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Differentially expressed cDNAs at the early stage of banana ripening identified by suppression subtractive hybridization and cDNA microarray

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Abstract The banana (*Musa acuminate* L. AAA group) fruit undergoes a postharvest ripening process, which plays an important role in improving the quality and extending the shelf life of bananas. To manipulate postharvest banana ripening, a better understanding of the mechanism of postharvest ripening is necessary. The isolation of mRNA transcripts encoding proteins associated with the ripening process is a powerful tool for this purpose. To isolate differentially expressed genes at the early stage of postharvest banana ripening, a forward suppression subtractive hybridization (SSH) cDNA library was constructed. SSH was performed with cDNA from banana fruit on the day of harvest as the "driver" and cDNA from banana fruit 2 days postharvest (DPH) as the "tester." A total of 289 clones in the SSH library were sequenced. BLASTX results revealed that 191 cDNAs had significant sequence homologies with known sequences in the NCBI database. Of the 191 cDNAs, 138 were singletons, and 53 belonged to divergent clusters containing 2–8 sequences. The identified cDNAs encoded proteins involved in cellular processes such as: metabolism; protein destination and storage; protein synthesis; signal transduction; transport and intracellular traffic; cell structure, growth, and division; transcription and post-transcription; and disease and defense. To characterize differentially expressed cDNAs in the SSH library, cDNA microarray analysis was conducted. A total of 26 cDNAs in the 2-DPH banana fruit were found to be up-regulated and these results were confirmed by using reverse transcriptase-polymerase chain reaction (RT-PCR). The information generated in this study provides new clues to aid in the understanding of banana ripening.

Keywords Banana (*Musa* AAA group cv Brazilian) · cDNA microarray · Postharvest banana ripening · Suppression subtractive hybridization

Abbreviations

SSH	Suppression subtractive hybridization
RT-PCR	Reverse transcriptase-polymerase chain reaction

Introduction

The banana (*Musa acuminate* L. AAA group), a typical climacteric fruit, undergoes a postharvest ripening process characterized by a green-storage phase, followed by a burst in ethylene production that signals the beginning of the climacteric period. Coincident with this respiratory climacteric, numerous physiological, and biochemical changes occur in the banana fruit including conversion of starch to sugars in the pulp (Hill and ap Rees 1995a, b), enzymatic degradation of structural carbohydrates (Kojima et al. 1994), degradation of polyphenols (Mendoza et al. 1994), an

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increase in isoamyl acetate (Harada et al. 1985), and chlorophyll breakdown (Thomas and Janave 1992). These changes influence qualities of banana fruit such as firmness, astringency, aroma, color, and shelf life. As a result, manipulating postharvest ripening plays an important role in improving the quality and extending the shelf life of bananas.

An essential first step for improving the postharvest banana ripening process is to gain a better understanding of the mechanism of postharvest ripening. A powerful method for exploring this mechanism is the isolation of mRNA transcripts encoding proteins that are associated with the ripening process (Dominguez-Puigjaner et al. 1992; Mugugaiyan 1993). Expressed mRNA transcripts at different stages of postharvest ripening have different levels of abundance that can be detected by using differential screening (Clendennen and May 1997; Medina-Suárez et al. 1997). Differential screening of cDNA libraries from banana pulp at ripening stages 1 and 3 revealed that the following cDNAs were down-regulated in pulp during the ripening process: starch synthase, granule-bound starch synthase (GBSS), chitinase, lectin, and a type-2 metallothionein (Clendennen and May 1997). In contrast, the following cDNAs showed increased abundances early in ripening: endochitinase, β-1,3-glucanase, a thaumatin-like protein, ascorbate peroxidase, metallothionein, and a putative senescence-related protein (Clendennen and May 1997). In another study, cDNA libraries from banana pulp at different ripening stages were differentially screened using a nove1 microtiter plate method. The identified ripening-related cDNAs encoded enzymes involved in ethylene biosynthesis, respiration, starch metabolism, cell wall degradation, and severa1 other key metabolic events (Medina-Suárez et al. 1997). Most of the cDNAs isolated in these studies are abundantly expressed in bananas, reflecting a limitation of differential screening.

To isolate genes that are differentially expressed at lower levels, suppression subtractive hybridization (SSH; Diatchenko et al. 1996) is a powerful tool. SSH combines SSH with PCR to generate a population of PCR fragments enriched for sequences that are differentially expressed. This technique, in combination with cDNA arrays, has been successfully used to isolate differentially expressed genes in different plant systems including potato (Faivre-Rampant et al. 2004), *Arabidopsis* (Hinderhofer and Zentgraf 2001), alfalfa (Hays and Skinner 2001), rice (Chen et al. 2002), wheat (Luo et al. 2002), apple (Degenhardt et al. 2005), maize (Zheng et al. 2004), soybean (Liao et al. 2003), poplar (Rishi et al. 2004), *Eucalyptus* (Paux et al. 2004), cotton (Ji et al. 2003), barley (Jang et al. 2003), and sugarcane (Watt 2003). However, to our knowledge, there is only one report identifying differentially expressed genes at the late stage of postharvest banana ripening using SSH, followed by differential screening of cDNA (Manrique-Trujillo et al. 2006).

The manipulation of postharvest banana ripening to improve banana quality and shelf life is increasingly viewed as a desirable adjunct to existing agronomic practices. However, knowledge of the genetic mechanisms of postharvest banana ripening is fundamental to the deployment of such technology. In this study, we used SSH combined with a cDNA microarray to isolate low-abundance cDNAs that are differentially expressed in the early stage of postharvest banana ripening.

Materials and methods

Plant materials

Banana fruits (*Musa* spp. AAA group, cv. Brazilian) were obtained from the banana plantation of the State Key Biotechnology Laboratory of Tropical Crops (Chengmai, Hainan province, China). Fully mature and ready-to-market fruits from the same hand were harvested and washed with distilled water. Each banana hand was divided into two groups, each consisting of three banana fingers. Fingers from the same hand were used as a sample group to avoid variation in ripening behaviors among different hands (Inaba and Nakamura 1986). Of these two groups of samples, one group was frozen quickly in liquid nitrogen. cDNA from this group represented mRNA from banana fruit 0 days postharvest (DPH). The other group was stored in a controlled environment incubator (New Brunswick Scientific Co. Inc., Edison, NJ, USA) at 28 °C for 48 h. cDNA from this group represented mRNA from bananas 2 DPH.

RNA extraction and cDNA synthesis

Total RNA from banana fruit (pulp and peel) 0 to 2 DPH was separately isolated following the method described by Wan and Wilkins (1994). Total RNA was purified using RNeasy mini spin columns (Qiagen, Valencia, CA, USA). Purified mRNA from bananas 0 and 2 DPH was reverse transcribed to cDNA by using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA), following the manufacturer's instructions. The cDNA from banana fruit 0 DPH was used as the "driver" cDNA pool and the cDNA from banana fruit 2 DPH was used as the "tester" cDNA pool in the SSH experiment.

SSH and construction of a subtractive cDNA library

SSH was conducted by using PCR-Select™ cDNA Subtraction Kit (Clontech). The tester and driver cDNA populations were digested with the restriction enzyme *Rsa*I (included in the kit) to obtain short, blunt-ended fragments. The tester pool was then divided into two populations. One population was ligated to adaptor 1 and the other to adaptor 2R (both provided in the kit). Each tester pool was then hybridized separately with an excess of driver cDNA, and the two reactions were mixed together for a second SSH. Fragments in the tester (banana fruit 2 DPH) cDNA, but not in the driver (banana fruit 0 DPH) cDNA, were then specifically amplified in two PCRs, according to the manufacturer's recommendations. The subtracted PCR products generated by SSH were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* DH5α. Plasmids were extracted from 305 randomly selected clones, and the presence of recombinant plasmid was confirmed by PCR with nested PCR primers provided in the PCR-Select cDNA Subtraction Kit (Clontech).

Sequence analysis

Nucleotide sequences of the inserted cDNA fragments were determined using an ABI PRISM™ 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) using the BigDye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). All sequences were compared to the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Functional categorization of sequences was performed by comparison with sequences in the NCBI database.

cDNA microarray preparation

A total of 265 recombinant clones were confirmed. The insert cDNAs were amplified by PCR using nested PCR primers (5′-TCGAGCGGCCGCCCGGGCAGGT-3′ and 5′-AGCGTGGTCGCGGCCGAGGT-3′) provided in the PCR-Select cDNA Subtraction Kit (Clontech). Reactions were performed for 40 cycles of denaturation at 95 °C for 45 s, annealing at 68 °C for 60 s, and extension at 72 °C for 1 min, followed by a final 7-min extension at 72 °C. The PCR products were purified using the QIAquick PCR purification Kit (Qiagen) and 3 μl of each reaction was fractionated in an agarose gel to confirm amplification quality and quantity. The remaining cDNA was precipitated by the addition of 100 μl anhydrous ethanol and resuspended in 15 μl 50% DMSO. Each cDNA sequence was arrayed in duplicate on two aluminum-coated and DMSO-optimized glass slides of $75 \times 25 \times 1$ mm^3 using the Array Spotter Generation III (Genomic Instrumentation Services, Piscataway, NJ, USA).

Two housekeeping genes from banana (actin and ubi) were used as internal controls (GenBank accession numbers AY904067 and DQ885479). Three sequences from *Arabidopsis* (c104, c125, and c127; GenBank accession numbers AC004146, AC007661, and U09332) that cannot hybridize to genome sequences of banana were used as external controls. A synthetic oligonucleotide labeled with hexachlorofluorecein (5′-GTCACATGCGATGGATCGAGCTCCTTTATCATCGTTCCCACCTTAATGCA-3′), which yields a fluorescent signal in microarray scanning whether or not it is hybridized to probes, was used to confirm successful spotting of samples onto the array. These control sequences were spotted 12 times in different subgrids on the two microarrays.

Preparation of fluorescent probes and hybridization

Fluorescent probes were prepared by using single primer amplification (SPA) of cDNA as described previously (Smith et al. 2003). For production of SPA cDNA, 10 μg total RNA from banana fruit 0 and 2 DPH was separately used for synthesis of first- and second-strand cDNA using the cDNA Synthesis Kit (TAKARA, Dalian, China). A modified oligonucleotide (5′-AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGCTTTTTTTTTTTTTTTV-3′) was used in place of the oligo(dT) primer provided in the kit to prime first-strand synthesis. After completion of second-strand synthesis, tester and driver cDNA were purified using a PCR purification kit (Qiagen) and eluted with 60 μl water. Amplification reactions of tester and driver cDNA were set up as follows. A volume of 10 μl of 10× PCR buffer (including a final Mg^{2+} concentration of 1.5 mM), 10 μl dNTPs at 2 mM concentration, 100 pmol heel primer (5′-CGGCCAGTGAATTGTAATACGACTCACTATAGGCG-3′), 12.5 U *Taq* DNA polymerase (NAKADA, Dalian, China), and cDNA template were combined in water to a 100 μl total volume. The reactions were placed in a GeneAmp RCR system 2400 (Perkin Elmer, Boston, MA, USA) and incubated at 94 °C for 3 min, then subjected to 40 cycles at 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 2 min. The amplified tester and driver cDNA was again purified using the Qiaquick PCR purification Kit (Qiagen).

The amplified tester and driver cDNA were then fluorescently labeled using the Klenow fragment. Briefly,

21 μl tester or driver cDNA was mixed with 20 μl random octamer oligonucleotides provided in the Bioprime Kit (Invitrogen, Carlsbad, CA, USA). The samples were heated at 90 °C for 5 min and snap-cooled on ice. A volume of 5 μl of 10× dNTP mix was added to each sample (1.2 mM each dATP, dGTP, and dTTP and 0.6 mM dCTP), followed by 3 μl Cy5-dCTP or Cy3-dCTP from 1 mM stocks (Amersham Biosciences, Piscataway, NJ, USA). Then, 1 μl high-concentration Klenow fragment (50 U/μl; Amersham Biosciences) was added, and the samples were incubated at 37 °C for 2 h. Reactions were stopped by the addition of 5 μl 0.5 M EDTA, pH 8.0. Labeled cDNA was purified with a PCR purification kit (Qiagen) and resuspended in the elution buffer. The labeled controls and tester and driver cDNA samples were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed with 30 μl hybridization solution containing 50% formamide, 1× hybridization buffer (Amersham Biosciences). DNA in the hybridization solution was denatured at 95 °C for 3 min prior to loading onto a microarray. One slide was hybridized with a Cy3/Cy5-labeled pair of cDNA from 0/2-DPH banana fruits. For the other slide, we reversed the assignment of Cy3/Cy5 dyes for 2/0-DPH banana fruits. The arrays were hybridized at 42 °C overnight and washed with two consecutive solutions (0.2% SDS, 2× SSC at 42 °C for 5 min, and 0.2× SSC for 5 min) at room temperature.

Data analysis

After washing, the microarray slides were dried briefly by centrifugation. The two microarrays were scanned with a ScanArray Express scanner using ScanArray 2.0 software (Packard Bioscience, Boston, MA, USA). We quantified signal intensities of individual spots from the 16-bit TIFF images using GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA). The linear normalization method was used for data analysis, based on the expression levels of ubi and actin (banana housekeeping genes) in combination with the *Arabidopsis* external controls. Expression levels of cDNAs in the array were represented as the intensity ratio of 2-DPH/0-DPH banana fruits based on the *t*-test. cDNAs with intensity ratios ≥1.5 ($P < 0.05$) were considered to be differentially expressed. These cDNAs were selected for reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm the results from the cDNA microarray.

RT-PCR for expression pattern of differentially expressed cDNA

To confirm the cDNA microarray result, 4 μg total RNA from 0- to 2-DPH banana fruit was used to synthesize cDNA using the SMART PCR cDNA Synthesis Kit (Clontech). Each PCR reaction contained 2 μl cDNA template along with selected highly expressed gene-specific primers (Table 1). The gene-specific primers used in this study were designed by using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA). All gene-specific primers amplified cDNA fragments of the predicted sizes (Table 1). Actin was used as an internal standard. The PCR conditions were as follows: 1 cycle at 94 °C for 4 min; 26 cycles at 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s; and an additional cycle at 72 °C for 10 min. To quantitate the RT-PCR bands, the Scion Image (Scion Corporation, Frederick, MD, USA) was used. Relative expression level of each target cDNA was represented as the value of band intensity ratio of target cDNA/Actin following manufacturer's instructions. The fold expression was represented as the value of each target cDNA relative expression level ratio of 2 DPH/0 DPH.

Results

Isolation and characterization of cDNA clones in SSH libraries representing mRNAs expressed in postharvest banana ripening.

To isolate genes differentially expressed in the postharvest banana, the SSH strategy was used for forward subtraction. cDNA from bananas 2 DPH was used as the tester and cDNA from bananas 0 DPH was used as the driver to enrich and identify genes differentially expressed in banana fruit 2 DPH. The SSH library was constructed by ligation of subtracted cDNA fragments into the pGEM-T Easy vector. A total of 289 clones from 302 randomly selected clones in the SSH library were confirmed to be recombinant by using PCR with nested primers (data not shown). The recombinant plasmids of the 289 clones were isolated and sequenced. Sequence analysis of these 289 insert cDNAs showed that 265 cDNA sequences were readable. BLASTX results for the 265 cDNAs revealed that 79 cDNAs, 27.9% of the total cDNAs, did not have a match within the NCBI database. The other 191 cDNAs had significant sequence homologies with known sequences in the NCBI database. Of the 191 cDNAs, 138 cDNAs were singletons, and 53 cDNAs belonged to divergent clusters. Each cluster contained 2–8 sequences. Of the 191 cDNAs, 52 had unknown functions. Based on the highest BLASTX scores, functional classification of these cDNAs was conducted according to the EU *Arabidopsis* sequencing project, as described by Goldberg (http://www.mcdb.ucla.edu/Research/Goldberg/). The cDNAs in the SSH library

Table 1 Up-regulated genes at an early stage of postharvest ripening of banana

SSH Clone number	BLAST homologues[a]	Accession number	Numbers of clones in the SSH library	Ratio of signal intensity[b] (fluorescence units) 2 DPH/0 DPH
SSH-24	Alcohol dehydrogenase A (*Washingtonia robusta*)	ABA39598.1	1	2.4276
SSH-266	Complete chloroplast genome (*Acorus calamus*)	AJ879453.1	2	2.3894
SSH-53	ATP synthase CF1 epsilon chain (atpE) gene (*Yucca schidigera*)	DQ069396.1	3	2.3877
SSR-170	Metallothionein-like protein (*Musa acuminata*)	AF268393.1	7	2.2410
SSH-60	ATP synthase CF_0 C chain (*Yucca schidigera*)	DQ069384.1	1	2.1013
SSH–61	ABA- and ripening-inducible-like protein (*Oryza sativa*)	AF039573.1	1	2.0181
SSH-64	No homologues		1	2.0481
SSH-47	Granule-bound starch synthase (*Pennisetum glaucum*)	AAQ06271.1	1	2.0735
SSH-145	Putative polyphosphoinositide binding protein (*Arabidopsis thaliana*)	AAL86320.1	1	1.9702
SSH-184	Hypothetical protein (*Deinococcus radiodurans*)	F75297	1	1.9317
SSH–93	Putative protein (*Arabidopsis thaliana*)	CAB78914.1	3	1.8455
SSH-269	Senescence-associated protein-like (*Oryza sativa*)	XP_481260.1	1	1.8117
SSH-95	MADS-box transcription factor (*Asparagus virgatus*)	BAD83772.1	1	1.8012
SSH-196	Endochitinase (*Musa acuminata*)	AF416677.1	4	1.6689
SSH-9	No homologues		1	1.6508
SSH-76	DNA-directed RNA polymerase alpha chain (PEP) (*Australopyrum velutinum*)	CAB01381.1	1	1.6508
SSH-198	No homologues		1	1.5990
SSH-110	ATP-dependent clp protease (*Arabidopsis thaliana*)	NM_114746.2	1	1.5778
SSH-3	Unknown protein (*Arabidopsis thaliana*)	NP-568066.1	1	1.5640
SSH-256	Putative serine/threonine-protein kinase (*Oryza sativa*)	XP_467323.1	1	1.5566
SSH-271	No homologues		1	1.5425
SSH-105	Expressed protein (*Arabidopsis thaliana*)	NP_568066.1	1	1.5309
SSH-21	Ribosomal protein L22 (*Panax ginseng*)	YP_087005.1	1	1.5251
SSH-219	Endo-1,4-beta-D-glucanase (*Populus tremuloides*)	AY535003.1	4	1.5110
SSH-27	No homologues		1	1.5091
SSH-161	No homologues		1	1.5063

[a] BLASTN and BLASTX searches were conducted to determine homologous genes and putative functions of the cDNAs in the SSH library. The cut-off *e*-value used was 10^{-5} . Sequences with no significant hits were labeled “no homologues”

[b] Ratios of signal intensities were determined by cDNA microarray as described in Materials and methods ($P < 0.05$)

were involved in many processes including metabolism (8.1%), signal transduction (7.9%), protein synthesis (5.6%), transcription and post-transcription (7.2%), protein destination and storage (4.8%), transporter and intracellular traffic (5.2%), cell structure, growth and division (6.0%), and disease and defense (7.7%).

We amplified and purified the 265 inserted cDNAs in the SSH library. The purified fragments were arrayed in duplicate on glass slides. Two chips were made to identify the cDNAs that are differentially expressed in ripening bananas 2 DPH compared to 0 DPH. One chip was hybridized with mRNA from banana fruit 0 DPH, labeled with Cy3-dCTP, and from banana fruit 2 DPH, labeled with Cy5-dCTP. The other chip was hybridized with mRNA from banana fruit 0 DPH, labeled with Cy5-dCTP, and from banana fruit 2 DPH, labeled with Cy3-dCTP. The overlay images of the two chips are shown in Fig. 1. Expression levels of cDNAs in the arrays were represented as the intensity ratios of 2-DPH/0-DPH banana fruit. cDNAs with ratios ≥1.5 ($P < 0.05$) were considered to be up-regulated in 2-DPH bananas. A total of 26 cDNAs were found to be up-regulated (Table 2). These cDNAs were involved in metabolism, signal transduction, disease and defense, and chloroplasts. In addition, five cDNAs with unknown functions and four cDNAs with no matches in the NCBI database were up-regulated in 2-DPH bananas. Furthermore, several highly expressed genes whose functions have never been linked to banana ripening were found to be up-regulated, including a putative serine/threonine-protein kinase, a MADS-box transcription factor, a putative polyphosphoinositide binding protein, ATP synthase CF1 epsilon chain (atpE), and ATP synthase CF_0 C chain.

Expression patterns of up-regulated genes by RT-PCR

To confirm the results of the cDNA microarray, we randomly selected ten up-regulated cDNAs to analyze their expression patterns by RT-PCR. Five cDNAs (alcohol dehydrogenase A, ATP synthase CF1 epsilon chain, metallothionein-like protein, abscisic acid (ABA)- and stress-inducible protein, and GBSS) that showed greater than 2.0-fold up-regulation and five cDNAs (putative polyphosphoinositide binding protein, MADS-box transcription factor, expressed protein, ATP-dependent clp protease, and endo-1,4-beta-D-glucanase) that showed between 1.5-fold and 2.0-fold up-regulated expression were used to validate the microarray results, with actin as a positive control. RT-PCR was performed using total RNA isolated from 0 to 2 DPH banana fruit with the synthesized primers listed in Table 2. For all genes, the RT-PCR results (Fig. 2) were generally in accordance with the microarray data (Table 2).

Discussion

Postharvest ripening plays a crucial role in commercial banana fruit quality and shelf life. To gain a better understanding of postharvest ripening of the banana at the molecular level, we used SSH to isolate 265 cDNAs that were up-regulated in ripening bananas at 2 DPH. These cDNAs were classified into several categories based on results of BLASTX searches in the NCBI database. cDNAs isolated by SSH were involved in a variety of physiological and molecular events including: metabolism; protein destination and storage; protein synthesis; signal transduction; transport and intracellular traffic; transcription and post-transcription; cell structure, growth, and division; and disease and defense. These results imply that many complicated molecular processes occur in the early stage of postharvest banana ripening. It is anticipated that the information obtained from this study will guide further exploration to establish the connection between differentially expressed genes, the corresponding translated proteins, and their biological functions in the early stages of banana ripening.

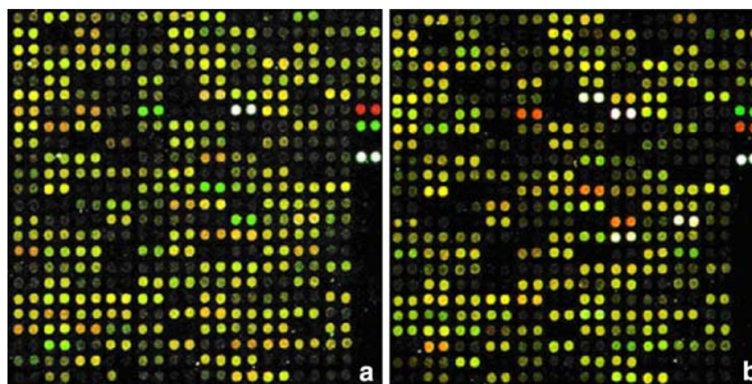

Fig. 1 Overlay images of cDNA microarrays screened by mRNA from 0- to 2-DPH bananas. **a** cDNA microarray hybridized with mRNA from banana fruit 0 DPH, labeled with Cy3, and from banana fruit 2 DPH, labeled with Cy5. **b** cDNA microarray hybridized with mRNA from banana fruit 0 DPH, labeled with Cy5, and from banana fruit 2 DPH, labeled with Cy3

Table 2 Primers used for RT-PCR

Clone number	Gene name	Primer set	Expected size (bp)
SSH-24	Alcohol dehydrogenase A	5′GTGCCTCACAAAGATGCTG3′ 5′GTACTAATCCCATATACC3′	329
SSH-47	Granule-bound starch synthase	5′CCAGTTACTGGGAAGAAGC3′ 5′CACTTTCAGGGAGTGGCC3′	520
SSH-53	ATP synthase CF1 epsilon chain (atpE)	5′TGACATTGATCCGCAAGAAGC3′ 5′TGGCAGAATCAACATGTGGTG3′	227
SSH-61	ABA- and ripening-inducible-like protein	5′CAAGCATCCCACACTCAATAC3′ 5′CACAAGCACAAGATCGAGG3′	285
SSH-95	MADS-box transcription factor	5′AGTTCGGATGCCATTGTGG3′ 5′TCCTGGAATAGCATCGTAC3′	350
SSH-105	Expressed protein	5′GCATGAAAGTGCCAACTC3′ 5′CTGCTGAGAAAAAGCAAGC3′	595
SSH-110	ATP-dependent clp protease	5′GTGAATTGCAGTGTATTGGAGC3′ 5′CTTGCCTCCTCAGGAAGC3′	323
SSH-170	Metallothionein-like protein	5′AGAGCTACGTCGACGAG3′ 5′TTAGAGTACATAGCCACAC3′	171
SSH-145	Putative polyphosphoinositide binding protein	5′CATGAGGGTTTGGAAGATC3′ 5′CACGAGTTCTCAGTTGACC3′	345
SSH-219	Endo-1,4-beta-D-glucanase	5′CATCTCAGTCTCAGCTTTATGC3′ 5′TACAACTGCAAAGGAGGATC3′	490
Control	Actin (access. No. AF285176)	5′TGTAGCAATTCAGGCTGTTCTT3′ 5′TCAGAGATGGCTGGAAGAGAAC3′	390

An oligonucleotide primer set was designed for selected genes using Primer Premier 5.0 and was used for RT-PCR analysis. The expected length of the PCR product for each gene is indicated

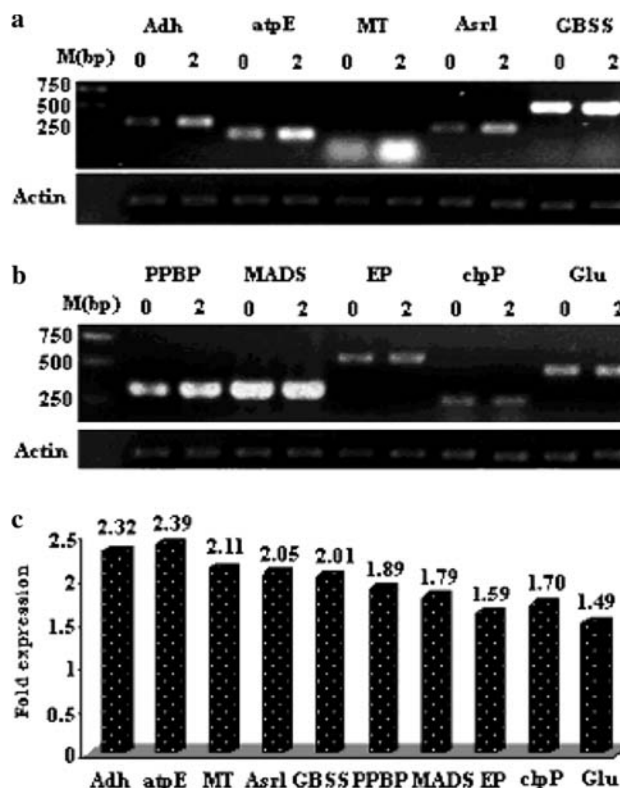

Fig. 2 RT-PCR of ten genes in banana that showed greater than 1.5-fold up-regulated expression at 2 DPH compared to 0 DPH. **a** Five genes that showed greater than 2.0-fold up-regulated expression at 2 DPH. **b** Five genes that showed greater than 1.5-fold up-regulated expression at 2 DPH. Actin was used as the control. **c** Fold expressions of ten up-regulated genes at 2 DPH. The fold expression of each gene is calculated by using Scion Image described in Materials and methods. M: DNA ladder. About 0 and 2 represent 0 and 2 DPH. *Adh* alcohol dehydrogenase, *atpE* ATP synthase CF1 epsilon chain, *MT* metallothionein-like protein, *Asr1* abscisic acid- and stress-inducible protein, *GBSS* granule-bound starch synthase, *PPBP* putative polyphosphoinositide binding protein, *MADS* MADS-box transcription factor, *EP* expressed protein, *clpP* ATP-dependent clp protease, *Glu* endo-1,4-beta-D-glucanase

There are three aspects of our study that distinguish it from similar studies on banana ripening (Medina-Suárez et al. 1997; Clendennen and May 1997; Manrique-Trujillo et al. 2006). First, of the 26 up-regulated cDNAs, some were linked for the first time to banana fruit ripening, even though they were present at low abundance. Second, six up-regulated cDNAs had deduced protein products with uncertain functions or were putative proteins, although the putative functions of these genes were deduced from the NCBI database. The identification of these genes with uncertain functions could provide new clues to understanding banana ripening. Third, of the 26 up-regulated cDNAs, three cDNAs were encoded by the chloroplast genome and were found for the first time to be expressed in the early stage of banana ripening (SSH-266, SSH-76, and

SSH-21). This result implies that genes encoded by the chloroplast genome may play roles in banana ripening. The relationship between the chloroplast genome and the nuclear genome in banana postharvest ripening remains an interesting aspect to be further researched.

To identify cDNAs differentially expressed at the early stage of postharvest banana ripening, cDNA microarrays were used. A total of 26 up-regulated cDNAs were identified, and the expression patterns of 10 cDNAs were further confirmed by RT-PCR. Some of the genes up-regulated during banana ripening were reported in previous studies, such as GBSS, endochitinase, metallothionein-like protein, and alcohol dehydrogenase (Clendennen and May 1997; Medina-Suárez et al. 1997; Manrique-Trujillo et al. 2006).

Alcohol dehydrogenases (Adh) have been found to be expressed in apples, strawberries, and tomatoes, with a suggested role in regulating ester biosynthesis during fruit ripening (Speirs et al. 1998; Beekwilder et al. 2004; Defilippi et al. 2005). In this study, a cDNA encoding an Adh was found to be strongly up-regulated (2.4 times) in the early stages of banana ripening. In a recent study, Adh was also found to be up-regulated at the later stages of banana ripening (Manrique-Trujillo et al. 2006). These results suggest that aroma production begins at the beginning of postharvest ripening of bananas and continues to the late phase of ripening.

A gene encoding a metallothionein-like protein was also found to be up-regulated, with seven clones in the SSH library. This cDNA showed high similarity in sequence to MT3, previously identified in banana (Liu et al. 2002). A recent report demonstrated that expression of MT3 was sharply up-regulated from PCI 1 to PCI 5, with a further increase at PCI 7 in the later stages of banana ripening (Manrique-Trujillo et al. 2006). Our results showed that this gene was up-regulated from 0 to 2 DPH prior to PCI 1 to PCI 5, because the banana fruits 2 DPH used in this study were at PCI 1 in terms of peel colors. Accordingly, our results suggest that the expression of this gene is up-regulated at an early stage. The activity of MTs in ripening banana fruit has been associated with the excess metal ions present during this process (Liu et al. 2002).

The endoglucanases (EGases; EC 3.2.1.4) are generally associated with the degradation of cellulose and are involved in cellulose biosynthesis in both the primary and secondary cell walls of *Arabidopsis* (Szyjanowicz et al. 2004). In a recent study, a full-length cDNA (*PtrKOR*) that matches the cDNA in our study was cloned from aspen xylem and was demonstrated to be expressed in response to tension stress when KOR (Korrigan endoglucanase) expression is suppressed (Bhandari et al. 2006). Moreover, three previously reported aspen cellulose synthase genes (*PtrCesA1*, *PtrCesA2*, and *PtrCesA3*) that are closely associated with secondary cell wall development in the xylem cells exhibited similar tension stress-responsive behavior. These results suggested that the coexpression of these four proteins is important for the biosynthesis of highly crystalline cellulose typically present in tension wood. The function of endo-1,4-beta-D-glucanase in ripening banana remains to be further researched.

A cDNA encoding GBSSshowed up-regulation in this study, in contrast to previous studies in which GBSS expression was down-regulated (Clendennen and May 1997; Medina-Suárez et al. 1997). These conflicting results may arise from the different banana materials used in the experiments. In our study banana fruit 2 DPH was used, but in the previous studies (Clendennen and May 1997; Medina-Suárez et al. 1997) ethylene-treated bananas at ripening stages 1 and 3 were used. Thus, the fruits used in these previous studies were stimulated by ethylene, which promoted the ripening process. The fruits used in our study ripened naturally. As a result, the fruits we analyzed were at an earlier stage of banana ripening than those in the previous studies. Our results indicate that starch synthesis proceeds at the early stage of natural postharvest ripening.

Two energy-related cDNAs were found to be up-regulated in our study. ATP synthase consists of the membrane-embedded sector F_0 and the extrinsic sector F_1. This enzyme catalyzes ATP synthesis by a proton-motive force across the membrane, which is generated by the respiratory chain or photosynthetic electron transport (McCarty et al. 2000). In this study, the ATP synthase CF1 epsilon chain (atpE) and ATP synthase CF_0 C chain genes were isolated for the first time and shown to be up-regulated in banana fruit 2 DPH. This finding suggests that energy production is required in the early stage of postharvest banana ripening. The elevated expression of energy production-related genes is consistent with the physiological and molecular events presumably occurring at this stage.

It should be noted that genes related to respiration and ethylene biosynthesis that were reported in a previous study on banana ripening (Clendennen and May 1997; Medina-Suárez et al. 1997) were not up-regulated at 2 DPH, although they were found in the SSH library. The reason for this discrepancy is that the subtraction library was constructed from bananas 2 DPH, the very early stage of banana ripening. Because small differences in gene expression between 0- and 2-DPH bananas can be detected by this protocol, genes related to respiration and ethylene biosynthesis do not appear

to be differentially expressed at 2 DPH compared to 0 DPH.

The MADS-box gene is a member of the large MADS-box family of transcription factors that play important roles in flower and fruit development (Robles and Pelaz 2005). In terms of fruit ripening, the identification of a fruit-specific strawberry MADS-box cDNA homologous to a tomato MADS-box gene (LeMADS-RIN) suggests the tantalizing possibility that MADS-box proteins may have a conserved function in fruit ripening in both climacteric and non-climacteric species (Vrebalov et al. 2002). More than 30 different members of the tomato MADS-box family were isolated and are available as ESTs (for tomato EST resources, see http://www.tigr.org/tdb/tgi/lgi/ and http://www.sgn.cornell.edu/). However, to our knowledge, no MADS-box gene was previously isolated from banana. All of the MADS-domain proteins examined to date dimerize or multimerize with other MADS-box proteins through homomeric or heteromeric interactions and then bind to DNA. The CArG box [CC(A/T)6GG] is a DNA consensus sequence that is recognized by MADS-domain proteins encoded by plant homeotic genes involved in the development of flowers and fruits. Accordingly, additional MADS-box genes may participate in postharvest banana ripening, because the MADS-box genes have been shown to act as dimers or heterogeneous higher-order multimers. All genes that contain CArG boxes in their promoters are potential targets of MADS-box proteins. The MADS-box gene described in this report will provide new insights into the mechanism of banana ripening in terms of transcription factors.

A cDNA encoding ABA- and ripening-inducible-like protein was found to be up-regulated in bananas 2 DPH. The deduced protein sequence showed that it had high homology with the ASR proteins first described in tomato, which are induced by ABA, stress, and ripening (Iusem et al. 1993; Amitai-Zeigerson et al. 1994; Rossi and Iusem 1994). *ASR* genes are expressed in various fruits such as the tomato, pomelo, apricot, and grape (Iusem et al. 1993; Canel et al. 1995; Mbeguie-A-Mbeguie et al. 1997; Çakir et al. 2003). *ASR* genes appear to be involved in processes of plant development such as senescence and fruit development and are expressed in response to abiotic stresses such as water deficit, salt, cold, and limited light (Schneider et al. 1997; de Vienne et al. 1999; Jeanneau et al. 2002). In grape, an *ASR* gene (*VvMSA*) up-regulated in the early stages of fruit development, and at late grape ripening, is inducible by sucrose, and this sugar induction is enhanced strongly by ABA (Çakir et al. 2003). However, to our knowledge, no *ASR* gene was previously cloned in banana, and there exists no precise information concerning the biological functions of ASR proteins.

In this study, a discrepancy between the number of clones in the SSH for the individual genes (Table 2) and the -fold changes in gene expression by RT-PCR (Fig. 2) was noted. Genes that are represented several times in the SSH (e.g., the metallothionein-like gene or endo-1,4-beta-D-glucanase) would be expected to show high-expression changes. The discrepancy may arise from the SSH and/or RT-PCR methods. The clone numbers of the individual genes in the SSH library may not represent the actual numbers of the individual gene transcripts in a normal cDNA library, possibly due to incomplete SSH. As a result, more clone numbers than expected would appear in the SSH library. We confirmed the results of the cDNA microarray screening by using RT-PCR. In this study, RT-PCR generally demonstrated the validity of the cDNA microarray screening, although RT-PCR as a method of relative quantification of gene expression is not faultless.

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