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Construction and Profiling of a cDNA Library from Young Fruit of Satsuma Mandarin

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To profile gene expression in the early stage of fruit development from 'Nichinan No. 1' satsuma mandarin (*Citrus unshiu* Marc.), we isolated total mRNA at 30 d after flowering. A cDNA library was prepared from mature mRNAs and a total of 2350 cDNA clones were partially sequenced. In all, 1914 ESTs were acquired after the removal of the vector sequence and filtering over a minimum length of 150 nucleotides. A total of 763 unigenes, consisting of 138 contigs and 625 singletons, was identified after assembly of those ESTs. According to our homology search with BLASTX against the NCBI database, the deduced amino acid sequences of 253 unigenes were homologous to proteins with known function and 242 unigenes were significantly matched to proteins with putative or unknown functions. The remaining 268 showed no significant similarity to any protein sequences found in the public database with matches higher than an E value of 10^{-5}. The 253 unigenes matched to proteins with known function were then manually assigned to 10 cellular functional categories using a modified MIPS MATDB classification. The expression level of each gene was analyzed based on the redundancy of cDNA clones in each contig that comprised more than 10 ESTs. Here, the most abundant gene expressed in young fruits was for a chitinase precursor. A miraculin-like protein and a lectin-related protein precursor were also abundant.

Keywords: cDNA library, *Citrus*, ESTs, gene, young fruit

The characterization of gene expressions in plants is very important not only for understanding their physiology but also for implementation in breeding programs. For example, because of the difficulty encountered in conventional citrus breeding and the lack of large segregating populations for marker-assisted selection methods, the introduction of identified genes into a plant seems to be a valuable alternative for crop improvement strategies. In addition, researchers can utilize sequence information as tools for manipulating the plant system.

More than 200000 citrus ESTs from 16 species and cultivars have now been registered in the GenBank dbEST. The greatest number of these (94704) has been isolated from *Citrus sinensis*, followed by *C. clementina* (61905) and *Poncitrus trifoliate* (28737). In contrast, only 2575 ESTs from *C. unshiu* have been deposited in the Genbank database due to small-scale EST sequencing (http://int-citrusgenomics. org).

cDNA libraries for *C. unshiu* have been constructed from young seeds (Hisada et al., 1996; Omura et al., 2000), fruit at the rapid cell development phase (Hisada et al., 1997, 1999; Omura et al., 2000), and mature fruit pulp and albedo (Moriguchi et al., 1998; Kita et al., 2000; Omura et al., 2000). However, a cDNA library has not been profiled for whole fruits, including the pulp and peel, at an early developmental stage.

Recent advances in plant biotechnology have enabled scientists to develop efficient techniques for constructing and analyzing cDNA libraries. Such research in our laboratory has been focused on satsuma mandarin, one of the most important crops in Jeju, Korea. We have previously constructed a cDNA library derived from whole young fruits, and have performed single-pass sequencing to identify their genes. Here, we have characterized individual clones, based on partial sequences, and identified the most highly expressed genes.

MATERIALS AND METHODS

Plant Material

Young fruits (about 10 mm diam.) were collected from a germplasm plant of 'Nichinan No. 1' satsuma mandarin (*Citrus unshiu* Marc.) that was cultivated at Jeju-do Agricultural Research and Extension Services (Fig. 1). These were immediately frozen in liquid nitrogen and stored at -80°C.

Construction of cDNA Library

Total RNA was isolated from whole fruits using an RNeasy Maxi kit (Qiagen, USA), and poly(A) RNA was purified with an Oligotex kit (Qiagen) according to the manufacturer's instructions. A GeneRacer kit (Invitrogen, USA) was used for cDNA library construction. Prior to reverse-transcription of the mRNA, total RNA was treated with intestinal phosphatase to dephosporylate all non-protected polynucleotides. Then, the mRNA was treated with tobacco acid pyrophosphatase to expose a reactive phosphate group at the 5′ end of the cap-bearing mRNAs. This allowed them to ligate to the linker oligonucleotide (5′-CGACUGGAGCACGAGGA-CACUGACAUGGACUGAAGGAGAGAA-3′). The ligated RNA was reverse-transcribed with the tagged oligo-dT (5′-GCT-GTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$-3′). Afterward, cDNAs were synthesized by PCR from single-

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Figure 1. Young fruits used for cDNA library construction. Bar = 1 cm.

strand cDNAs that had been reverse-transcribed from mature mRNAs with both a 5′ cap and a polyA tail. PCR was carried out for 30 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min. The PCR products were cloned into the pCR4-TOPO vector and the plasmids were transformed into *Escherichia coli* cells. Our cDNA libraries were plated onto LB-ampicillin plates containing IPTG and X-gal, and the white colonies were picked into 96-well blocks containing 1.3 mL of an LB-ampicillin medium.

cDNA Sequencing and Sequence Analysis

Bacterial culturing and plasmid extractions were done in a 96-well format. The sequencing reactions were performed using a Big Dye Termination Mix (Applied Biosystems, USA). Reaction products were analyzed with an ABI 3700 DNA Sequencer (Applied Biosystems). Vectors and ends were trimmed and removed along with sequences shorter than 150 nucleotides. The remaining ESTs -- high-quality sequences longer than 150 bp -- were then subjected to database searches. A CAP3 sequence assembly program was used for clustering the cDNAs into contigs and singletons in order to screen for unigenes that had their own unique nucleotide sequences. Their functions were then identified based on amino acid sequence homology to the cDNAs of known genes. Database searches were carried out using BLASTX software with default parameters. Homologies with E values of $\leq 10^{-5}$ were divided into known function, putative function, and unknown function; the other unigenes (no hit or an E value $>10^{-5}$) were classified as non-significant matches. Unigenes that matched proteins with known functions were categorized manually according to a modified MIPS MATDB classification scheme first adopted for *Arabidopsis* (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html). The expression levels of individual genes were examined based on the redundancy of cDNA clones found in each contig that comprised more than 10 ESTs.

RESULTS

Sequencing and Clustering of ESTs

We generated a cDNA library from whole young fruits of 'Nichinan No. 1' satsuma mandarin. A total of 2350 cDNAs were randomly selected for partial sequencing. The following vector and low-quality sequence trimming resulted in 1914 high-quality sequences, with an average EST read-length of 422 nucleotides. These 1914 sequences were then analyzed with a CAP3 sequence assembly program to identify the unigenes.

In all, 138 contigs clustered from 1289 clones and 625 singletons were formed. Among those 138 contigs, 105 consisted of 2 to 5 ESTs, while 13 others had at least 21 ESTs. The combined set of contigs and singletons resulted in 763 assembled sequences that represented the putative transcripts found in young fruit of satsuma mandarin. We have summarized this library analysis in Table 1.

Functional Annotation and Classification of Unigenes

A homology search of the cDNAs was carried out with a

Table 1. Summary of EST sequencing and contigs assembly.

EST sequencing	Number of cDNAs sequenced	2350
	Number of high quality sequences (>150 bp)	1914
Contig assembly results	Number of ESTs assembled	1914
	Number of contigs	138
	Number of singletons	625
	Number of unigenes	763
Redundancy	2~5 ESTs	105
	6~10 ESTs	12
	11~15 ESTs	5
	16~20 ESTs	3
	>21 ESTs	13

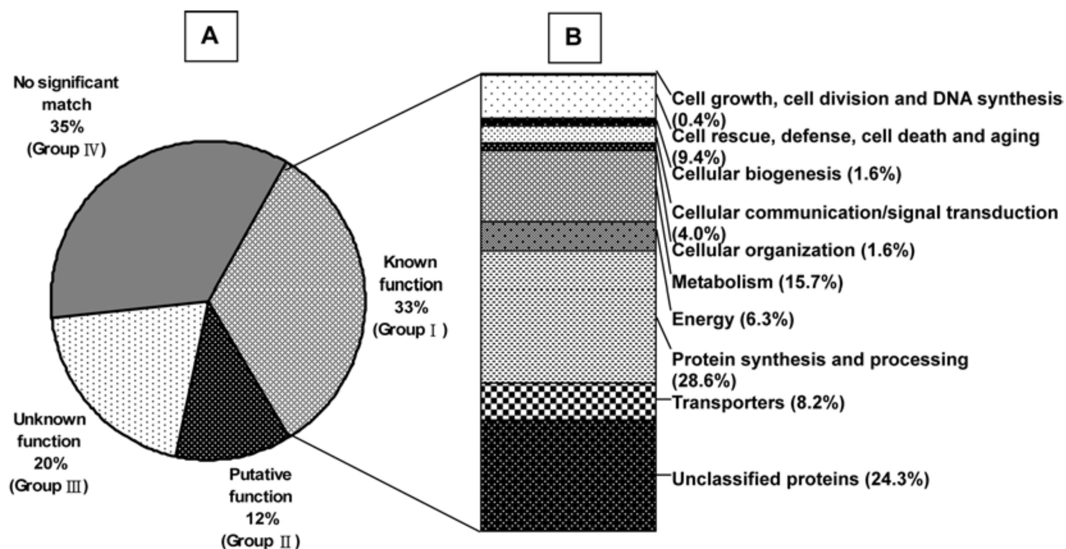

Figure 2. Graphical representations for functional annotation and classification of unigenes. (**A**) Functional annotation of unigenes with BLASTX program in NCBI. (**B**) Distribution of Group 1 ESTs using functional categories based on modified MIPS classification scheme.

BLASTX program to assign the functions of 763 unigenes. Examination of the initial BLASTX matches showed that these could be categorized into four groups: Group I, 253 (33%) unigenes matched to proteins with known function in the public databases; Group II, 90 (12%) unigenes matched to proteins with putative function; Group III, 152 (20%) unigenes matched to proteins with unknown function; and Group IV, 268 (35%) unigenes had no significant similarity to any protein sequences had matches higher than an E value of 10^{-5} in the NCBI database (Fig. 2A). Therefore, Group IV unigenes seemed to be novel genes expressed in the young fruit of satsuma mandarin.

According to a modified MIPS MATDB *Arabidopsis* classification scheme, the Group I ESTs could be assigned to a functional category based on BLASTX matches (Fig. 2B). In that analysis, the 253 unigenes were manually classified into 10 cellular functional categories: ‘cellular biogenesis’, ‘cellular communication/signal transduction’, ‘cell growth, cell division and DNA synthesis’, ‘cellular organization’, ‘cell rescue, defense, cell death and aging’, ‘energy’, ‘metabolism’, ‘protein synthesis and processing’, ‘transporters’, and ‘unclassified proteins’. ‘Protein synthesis and processing’ was a combination of the following MIPS categories: ‘protein synthesis’, ‘transcription’, and ‘protein destination’. Finally, the category of ‘transporters’ combined ‘cellular transport’, ‘transport mechanisms’, and ‘transport facilitation’. Overall, the largest category of Group I ESTs (28.6%) was ‘protein synthesis and processing’, followed by ‘metabolism’ (15.7%) and ‘cell rescue, defense, cell death and aging’ (9.4%). The category ‘transporters’ consisted of 8.2% of the Group I ESTs and ‘energy’ contained 6.3%. In the latter, all members were involved in photosynthesis. Lastly, the Group I ESTs comprised 4.0% for ‘cellular communication/signal transduction’, 1.6% for ‘cellular biogenesis’, 1.6% for ‘cellular organization’, and 0.4% for ‘cell growth, cell division and DNA synthesis’. These functional categories are listed in Table 2.

Highly Expressed Genes in Young Fruits of Satsuma Mandarin

The extent of gene expression depends on the abundance of mRNA. Therefore, the number of cDNA clones in a contig represents the level of mRNA transcription as well as expression. Here, we analyzed individual genes based on the number of cDNA clones in each contig that comprised more than 10 ESTs. Among these, 12 contigs were homologous to known functional genes; their most significant matches as well as their E values are displayed in Table 3.

The most highly abundant genes were for a chitinase precursor, a miraculin-like protein, and a lectin-related protein precursor. These three kinds showed high homology to corresponding genes already reported in other citrus species, including sweet orange and grapefruit. Of these, the largest representation was Contig 46 with 129 ESTs identifying the chitinase precursor. The predicted proteins of Contigs 53 and 73 were also homologous to that same chitinase precursor. Likewise, five kinds of contigs were matched to two classes of miraculin-like protein: Contigs 134 and 54, to a miraculin-like protein and Contigs 31, 20, and 74, to miraculin-like protein 2. Finally, Contigs 86, 88, and 123 corresponded to one class of a lectin-related protein precursor.

DISCUSSION

Our analysis of 2350 cDNAs from the whole young fruits of *C. unshiu* revealed 1914 high-quality ESTs, with an average sequence length of 422 b. A total of 763 assembled sequences (sum of contigs and singletons) were identified here, with 138 being contigs of two ESTs or more, and the remaining 625 being singletons (Table 1). Cluster analysis of these 1914 sequences via a CAP3 sequence assembly program showed that 625 clones were singletons and 1289 clones were clustered into 138 contigs. Their redundancy

Table 2. Classification of ESTs matched to known functional genes into functional categories.

Cell growth, cell division and DNA synthesis
Skp1
Cell rescue, defense, cell death and aging
Ascorbate peroxidase
Avr9 elicitor response-like protein
Catalase-1
Crystal structure of recombinant ascorbate peroxidase
Chitinase precursor(6)
Copper/zinc superoxide dismutase
DnaJ-like protein
Glutaredoxin
Glutathione S-transferase GST 23
Heat shock protein 70
Lectin-related protein precursor (5)
LMW heat shock protein
Metallothionein-like protein
Peroxidase (2)
Cellular biogenesis
Actin depolymerizing factor 3-like protein
Caffeoyl-CoA 3-O-methyltransferase (2)
Glycine-rich protein
Cellular communication/signal transduction
Calmodulin (2)
Elicitor-inducible LRR receptor-like protein EILP
GDP dissociation inhibitor
GTP-binding-like protein
GTP-binding protein
Ras-related GTP-binding protein RHA1
Response regulator 5
Small GTP-binding protein
Type 2A protein phosphatase-3
Cellular organization
Annexin
Beta-tubulin R2242
Tubulin alpha chain
Vacuole-associated annexin VCaB42
Metabolism
2-Oxoglutarate dehydrogenase E2 subunit
3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
6-Phosphogluconolactonase-like protein (2)
8-Amino-7-oxononanoate synthase-like protein
Acid phosphatase
Adenosine-5'-phosphosulfate kinase
ATP citrate lyase b-subunit
Brassinosteroid-regulated protein BRU1 precursor
Caffeic acid O-3-methyltransferase (3)
Chalcone synthase 2 (2)
Cytochrome P450 98A2
dUTP pyrophosphatase-like protein
Enolase
Ferrodoxin precursor
Flavonol synthase
Fructokinase-like protein
Geranylgeranyl diphosphate synthase
Glucosyltransferase NTGT3
Glyceraldehyde-3-phosphate dehydrogenase (2)
Glycine decarboxylase complex H-protein
Laccase
NAD-malate dehydrogenase
Ornithine carbamoyltransferase
Phosphoethanolamine N-methyltransferase
Phospholipase-like protein
Phosphoribosylformylglycinamidine cyclo-ligase
Ribulose-phosphate 3-epimerase
S-adenosylmethionine synthetase
Serine O-acetyltransferase
Transketolase TKT1 precursor
Triosephosphate isomerase
Tryptophan synthase alpha 1-like protein
UDP-glucose 4-epimerase GEPI48
Xyloglucan endotransglycosylase precursor (2)
Energy
Chlorophyll a/b binding protein C
Chlorophyll a/b binding protein CP24 precursor (2)
Chlorophyll a/b binding protein CP29
Chlorophyll a/b binding protein precursor
Chlorophyll a/b binding protein type I
Chlorophyll a/b binding protein (3)
Light harvesting chlorophyll a/b binding protein
Oxygen-evolving enhancer protein 2
Photosystem I reaction center subunit X
Photosystem II 10 kDa polypeptide
Photosystem II reaction center W protein
PS I antenna protein Lhca2 fragment
Ribulose bisphosphate carboxylase small chain
Protein synthesis and processing
14-3-3 protein
14-3-3 protein GF14
20S proteasome beta subunit
40S ribosomal protein
40S ribosomal protein S2
40S ribosomal protein S3A
40S ribosomal protein S4 (2)
40S ribosomal protein S5
40S ribosomal protein S6
40S ribosomal protein S8 (2)
40S ribosomal protein S12
40S ribosomal protein S16
50S ribosomal protein L12-A
60S acidic ribosomal protein PO
60S ribosomal protein L6
60S ribosomal protein L10
60S ribosomal protein L12
60S ribosomal protein L13a
60S ribosomal protein L21
60S ribosomal protein L30
60S ribosomal protein L35
60S ribosomal protein L38
60S ribosomal protein L4
60S ribosomal protein L44 (2)
CCHH finger protein 2-like protein
Chaperonin 21 precursor
Cleavage stimulation factor subunit 1-like protein
Cyclophilin
Elongation factor 1-alpha (2)
Elongation factor 1-alpha 1
Eukaryotic translation initiation factor
Glycine-rich RNA binding protein
Histone H1 variant
Homeobox protein
Homeotic protein HAT4
MADS1
MADS-box protein MADS3
Mitochondrial chaperonin
MYB-related transcription factor PHAN1
Nascent polypeptide associated complex alpha chain
Palmitoyl-protein thioesterase precursor-like

was relatively high (67%; 1289 of 1914 ESTs) when compared with the cDNA library profiles for the fruits, seeds, and ovaries of Miyagawa wase satsuma mandarin (*C. unshiu* Marc) (Omura et al., 2000). This variability may have been due to differences in the PCR methods used for constructing each cDNA library.

Table 2. Continued.

Papain-like cysteine proteinase isoform I	Aluminum-induced protein-like
Patatin-like protein 3	Aminolevulinate dehydratase
Polyubiquitin (2)	Anti-silencing factor 1-like protein
Ribosomal protein (2)	Beta-ureidopropionase
Ribosomal protein L2	Bis(5'-adenosyl)-triphosphatase-like (2)
Ribosomal protein L5-like	Coatomer-like protein
Ribosomal protein L7	Copia-like retroelement pol polyprotein
Ribosomal protein L7Ae-like	Cyclase
Ribosomal protein L23	Cystatin
Ribosomal L23a-like protein	DCL protein
Ribosomal protein L28-like	Dicarboxylate/tricarboxylate carrier
Ribosomal protein L32	Early light-induced protein
Ribosomal protein L33	Epsilon subunit of mitochondrial F1-ATPase
Ribosomal protein S19 (2)	ER lumen protein-retaining receptor-like protein
Ribosomal protein S27	ERD15 protein
Ribosomal protein S28	Fe-superoxide dismutase precursor
Ribosomal S29-like protein	FH protein interacting protein FIP2
Ribosome-associated protein	Fiber protein E6
RING finger protein	G2/mitotic-specific cyclin 1
RNA polymerase II fifth largest subunit-like protein	Gamma hydroxybutyrate dehydrogenase
Ubiquitin/ribosomal protein CEP52	GF14omega isoform
Ubiquitin conjugating protein	HSP associated protein-like
Ubiquitin extension protein (2)	In2-1 protein
Vf14-3-3c protein	Iron sulfur subunit of succinate dehydrogenase and ribosomal protein S14
Transporters	Lactoylglutatione lyase
34 kDa outer mitochondrial membrane protein porin	Late embryogenesis-like protein
36 kDa outer mitochondrial membrane protein porin	LTCOR11
Adenosine triphosphatase (2)	Minor allergen hazelnut profiling
ADP-ribosylation factor	Miraculin-like protein (2)
ADP-ribosylation factor-like protein	Miraculin-like protein 2 (5)
Aquaporin-like protein	mRNA cleavage factor subunit-like protein
Gamma tonoplast intrinsic protein	NAM-like protein (2)
Nonspecfic lipid-transfer protein 3 precursor (2)	N-hydroxycinnamoyl/benzoyltransferase
Phosphate transport protein G7	O-linked GlcNAc transferase-like
Plasma membrane intrinsic protein 1-1	PAPS-reductase-like protein
Plasma membrane intrinsic protein 1C	Phi-1-like protein
Plasma membrane intrinsic protein 2-2	Phospholipid transfer protein
Plasma membrane MIP protein	Plastid ribosomal protein S9 precursor
Suppressor of K+ transport growth defect-like protein	QM-like protein
SYBL1-like protein	Remorin
Tonoplast intrinsic protein bobTIP26-2	Reversibly glycosylated polypeptide
Vacuolar ATP synthase subunit G	RUB1 conjugating enzyme
Vacuolar H+-pumping ATPase 16 kDa proteolipid	Rurm1
Water channel-like protein	Salt-tolerance protein
Unclassified proteins	Sinapyl alcohol dehydrogenase
(3R)-Hydroxymyristoyl-dehydratase-like protein	Stem secoisolariciresinol dehydrogenase
1-Aminocyclopropane-1-carboxylate oxidase	TGF-beta receptor-interacting protein 1
24-Sterol C-methyltransferase	Thiazole biosynthetic enzyme
2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	Thymidine kinase
3-Oxoacyl-reductase	Translationally controlled tumor protein homolog

*( ), The number of unigenes matched to the same functional gene.

In the current study, a homology search of 763 unigenes was carried out with the BLASTX program against the NCBI database. Here, the deduced amino acid sequences of 495 unigenes (65%) were matched to protein sequences in that database, while the other 268 (35%) had no significant similarity to any other peptide sequences with matches higher than an E value of 10^{-5} (Fig. 2A). This percentage is lower than that calculated in a whole-seedling EST project with *C. sinensis* (Bausher et al., 2003) but close to that obtained in research with citrus fruit/seed (Omura et al., 2000).

The 253 unigenes matched to proteins with known function were manually classified into 10 cellular functional categories using a modified MIPS MATDB classification (Fig. 2B). The largest category was 'protein synthesis and processing', with 28.6%. The second and the third most prominent were related to 'metabolism' and 'cell rescue, defense, cell death and aging', with 15.7 and 9.4%, respectively. This demonstrates that the distributions of functional categories differ from those for fruit pulp at the development stage and for the mature pulp of *C. unshiu*. In that earlier study, Omura et al. (2000) found that the largest category pertained to 'cell growth and structure', followed by 'environment response', 'metabolism', and 'protein synthesis/processing', the last including 'protein regulation/signal transduction', 'transcription', and 'translation'. However, our results here were similar to those reported for the

Table 3. Redundancy of ESTs matched to known functional genes.

Clone	Accession number[a]	Putative function	Organism[b]	E-value[c]	No. of clones
Contig 46	T10106	Chitinase precursor	Citrus sinensis	8.44E-56	129
Contig 134	AAG38517	Miraculin-like protein	Citrus x paradisi	3.87E-61	54
Contig 88	AAG38522	Lectin-related protein precursor	Citrus x paradisi	1.11E-24	50
Contig 31	AAG38518	Miraculin-like protein 2	Citrus x paradisi	7.14E-124	43
Contig 123	AAG38522	Lectin-related protein precursor	Citrus x paradisi	1.05E-24	36
Contig 54	AAG38517	Miraculin-like protein	Citrus x paradisi	7.91E-129	23
Contig 53	T10106	Chitinase precursor	Citrus sinensis	5.65E-60	22
Contig 60	Q40250	Ribulose bisphosphate carboxylase small chain	Lactuca sativa	3.80E-80	20
Contig 73	T10106	Chitinase precursor	Citrus sinensis	5.17E-63	13
Contig 20	AAG38518	Miraculin-like protein 2	Citrus x paradisi	4.61E-50	12
Contig 74	AAG38518	Miraculin-like protein 2	Citrus x paradisi	4.40E-40	12
Contig 86	AAG38522	Lectin-related protein precursor	Citrus x paradisi	8.78E-85	11

[a]Accession number of the most significant BLASTX match.
[b]Source organism of the most significant BLASTX match.
[c]E-value of the most significant BLASTX match.

mature albedo of *C. unshiu*, where the largest categories were 'protein synthesis/processing', then 'cell growth and structure' (its proportion being very low in the current work), 'environment response', and 'metabolism'. This seems to suggest that the peel containing the albedo of immature *C. unshiu* fruit is actively engaged in protein synthesis and processing.

The most abundant gene expressed in our young fruit was associated with chitinases (Table 3), which are widely distributed in plants, fungi, and bacteria. Chitinases are pathogenesis-related (PR) proteins that can be induced by a variety of chemical elicitors or pathogen infection, and they are believed to be involved in the plant defense response (Porat et al., 2001). They are well known for their protective properties against several phytopathogenic fungi, where they inhibit both spore germination and hyphal growth, either alone or synergistically with other PR proteins, e.g., β-1,3-glucanase, as well as with ribosome-inactivating proteins (Vannini et al., 1999).

The second most abundant gene in our cDNA library was related to the miraculin-like protein (Table 3). Such a protein isolated from the legumes of sugar snap *Pisum sativum* var. macrocarpon reportedly has antifungal activity (Ye et al., 2000). Miraculin is called "a taste-modifying protein" because it has the unusual ability to modify a sour taste into something sweet (Theerasilp and Kurihara, 1988; Gibbs et al., 1996).

The third most abundant gene expressed in the young fruit was lectin-related (Table 3). Lectin has an entomotoxic effect when fed to insects from the Coleoptera, Homoptera, and Lepidoptera orders. However, application of such lectins for engineering insect-resistant plants is still largely restricted by their toxicity to mammals and/or non-target insects (Carlin and Grossi-de-sá, 2002), such that more screening is required in the plant kingdom.

As mentioned above, the three kinds of highly expressed genes -- chitinase, miraculin-like protein, and a lectin-related protein precursor -- are known to be involved in plant biotic and/or abiotic responses. Based on previous investigations, these genes seem to be very common in citrus species, playing important roles in protecting plants, especially their fruits at an early developmental stage, from attacks by pests and pathogens. Therefore, those genes may prove useful as molecular markers for conventional breeding as well as target genes for molecular breeding of insect- and pest-resistant crops.

Sequences for the 1914 high-quality ESTs have now been registered in the Genbank dbEST (http://www.ncbi.nlm.nih.gov) and the Gene Bank of Subtropical/Tropical Organisms at Cheju National University (http://genebank.cheju.ac.kr.). These data provide information about the genes expressed in the young fruit of satsuma mandarins. We are currently utilizing these data and others in Genbank for microarray analysis and molecular approaches toward understanding the physiological events within citrus crops.

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