'Miyagawa-wase' satsuma mandarin cultivated at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan, were harvested 10 weeks following anthesis. Fruit tissues were separated into juice sacs and pulp segments, immediately frozen, and stored at -80°C until use. Tissues were ground to a fine powder in liquid nitrogen, and polysomal poly(A)⁺RNAs were isolated according to Komatsu et

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al. (1996). A directional cDNA library was constructed in a Lambda Uni-ZAP XR vector (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Phage was packaged using an in vitro packaging kit, LAMBDA INN (Nippon Gene, Tokyo). The cDNA clones were excised as Bluescript SK⁻ plasmids (Stratagene) in the bacterial host SOLR strain according to the in vivo excision protocol supplied by Stratagene.

NUCLEOTIDE SEQUENCING AND DATA ANALYSIS. Plasmid DNA for nucleotide sequencing was prepared by the alkaline-lysis method of Birnboim and Doly (1979). Insert size was estimated by 1.0% agarose gel electrophoresis after digestion with *EcoRI* and *XhoI*. Partial nucleotide sequence was determined by the fluorescence detection method of Smith et al. (1986) using labeled dye primers. Cycle sequencing was performed with a Catalyst 800 Molecular Biology LabStation (Applied Biosystems, Foster City, Calif.). A sequencer (model 373A; Applied Biosystems) was used for automated sequence analysis. The cDNA sequence (from the 5' to 3' direction) of the insert was analyzed to simplify analysis of putative translation products.

DATA ANALYSIS. Sequences were edited manually by removing vector and ambiguous sequences. Partial sequences with an average length of 320 bp were used for database search. Comparisons with nucleic acid sequences deposited in DDBJ, GenBank, and EMBL databases using the FASTA (Pearson and Lipman, 1988) e-mail server were made. Data analysis was carried out according to Hisada et al. (1996). Briefly, the higher the degree of nucleotide sequence similarity, the higher was the optimized (OPT) score value and 50% of sequence similarity corresponded to OPT = 200. For low-homology-sequence clones, the GENETYX-MAC/ATSQ program, based on FASTA algorithm, was used to align overlapping sequences by aligning scores of ≥ 80 (Newman et al., 1994).

Results and Discussion

A total of 950 cDNA clones from a cDNA library derived from fruit tissue of *Citrus unshiu* during the rapid cell enlargement phase was analyzed. Clones were classified into three groups based on their homology scores to genes registered in database gene banks according to Fujiwara et al. (1995). These included a high-homology score group (HSG) with an OPT score of ≥ 400 , a medium-homology score group (MSG) with an OPT score of 200–399, and a low-homology score group (LSG) with an OPT score of < 200 . About 22.3%, 22.5%, and 55.2% of the cDNA clones were classified into HSG, MSG, and LSG, respectively. Because clones with an OPT score of ≥ 200 in the FASTA algorithm were possibly homologous to registered genes (Hisada et al., 1996; Sasaki et al., 1994; Uchimiya and Kato, 1992), it was estimated that 426 clones (44.8%) matched previously identified genes with $> 50\%$ of sequence similarity on the nucleotide sequence level (Table 1). Among those, 311 clones matched functionally characterized genes, whereby 258, 36, and 17 clones showed significant homology to plant-, other organism- and mitochondrial homologue-genes, respectively. Among clones homologous to plant genes, 29 showed homology with genes derived from fruit trees; among these, 18 and 3 clones matched genes from *Citrus* and *Poncirus*, respectively. Moreover, 8 clones were similar to metallothionein genes from kiwifruit. In addition to clones homologous to identified genes, there were 115 cDNA clones that exhibited homology with expressed sequence tags (ESTs) with an OPT score of ≥ 200 (Table 1). Among those, 113 clones were homologous to plant ESTs, mainly from *Arabidopsis thaliana*; rice, *Rucinus communis*, and the remaining two were homologous to ESTs from other organisms, including human (data not shown).

Recently, Rounsley et al. (1996) reported the application of a

Table 1. Grouping of clones from a *Citrus* fruit cDNA library during rapid cell development phase.²

Grouping	Clones (no.)	Homologous to genes derived from fruit tree
Gene identified	311 (32.7%)	
Metabolism	20 (2.1%)	
Amino acid	4	
Carbohydrate	2	Myo-inositol-1-phosphate synthase from <i>Citrus paradisi</i> (Z32632)
Nucleic acid	2	
Secondary	4	
Lipid and membrane	8	
Responsible gene	22 (2.3%)	
Hormone and ripening	7	
Cold/dry	8	Cold stress protein from <i>Poncirus trifoliata</i> (L39005), Lea protein from <i>Citrus sinensis</i> (Z46824, Z46657)
Defence/others	7	Heat-shock protein from <i>Citrus maxima</i> (U19461)
Protein regulation and signal transduction	6 (0.6%)	Serine/threonine protein phosphatase from <i>Malus domestica</i> (Z47076)
Nuclear structure	8 (0.8%)	
Transcription	17 (1.9%)	
Translation	21 (2.2%)	26S or 18S ribosomal RNA from <i>Citrus</i> (X05910, X03641, U38312)
Growth	16 (1.7%)	Metallothionein-like protein from <i>Actinidia deliciosa</i> (L27813)
Energy	5 (0.5%)	
Morphogenesis	4 (0.4%)	
Cell structure	93 (9.8%)	
Ubiquitin	2 (0.2%)	
Unclassified	23 (2.4%)	
Expressed sequence tag (EST)	115 (12.1%)	
No homology	524 (55.2%)	
Total	950	

²DDBJ Data Library accession numbers of identified genes and ESTs are C21915–C22340, and of no-homology clones (448 clones) are C23872–C24319. The remaining 76 no-homology clones are not registered because of their short length in nucleotide sequence (< 150 bp).

method for reducing redundancy and increasing information content by grouping overlapping ESTs representing the same gene into a contig or assembly. As to frequency of occurrence in 950 clones analyzed, 30 putatively identified genes appeared in more than one clone (Table 2). For reducing redundancy, these clones were evaluated by generating contig maps. Among those, clones homologous to glycine-rich proteins (GRPs) appeared 87 times, resulting in the most redundant gene and accounting for $\approx 9.2\%$ of the total 950 clones (Table 2). Using a program of contig sequence maps, these GRP clones belonged to one major group and five minor groups (data not shown). The major group, which was highly homologous to carrot GRPs, was notably abundant, while the other groups were homologous to GRPs from *A. thaliana* and *Nicotiana sylvestris*. GRPs have been considered to be constituents of plant cell walls (Varner and Cassab, 1986) and play an important role in providing developing plant cell walls with tensile strength without limiting tissue flexibility (Creelman and Mullet, 1991; Keller et al., 1988). The expression of GRPs is regulated by several biotic and abiotic stimuli, including plant hormones (de Oliveira et al., 1990; Reddy and Poovaiah, 1987), viral infection (Linthorst et al., 1990), water stress (de Oliveira et al., 1990; Keller et al., 1988), and wounding (Condit and Meagher, 1987). The degree of redundancy of this group of clones was one of the highest among plant cDNA libraries so far analyzed (Cooke et al., 1996; Höfte et al., 1993; Keith et al., 1993; Lim et al., 1996; Newman et al., 1994; Park et al., 1993; Sasaki et al., 1994; Shen et al., 1994; Uchimiya et al., 1992; van de Loo et al., 1995), constituting a peculiar feature of this library.

Three clones were homologous to late-embryogenesis-abundant (LEA) proteins, and another three clones were homologous to cold-stress proteins (a LEA-like protein) derived from *Poncirus trifoliata* (Table 2). These six clones belonged to two groups based on their nucleotide sequence similarity (data not shown). LEA proteins are reported to be induced by stress, especially low temperatures, drought, and osmotic stress (Neven et al., 1993). Cai et al. (1995) cloned a LEA2-like gene from citrus under low-temperature treatment and confirmed that expression of this gene in leaf tissue was induced in response to cold acclimatization, but that it was repressed during drought and flooding stress. Although in the present study the function of LEA homologous clones was not ascertained, *Citrus* trees grown in Japan are subjected to some drought stress during fruit growth due to midsummer seasonal water shortage and competition between leaf and fruit tissues for water use. Expression of LEA genes in *Citrus* fruit may be induced in response to drought stress.

Ribosomal proteins, small nuclear ribonucleoprotein particles (snRNPs)-U2, and ubiquitin, which play important roles in translation, RNA splicing, and protein degradation process were also redundantly transcribed. These genes are homologous even to unrelated species such as *Homo sapiens* (Adams et al., 1991, 1992), suggesting conservation of nucleotide sequences over a long evolutionary period of housekeeping standard genes.

Furthermore, nuclear antigen, which is correlated to cell proliferation rate (Dudits et al., 1991; Hirt et al., 1991; Smith et al., 1988), metallothionein, which is involved with heavy metal detoxification (Hamer, 1986), and glyoxalase-I, which reversely cata-

Table 2. Redundancy of cDNA clones in *Citrus* fruit cDNA library.

Putatively identified gene name	Total number of clones	Number of identical groups ^z
Glycine-rich protein	87	6
Ribosomal RNA	46	8
Ribosomal protein	20	10
Metallothionein-like protein	8	2
Chloroplast genome DNA	6	4
Auxin-induced protein (Glyoxalase I)	6	2
Autoantigen	5	1
U2 small nuclear RNA	5	1
ADP-ribosylation factors	4	2
Ubiquitin-conjugating enzyme	4	2
Aquaporin	3	2
Cold stress protein	3	1
Lea5 protein	3	2
Ligase I	3	1
Tumor suppressor	3	1
MADS box	3	2
Cytochrome C reductase	3	2
Nuclear antigen	3	1
Cysteine proteinase inhibitor	2	1
Chimeric proline-rich cell wall protein	2	2
Elongation factor 1 beta	2	1
Dehydrin	2	1
Glutathione reductase	2	1
Histone	2	2
Genome	2	2
Microsatellite sequence	2	1
Photosystem I psaH protein	2	1
Phospholipid transfer protein	2	1
Primary sigma factor	2	1
S-phase specific protein	2	1

^zSimilar sequences were grouped after calculating contig sequences using GENETYX-MAC/ATSQ program.

lyzes S-D-lactoylglutathione from methylglyoxal and glutathione (Thornalley, 1990), are also putatively identified along with elongation factor 1-beta, extensin, and expansin, which are all related to cell growth. Moreover, among four morphogenic genes detected including MADS, two are homologous to tomato *TDR5* and are expressed for a long period during fruit development. On the other hand, few homologous genes are related to cell cycle regulation are detected, except for those related to S-phase-specific protein genes found in *Brassica rapa*. Since cell cycle-related genes such as protein kinase have been detected at higher frequencies in a *Citrus* cDNA library developed from young seeds (Hisada et al., 1996), the profile of gene expression has shifted from cell division to cell enlargement.

Compared to housekeeping genes, the transcription of metabolism-related genes such as carbohydrate- and TCA cycle-related genes was quite limited. This might be due to a low frequency of occurrence rather than to low representation of the clones analyzed. For examples, sucrose-phosphate synthase gene could be isolated at a frequency of about three positive signals per 2.5×10^5

clones (Komatsu et al., 1996), therefore requiring analysis of larger numbers of clones. This also suggested that normalization of the cDNA library (Soares et al., 1994) was necessary for screening metabolic genes from developing *Citrus* fruit.

To characterize this cDNA library, we also determined the redundancy of EST homologous clones and LSG clones. To align identical clones, contig sequence maps were constructed among 115 ESTs of HSG and MSG. Among these, 57 ESTs fell into 22 contig groups, while the remaining 58 ESTs failed to form any contig group (Table 3). The largest contig group (no. 1) consisted of 10 ESTs, which corresponded to 14,072 *A. thaliana* cDNA clones (accession no. R65568) or transcribed sequences (accession no. ATTS1586). Among 524 LHG clones, 162 were successfully classified into 43 contig groups, while 362 clones failed to form any contig group, probably due to low frequency of expression. The largest contig group of LHG contained 34 clones, followed by two groups composed of 12 clones. These results suggested that highly transcribed genes of *Citrus* fruit were involved in normal fruit development. As some clones did not show

Table 3. Groups of expressed sequence tag (EST) contigs found in a *Citrus* fruit cDNA library when compared to those reported in other plant species.

Contig	Clone	Clones (no.)	Entry name	Species
1	pcMFRI804.04 pcMFRI706.41 pcMFRI621.63 pcMFRI622.99 pcMFRI727.29 pcMFRI802.138 pcMFRI622.102 pcMFRI717.146 pcMFRI802.28 pcMFRI802.94	10	Z26457 R65568	<i>Arabidopsis thaliana</i> EST
2	pcMFRI616.46 pcMFRI620.82 pcMFRI622.89 pcMFRI622.126	4	T24312	<i>Ricinus communis</i> ESTs
3	pcMFRI621.03 pcMFRI621.13 pcMFRI729.124	3	Z25495	<i>Arabidopsis thaliana</i> EST
4	pcMFRI802.60 pcMFRI621.136	2	Z26063	<i>Arabidopsis thaliana</i> EST
5	pcMFRI804.50 pcMFRI807.119	2	T46648 T04159	<i>Arabidopsis thaliana</i> EST
6	pcMFRI717.59 pcMFRI802.50	2	T76356 Z18468	<i>Arabidopsis thaliana</i> EST
7	pcMFRI717.79 pcMFRI719.138	2	T42446 N38349	<i>Arabidopsis thaliana</i> EST
8	pcMFRI719.11 pcMFRI719.123	2	T23005	<i>Arabidopsis thaliana</i> EST
9	pcMFRI727.45 pcMFRI622.106	2	T76809 Z17481	<i>Arabidopsis thaliana</i> EST
10	pcMFRI729.73 pcMFRI616.109 pcMFRI802.74	3	T21326 T13822	<i>Arabidopsis thaliana</i> EST
11	pcMFRI729.92 pcMFRI802.68	2	Z17454	<i>Arabidopsis thaliana</i> EST
12	pcMFRI802.119 pcMFRI729.121	2	R30306	<i>Arabidopsis thaliana</i> EST
13	pcMFRI802.124 pcMFRI706.110 pcMFRI729.30	3	T46779 L46544 D46996	<i>Arabidopsis thaliana</i> EST <i>Brassica campestris</i> EST Rice EST
14	pcMFRI802.63 pcMFRI729.144	2	T88450	<i>Arabidopsis thaliana</i> EST
15	pcMFRI620.40 pcMFRI622.147	2	T41851	<i>Arabidopsis thaliana</i> EST
16	pcMFRI727.104 pcMFRI719.03	2	T14197 Z17473	<i>Arabidopsis thaliana</i> EST
17	pcMFRI706.40 pcMFRI622.62	2	H37220	<i>Arabidopsis thaliana</i> EST
18	pcMFRI616.27 pcMFRI727.128	2	D15600	Rice EST
19	pcMFRI622.46 pcMFRI622.91	2	D15229	Rice EST
20	pcMFRI719.78 pcMFRI804.108	2	D41341	Rice EST
21	pcMFRI622.117 pcMFRI729.110	2	Z34050 T42831	<i>Arabidopsis thaliana</i> EST
22	pcMFRI727.35 pcMFRI807.34	2	T24153 D22538	<i>Ricinus communis</i> ESTs Rice EST

homology with any of the registered genes, further molecular and biochemical analysis would provide knowledge of their role in *Citrus* fruit development.

The results of random sequencing analysis of the cDNA library indicated that this repertoire of genes was apparently active in transcription and translation in *Citrus* fruit at this developmental stage. It also suggested that fruit tissues were partially under stress. Although it was not possible to assign the exact function of these genes, they might play important roles in fruit enlargement in *Citrus*. Characterizing genes of differentially expressed in fruit tissue during the rapid developing stage would be useful.

This cDNA library will provide information on the sequence of each clone, which may be useful in mapping genes. In *Citrus* genome analysis, linkage maps of randomly amplified polymorphic DNA markers, restriction fragment length polymorphisms, and isoenzyme markers have been reported by Jarrell et al. (1992), Durham et al. (1992), and Cai et al. (1994), where cDNA clones from citrus leaves were used as probes. Mapping with characterized markers would provide excellent landmarks to expedite *Citrus* genomic analysis, and these clones will be useful in genome mapping of *Citrus*.

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