

# Chapter 2

## Suppression Subtractive Hybridization as a Tool to Identify Anthocyanin Metabolism-Related Genes in Apple Skin

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### Abstract

The pigmentation of anthocyanins is one of the important determinants for consumer preference and marketability in horticultural crops such as fruits and flowers. To elucidate the mechanisms underlying the physiological process leading to the pigmentation of anthocyanins, identification of the genes differentially expressed in response to anthocyanin accumulation is a useful strategy. Currently, microarrays have been widely used to isolate differentially expressed genes. However, the use of microarrays is limited by its high cost of special apparatus and materials. Therefore, availability of microarrays is limited and does not come into common use at present. Suppression subtractive hybridization (SSH) is an alternative tool that has been widely used to identify differentially expressed genes due to its easy handling and relatively low cost. This chapter describes the procedures for SSH, including RNA extraction from polysaccharides and polyphenol-rich samples, poly(A)<sup>+</sup> RNA purification, evaluation of subtraction efficiency, and differential screening using reverse northern in apple skin.

**Key words:** Anthocyanin, apple (*Malus domestica*) skin, differential screening, gene expression, poly(A)<sup>+</sup> RNA purification, RNA extraction, suppression subtractive hybridization (SSH).

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### 1. Introduction

Anthocyanins, which impart red coloration in apple skin, belong to the diverse group of ubiquitous secondary metabolites known as flavonoids. The pigmentation of anthocyanins is an important determinant of consumer preference and marketability as well as an attractive factor to pollinators. Anthocyanin biosynthesis has been well characterized in the flowers of petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*) and in the kernels of maize (*Zea mays*) (1). As such, the biosynthetic pathway is one of the well-known pathways in plants (2). It has been known that anthocyanin metabolism is induced by various environmental

factors (3) and controlled through the coordinated expression of biosynthetic genes in many plants (1). For example, in apple, both ultraviolet (UV)-B (280–320 nm) irradiation and low temperatures stimulate the production of anthocyanins (4, 5). The mRNA levels of *chalcone synthase* (*CHS*), *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*), and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UGT*) were up-regulated by both low temperature and UV-B irradiation (6). In addition, the expression of *MdMYBA*, a key transcription factor for anthocyanin accumulation, was also induced by low temperature and UV-B irradiation (7). Therefore, it can be considered that most of the genes involved in anthocyanin biosynthesis appear to participate in the development of pigmentation under the regulation of certain environmental factors. In this sense, the identification of genes whose expression is affected by the relevant environmental factors is a useful strategy for elucidating the mechanisms underlying the physiological process leading to pigmentation.

In order to isolate differentially expressed genes, microarrays have been widely used in plants. However, this technique needs special apparatus and is highly expensive. Thus, availability of microarrays is often limited and not commonly used. In addition, availability of commercial microarrays is restricted to model plants, although anthocyanins are widely distributed in numerous plant species. In apple, although about 180,000 ESTs have been deposited in public databases (8, 9), no commercial microarrays for the species have become available so far. Therefore, it is difficult to utilize microarray technology in many studies involving anthocyanins. Suppression subtractive hybridization (SSH) is an alternative tool that has been widely used to identify differentially expressed genes due to its easy handling and relatively low cost (10). SSH is a powerful technique to compare two populations of mRNA and obtain clones of genes that are differentially expressed. Although there are many steps in the method of SSH, the basic theory is simple. First, cDNA is synthesized from 2  $\mu$ g of poly(A)<sup>+</sup> RNA generated from the two types of tissues being compared: we refer to the cDNA that contains specific (differentially expressed) transcripts as tester and the reference cDNA as driver. The tester and driver cDNAs are then digested with *Rsa*I. The digested tester cDNA is subdivided into two portions, and each is ligated with a different cDNA adaptor. The driver cDNA has no adaptors. Tester and driver cDNAs are then hybridized. The hybridized sample is subjected to PCR that permits exponential amplification of cDNAs which differ in abundance, whereas amplifications of sequences of identical abundance in the two populations are suppressed. The amplified cDNAs can be directly inserted into a T/A cloning vector. This cloning allows identification of differentially expressed RNAs by sequence and/or hybridization analysis.

This chapter describes the procedures for SSH including the steps from RNA extraction to differential screening, based on our previous study (11), in which we performed SSH with the apple skin irradiated with UV-B to isolate other genes participating in anthocyanin biosynthesis apart from those located in the flavonoid biosynthetic pathway and MYB. The protocols described in this chapter can be utilized for polysaccharide- and polyphenol-rich samples such as fruits and flowers.

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## 2. Materials

### 2.1. Total RNA Extraction

1. Miracloth<sup>®</sup> (Calbiochem, San Diego, CA).
2. RNase-free water, made by adding 0.001% (v/v) diethylpyrocarbonate (DEPC), stirring overnight, and then autoclaving.
3. Extraction buffer (*see Note 1*): 200 mM sodium borate (pH 9.0), 30 mM ethylene glycol tetraacetic acid (EGTA), and 1% (w/v) sodium dodecyl sulfate (SDS). Use RNase-free water and store at room temperature. The extraction buffer is supplemented with 10 mM dithiothreitol (DTT), 2% (w/v) polyvinylpyrrolidone (PVP, MW 40,000), and 1% (v/v) Nonidet P-40 (NP-40) just prior to use.
4. Proteinase K (10 mg/mL). Use RNase-free water and store at  $-20^{\circ}\text{C}$ .
5. 2 M potassium chloride (KCl). Use RNase-free water, autoclave, and store at  $4^{\circ}\text{C}$ .
6. 10 and 2 M lithium chloride (LiCl). Use RNase-free water, autoclave, and store at  $4^{\circ}\text{C}$ .
7. 10 mM Tris-HCl (pH 7.5). Use RNase-free water, autoclave, and store at  $4^{\circ}\text{C}$ .
8. 2 M K-acetate (pH 5.5). Use RNase-free water, autoclave, and store at  $4^{\circ}\text{C}$ .
9. Absolute ethanol.
10. 70% ethanol. Use RNase-free water and store at  $4^{\circ}\text{C}$ .

### 2.2. Poly(A)<sup>+</sup> RNA Purification

1. RNase-free water.
2. Oligotex<sup>™</sup>-dT30 <Super> (Takara Bio Inc., Shiga, Japan) (mRNA purification kit).
3. SUPREC<sup>™</sup>-01 (Takara Bio Inc.) (DNA purification kit).
4. Washing buffer: 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 7.5), 0.5 M sodium chloride (NaCl), and 0.1% (w/v) SDS. Use RNase-free water and store at room temperature.

5. 5 M NaCl. Use RNase-free water, autoclave, and store at room temperature.
6. 3 M Na-acetate (pH 5.2). Use RNase-free water, autoclave, and store at room temperature.
7. Dr. GenTLE™ precipitation carrier (Takara Bio Inc.).
8. Absolute ethanol.
9. 70% ethanol. Use RNase-free water and store at 4°C.

### **2.3. Suppression Subtractive Hybridization (SSH)**

1. PCR-Select subtractive hybridization kit (Clontech, Palo Alto, CA): the kit includes AMV reverse transcriptase, cDNA synthesis primer, first-strand buffer, second-strand enzyme cocktail, second-strand buffer, T4 DNA polymerase, *Rsa*I, *Rsa*I restriction buffer, T4 DNA ligase, adaptors 1 and 2R, DNA ligation buffer, dilution buffer, hybridization buffer, PCR primer 1, nested PCR primers 1 and 2R, dNTP mix, EDTA/glycogen mix, ammonium acetate, and sterile water.
2. Advantage 2 polymerase mix (Clontech).
3. Absolute ethanol.
4. 70% ethanol. Use RNase-free water and store at 4°C.
5. Phenol:chloroform:isoamyl alcohol (25:24:1). Store at 4°C.
6. Chloroform:isoamyl alcohol (24:1). Store at 4°C.

### **2.4. Evaluation of Subtraction Efficiency**

1. Primer set for the reference gene which is not differentially expressed such as *ubiquitin* and *actin* (see **Note 2**).
2. AmpliTaq Gold and GeneAmp PCR buffer II (Applied Biosystems, Foster City, CA).
3. Tris-acetate-EDTA (TAE) buffer: 40 mM Tris-acetate and 1 mM EDTA.
4. Agarose (electrophoresis grade).

### **2.5. Differential Screening Using Reverse Northern**

#### **2.5.1. Preparation of Membrane**

1. TA cloning kit (Invitrogen, San Diego, CA).
2. Luria–Bertani (LB) medium, ampicillin, isopropyl-beta-thio galactopyranoside (IPTG), and 5-bromo-4-chloro-indoly-β-D-galactoside (X-gal).
3. 10×12 cm nylon membrane (Hybond N<sup>+</sup>, Amersham Biosciences, Piscataway, NJ).
4. Nested PCR primers 1 and 2R (Clontech) (see **Note 3**).
5. TaKaRa Ex Taq (Takara Bio Inc.).
6. 0.6 N NaOH; prepare just prior to use.
7. 0.25% (w/v) bromophenol blue (BPB).
8. 0.5 M Tris–HCl (pH 7.5)

### 2.5.2. Labeling Probes

1. SuperScript first-strand synthesis system II for RT-PCR (Invitrogen): the kit includes RNase-free water, oligo(dT) primer, 10× RT buffer, MgCl<sub>2</sub>, DTT, RNase OUT™ Superscript II, and RNase H.
2. DIG DNA labeling mix (Roche Diagnostics, Mannheim, Germany).

### 2.5.3. Hybridization, Washing, and Detection

1. 20× saline sodium citrate (SSC): 750 mM NaCl and 75 mM tri-sodium citrate (pH 7.0), autoclave, and store at room temperature.
2. Maleic acid buffer: 0.1 M maleic acid and 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5, autoclave, and store at room temperature.
3. 10× blocking stock solution: dissolve blocking reagent (Roche Diagnostics) 10% (w/v) in maleic acid buffer under constant heat in a microwave oven, autoclave, and store at −20°C.
4. 1× blocking solution: dilute 10× solution 1:10 with maleic acid buffer. Prepare just prior to use.
5. 10% (w/v) SDS.
6. High SDS hybridization buffer: 50% formamide, 5× SSC, 2× blocking solution, 0.1% lauroylsarcosine and 7% SDS.
7. CSPD solution: dilute CSPD (Roche Diagnostics) (chemiluminescent substrate for alkaline phosphatase) 1:100 in detection buffer, and store at 4°C with dark condition.
8. Washing buffer: maleic acid buffer, 0.3% (v/v) Tween 20.
9. Detection buffer: 0.1 M Tris-HCl and 0.1 M NaCl; adjust to pH 9.5, autoclave, and store at room temperature.
10. Antibody solution: centrifuge anti-digoxigenin-AP, Fab fragments (Roche Diagnostics) at 9,500×g for 5 min at 4°C in the original vial prior to use, and pipette the necessary amount carefully from the surface. Dilute anti-digoxigenin-AP 1:5,000 (150 mU/mL) in blocking solution.
11. X-ray film.

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## 3. Methods

### 3.1. Total RNA Extraction (see Note 4)

1. Pipette 35 mL of extraction buffer into a 50-mL tube. Warm in a water bath to 80°C.
2. Grind 10 g of tissue to a fine powder in liquid nitrogen using mortar and pestle. Transfer the ground tissue into an empty 50-mL tube.

3. Add the heated buffer to the ground tissue by decanting. Vortex well until the powder is fully dispersed and thawed in the buffer.
4. Add 2.5 mL of proteinase K, mix gently, and incubate with mild agitation ( $\sim 100$  rpm) on a shaker at  $42^{\circ}\text{C}$  for 1.5 h.
5. Add 3.6 mL of 2 M KCl, mix gently, and incubate on ice for 1 h.
6. Centrifuge at  $11,900\times g$  for 20 min at  $4^{\circ}\text{C}$  and filter the supernatant through Miracloth into a fresh 50-mL tube.
7. Add 0.25 volume of 10 M LiCl (final concentration of 2 M LiCl), mix gently, and incubate on ice at  $4^{\circ}\text{C}$  overnight.
8. Centrifuge at  $11,900\times g$  for 20 min at  $4^{\circ}\text{C}$ . Discard the supernatant by gentle decanting without dislodging the pellet.
9. Suspend the pellet with 2.5 mL of ice-cold 2 M LiCl. Separate into two aliquots and transfer to fresh 2.0-mL tubes.
10. Centrifuge at  $16,100\times g$  for 20 min at  $4^{\circ}\text{C}$ . Discard the supernatant by gentle decanting.
11. Repeat steps 9 and 10 once.
12. Suspend the pellet with 750  $\mu\text{L}$  of 10 mM Tris-HCl (pH 7.5) for each one of the separate tubes (*see Note 5*).
13. Centrifuge at  $16,100\times g$  for 10 min at  $4^{\circ}\text{C}$ . Transfer the supernatant to a fresh 1.5-mL tube by pipetting.
14. Add 75  $\mu\text{L}$  of 2 M K-acetate (pH 5.5) to each one of the separated tubes, mix gently, and incubate on ice for 15 min.
15. Centrifuge at  $16,100\times g$  for 10 min at  $4^{\circ}\text{C}$ . Transfer the supernatant to a fresh 2.0-mL tube by pipetting.
16. Add 2.5 volume of absolute ethanol. Incubate at  $-80^{\circ}\text{C}$  for 1 h.
17. Centrifuge at  $16,100\times g$  for 30 min at  $4^{\circ}\text{C}$ . Discard the supernatant by gentle decanting without dislodging the pellet.
18. Add 1.5 mL of 70% ethanol and centrifuge at  $16,100\times g$  for 5 min at  $4^{\circ}\text{C}$ .
19. Discard the supernatant by gentle decanting and dry up the pellet.
20. Dissolve the pellet with appropriate volume of RNase-free water.
21. RNA quantification is performed spectrophotometrically at wavelengths of 230, 260, and 280 nm. To confirm the RNA quality, the RNA is electrophoresed on a 1.2% agarose gel containing formaldehyde. Intact total RNA typically exhibits two bright bands corresponding to ribosomal 28S and 18S RNA with a ratio of intensities of  $\sim 1.5\text{--}2.5:1$ .

### **3.2. Poly(A)<sup>+</sup> RNA Purification**

1. Prepare 600  $\mu\text{L}$  of total RNA solution (the amount of total RNA should be more than 350  $\mu\text{g}$ ) with RNase-free water.
2. Add 150  $\mu\text{L}$  of Oligotex<sup>TM</sup>-dT30 <Super> and mix well. Incubate at 70°C for 10 min.
3. Cool the mixture immediately on ice with absolute ethanol ( $-20^{\circ}\text{C}$ ).
4. Add 75  $\mu\text{L}$  of 5 M NaCl and mix well. Incubate at 37°C for 10 min.
5. Centrifuge at 13,800 $\times g$  for 5 min at room temperature. Discard the supernatant by gentle pipetting.
6. Add 500  $\mu\text{L}$  of wash buffer and mix well.
7. Centrifuge at 13,800 $\times g$  for 5 min at room temperature. Discard the supernatant by gentle pipetting.
8. Add 100  $\mu\text{L}$  of RNase-free water and mix well. Incubate at 65°C for 5 min.
9. Apply the mixture to the SUPREC<sup>TM</sup>-01.
10. Centrifuge at 3,900 $\times g$  for 10 min at room temperature and collect the flowthrough.
11. Add 100  $\mu\text{L}$  of RNase-free water (pre-heated to 65°C) to mix by pipetting.
12. Centrifuge at 3,900 $\times g$  for 10 min at room temperature and collect the flowthrough.
13. Add 400  $\mu\text{L}$  of RNase-free water to collected flowthrough.
14. Repeat steps 2–12 once.
15. Add 0.1 volume of 3 M Na-acetate (pH 5.2) to the collected samples and vortex.
16. Add 4  $\mu\text{L}$  of Dr. GenTLE<sup>TM</sup> precipitation carrier and vortex.
17. Add 2.5 volume of ethanol and vortex.
18. Centrifuge at 16,100 $\times g$  for 15 min at 4°C and discard the supernatant by gentle decanting without dislodging the pellet.
19. Add 1 mL of 70% ethanol and centrifuge at 16,100 $\times g$  for 5 min at 4°C.
20. Discard the supernatant by gentle decanting and dry up the pellet.
21. Dissolve the pellet with 5  $\mu\text{L}$  of RNase-free water.
22. Poly(A)<sup>+</sup> RNA quantification is performed spectrophotometrically at wavelengths of 230, 260, and 280 using 0.5  $\mu\text{L}$  of poly(A)<sup>+</sup> RNA solution. To confirm the quality, 0.5  $\mu\text{L}$  of poly(A)<sup>+</sup> RNA solution is electrophoresed on a

1.2% agarose gel containing formaldehyde. Poly(A)<sup>+</sup> RNA appears as a smear with no ribosomal RNA bands present (*see* **Note 6**).

### **3.3. Suppression Subtractive Hybridization (SSH)**

The protocols in this section are excerpted from PCR-Select™ cDNA subtraction kit user manual (Clontech). For the detailed protocols, it is strongly recommended to refer to the appropriate Clontech manuals. In this section, cDNA that contains specific (differentially expressed) transcripts and the control cDNA are referred to as tester and driver, respectively. For example, if the researcher wants to isolate UV-B-inducible genes, refer to the cDNA from UV-B-irradiated sample as tester and the cDNA from control sample as driver.

#### **3.3.1. First-Strand cDNA Synthesis**

1. For tester and driver, combine the following components in a sterile 0.5-mL microtube (do not use a polystyrene tube): poly(A)<sup>+</sup> RNA (2 µg) 4 µL and cDNA synthesis primer (10 µM) 1 µL. Mix contents and spin briefly in a centrifuge.
2. Incubate at 70°C for 2 min in a thermal cycler.
3. Cool on ice for 2 min and briefly centrifuge.
4. Add the following to each reaction: 5× first-strand buffer 2 µL, dNTP mix (10 mM each) 1 µL, sterile water 1 µL, and AMV reverse transcriptase (20 U/µL) 1 µL.
5. Gently vortex and briefly centrifuge the tubes.
6. Incubate the tubes at 42°C for 1.5 h in an air incubator.
7. Place on ice to terminate first-strand cDNA synthesis and immediately proceed to next section.

#### **3.3.2. Second-Strand cDNA Synthesis**

1. Add the following components to the first-strand synthesis reaction tubes (containing 10 µL): sterile water 48.4 µL, 5× second-strand buffer 16 µL, dNTP mix (10 mM) 1.6 µL, and 20× second-strand enzyme cocktail 4 µL.
2. Mix contents and briefly spin. The final volume should be 80 µL.
3. Incubate at 16°C for 2 h in water bath or in thermal cycler.
4. Add 2 µL of T4 DNA polymerase. Mix contents well.
5. Incubate at 16°C for 30 min in a water bath or in a thermal cycler.
6. Add 4 µL of 20× EDTA/glycogen mix to terminate second-strand synthesis.
7. Add 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1).
8. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.



9. Carefully collect the top aqueous layer and place in a fresh 0.5-mL microcentrifuge tube.
10. Add 100  $\mu$ L of chloroform:isoamyl alcohol (24:1).
11. Repeat steps 8 and 9.
12. Add 40  $\mu$ L of 4 M  $\text{NH}_4\text{OAc}$  and 300  $\mu$ L of 95% ethanol. Proceed to next step immediately.
13. Vortex thoroughly and centrifuge at 14,000 rpm for 20 min at room temperature.
14. Carefully collect the supernatant.
15. Overlay the pellet with 500  $\mu$ L of 80% ethanol.
16. Centrifuge at 14,000 rpm for 10 min.
17. Remove the supernatant.
18. Air-dry the pellet for about 10 min to evaporate residual ethanol.
19. Dissolve precipitate in 50  $\mu$ L of sterile water.

### 3.3.3. *RsaI* Digestion

1. Add the following reagents: dscDNA (double-stranded cDNA; from **Section 3.3.2** step 19) 43.5  $\mu$ L, 10 $\times$  *RsaI* restriction buffer 5  $\mu$ L, and *RsaI* (10 U/ $\mu$ L) 1.5  $\mu$ L.
2. Mix by vortexing and briefly centrifuge.
3. Incubate at 37°C for 1.5 h.
4. Add 2.5  $\mu$ L of 20 $\times$  EDTA/glycogen mix to 45  $\mu$ L of the digest mixture.
6. Add 50  $\mu$ L of phenol:chloroform:isoamyl alcohol (25:24:1).
7. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
8. Carefully collect the top aqueous layer and place in a fresh 0.5-mL tube.
9. Add 50  $\mu$ L of chloroform:isoamyl alcohol (24:1).
10. Repeat steps 7 and 8.
11. Add 25  $\mu$ L of 4 M  $\text{NH}_4\text{OAc}$  and 187.5  $\mu$ L of 95% ethanol. Proceed to next step immediately.
12. Repeat step 7.
13. Remove the supernatant.
14. Gently overlay the pellets with 200  $\mu$ L of 80% ethanol.
15. Centrifuge at 14,000 rpm for 5 min.
16. Carefully remove the supernatant.
17. Air-dry the pellets for 5–10 min.
18. Dissolve the pellet in 5.5  $\mu$ L of sterile water and store at  $-20^\circ\text{C}$ .

### 3.3.4. Adaptor Ligation (see **Note 7**)

1. Dilute 1  $\mu\text{L}$  of *Rsa*I-digested tester cDNA (from **Section 3.3.3** step 18) with 5  $\mu\text{L}$  of sterile water.
2. Prepare a ligation master mix by combining the following reagents in a 0.5-mL microcentrifuge tube: sterile water 3  $\mu\text{L}$ , 5 $\times$  ligation buffer 2  $\mu\text{L}$ , and T4 DNA ligase (400 U/ $\mu\text{L}$ ) 1  $\mu\text{L}$ . Tubes containing the master mix should be prepared in a set of two.
3. Ligate the two kinds of adaptors (adaptors 1 and 2R) separately as follows. The reaction mixtures containing adaptors 1 and 2R are designated as tester 1-1 and tester 1-2, respectively. Combine the following reagents in a 0.5-mL microcentrifuge tube in the order shown: tester 1-1: diluted tester cDNA 2  $\mu\text{L}$ , adaptor 1 (10  $\mu\text{M}$ ) 2  $\mu\text{L}$ , and master mix 6  $\mu\text{L}$  (from step 2); tester 1-2: diluted tester cDNA 2  $\mu\text{L}$ , adaptor 2R (10  $\mu\text{M}$ ) 2  $\mu\text{L}$ , and master mix 6  $\mu\text{L}$ . Pipette mixture up and down to mix thoroughly.
4. In a fresh microcentrifuge tube, mix 2  $\mu\text{L}$  of tester 1-1 and 2  $\mu\text{L}$  of tester 1-2. After ligation is complete, this will be unsubtracted tester control.
5. Centrifuge briefly the three kinds of tubes (tester 1-1, tester 1-2, and unsubtracted tester control) and incubate at 16°C overnight.
6. Add 1  $\mu\text{L}$  of EDTA/glycogen mix to stop ligation reaction.
7. Heat samples at 72°C for 5 min to inactivate the ligase.
8. Briefly centrifuge the tubes. Adaptor-ligated tester cDNAs and unsubtracted tester control are now complete.
9. Remove 1  $\mu\text{L}$  from unsubtracted tester control and dilute into 1 mL of sterile water. This sample will be used for PCR (**Section 3.3.7**).
10. Store samples at -20°C.

### 3.3.5. First Hybridization

1. Adaptor 1-ligated tester 1-1 (from **Section 3.3.4** step 10) and adaptor 2R-ligated tester 1-2 (from **Section 3.3.4** step 10) are separately hybridized with *Rsa*I-digested driver cDNA (from **Section 3.3.3** step 18). Combine the following reagents in a 0.5-mL microcentrifuge tube in the order shown: hybridization sample 1: *Rsa*I-digested driver cDNA (from **Section 3.3.3** step 18) 1.5  $\mu\text{L}$ , adaptor 1-ligated tester 1-1 (from **Section 3.3.4** step 10) 1.5  $\mu\text{L}$ , and 4 $\times$  hybridization buffer 1  $\mu\text{L}$ ; hybridization sample 2: *Rsa*I-digested driver cDNA (from **Section 3.3.3** step 18) 1.5  $\mu\text{L}$ , adaptor 2R-ligated tester 1-2 (from **Section 3.3.4** step 10) 1.5  $\mu\text{L}$ , and 4 $\times$  hybridization buffer 1  $\mu\text{L}$ .

2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples at 98°C for 1.5 min in a thermal cycler.
4. Incubate samples at 68°C for 8 h. Samples may hybridize for 6–12 h. Do not let the incubation exceed 12 h.

3.3.6. Second  
Hybridization (see  
**Note 8**)

1. The two samples from the first hybridization (hybridization samples 1 and 2 from **Section 3.3.5** step 4) are mixed together, and fresh denatured driver cDNA (from **Section 3.3.3** step 18) is added for further enrichment of differentially expressed sequences. Add the following reagents into a sterile tube: *Rsa*I-digested driver cDNA (from **Section 3.3.3** step 18) 1 µL, 4× hybridization buffer 1 µL, and sterile water 2 µL.
2. Place 1 µL of this mixture in a 0.5-mL microcentrifuge tube and overlay it with one drop of mineral oil.
3. Incubate at 98°C for 1.5 min in a thermal cycler.
4. Remove the tube of freshly denatured driver from the thermal cycler. Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (prepared in **Section 3.3.5** step 4).
  - a. Set a micropipettor at 15 µL.
  - b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
  - c. Carefully draw the entire sample partially into the pipette tip. Do not be concerned if a small amount of mineral oil is transferred with the sample.
  - d. Remove the pipette tip from the tube and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
  - e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket.
  - f. Transfer the entire mixture to the tube containing hybridization sample 1.
  - g. Mix by pipetting up and down.
5. Briefly centrifuge if necessary.
6. Incubate reaction at 68°C overnight.
7. Add 200 µL of dilution buffer and mix by pipetting.
8. Heat at 68°C for 7 min in a thermal cycler.
9. Store at –20°C.

### 3.3.7. PCR Amplification (see **Note 9**)

1. The first PCR reaction is performed in a total volume of 25  $\mu$ L comprising each diluted cDNA (i.e., subtracted sample from **Section 3.3.6** step 9 and the diluted unsubtracted tester control from **Section 3.3.4** step 9) 1  $\mu$ L, sterile water 19.5  $\mu$ L, 10 $\times$  PCR reaction buffer 2.5  $\mu$ L, dNTP mix (10 mM) 0.5  $\mu$ L, PCR primer 1 (10  $\mu$ M) 1  $\mu$ L, and 50 $\times$  Advantage cDNA polymerase mix 0.5  $\mu$ L.
2. Mix well by vortexing and briefly centrifuge the tube.
3. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)
4. Immediately commence thermal cycling. After pre-PCR heating at 94°C for 25 s, a reaction cycle of 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min is repeated 27 times (see **Note 10**).
5. Dilute 3  $\mu$ L of each primary PCR mixture in 27  $\mu$ L of sterile water.
6. The second PCR reaction is performed in a total volume of 25  $\mu$ L comprising each diluted primary PCR product mixture (from step 5) 1  $\mu$ L, sterile water 18.5  $\mu$ L, 10 $\times$  PCR reaction buffer 2.5  $\mu$ L, dNTP mix (10 mM) 0.5  $\mu$ L, nested PCR primer 1 (10  $\mu$ M) 1  $\mu$ L, nested PCR primer 2R (10  $\mu$ M) 1  $\mu$ L, and 50 $\times$  Advantage cDNA polymerase mix 0.5  $\mu$ L.
7. Mix well by vortexing and briefly centrifuge.
8. Immediately commence thermal cycling. A reaction cycle of 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min is repeated 10–12 times (see **Note 10**).
9. Store reaction products at –20°C.

### 3.4. Evaluation of Subtraction Efficiency

1. Dilute the subtracted and unsubtracted secondary PCR products (from **Section 3.3.7** step 9) about 10-fold with sterile water. The concentration of subtracted and unsubtracted products should be roughly equal (see **Note 11**).
2. The PCR reaction is performed in a total volume of 30  $\mu$ L comprising 1  $\mu$ L of each diluted secondary PCR product (from step 1), 200  $\mu$ M dNTPs, 150  $\mu$ M MgCl<sub>2</sub>, 0.5  $\mu$ M of non-differentially expressed gene primers, 0.75 U of AmpliTaq Gold and GeneAmp PCR buffer II.
3. Immediately commence thermal cycling. After pre-PCR heating at 95°C for 12 min, a reaction cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1.5 min is repeated 38 times.

4. 5  $\mu$ L of PCR reaction mixture is removed after 18, 23, 28, 33, and 38 cycles and placed in clean tubes. These samples are analyzed on a 2% agarose gel.
5. Example of the results using apple *ubiquitin* (*Ub*) is shown in **Fig. 2.1**. For the unsubtracted cDNA, the apple *Ub* was amplified after 18 cycles. However, for the subtracted cDNA, the *Ub* product was only detected after 38 cycles. The reduction of apple *Ub* abundance in the subtracted cDNA ensures that SSH was successfully achieved (*see Note 12*).

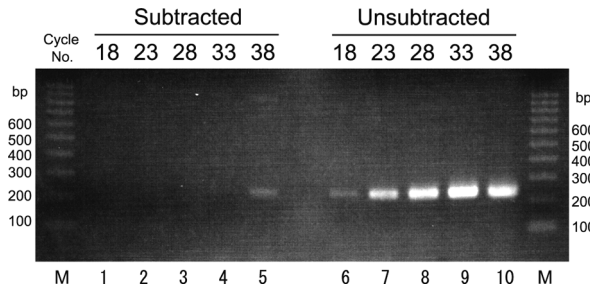


Fig. 2.1. Confirmation of SSH efficiency using apple *ubiquitin* (*Ub*). PCR was performed on the subtracted (lanes 1–5) and unsubtracted (lanes 6–10) cDNA with the *Ub* primers. Numbers of PCR cycles are indicated above the panel. Lane M indicates 100 bp ladder marker.

### 3.5. Differential Screening Using Reverse Northern (see Note 13)

#### 3.5.1. Preparation of Membrane

1. Subclone the subtracted cDNAs (from **Section 3.3.7** step 9) into the pCR2.1 vector and transform to *Escherichia coli* using a TA cloning kit. Spread the transformed *E. coli* to LB plate with ampicillin/IPTG/X-gal and incubate at 37°C overnight.
2. Pick up and culture white clones in 100  $\mu$ L of LB medium with ampicillin using 96-well cell culture plate. Incubate at 37°C overnight with continuous shaking at 150 rpm.
3. Inserts of cDNA clones are amplified by PCR using cultured LB medium as templates in 96-well PCR plate. The PCR reaction is performed in a total volume of 20  $\mu$ L comprising 1  $\mu$ L of cultured LB medium, 80  $\mu$ M dNTPs, 150  $\mu$ M  $\text{MgCl}_2$ , 0.5  $\mu$ M of nested PCR primers 1 and 2R, 0.5 U of TaKaRa Ex Taq and 1 $\times$  TaKaRa Ex Taq buffer. After pre-PCR heating at 94°C for 30 s, a reaction cycle of 95°C for 10 s and 68°C for 3 min is repeated 30 times. The remaining culture medium is stored as glycerol stocks at –80°C for further analysis (e.g., sequencing and expression analysis).
4. Dispense 5  $\mu$ L of 0.6 N NaOH and 2  $\mu$ L of 0.25% BPB and transfer 5  $\mu$ L of the PCR products to 96-well microtiter plate. Mix gently by pipetting.

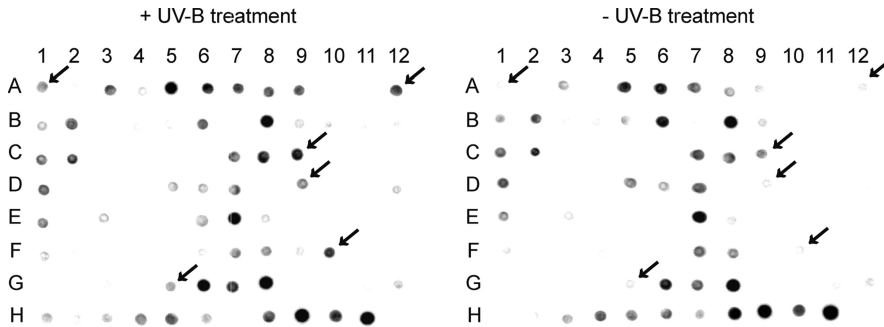


Fig. 2.2. Selection of cDNAs up-regulated in UV-B-treated apple skin by reverse northern. Two identical cDNA blots of PCR-amplified inserts derived from the subtracted cDNA library were hybridized with the probes from + UV-B-treated (tester) and – UV-B-treated (driver) skin. The *arrows* indicate the spots which show higher intensity in the tester sample.

5. Spot 1.2  $\mu\text{L}$  of the mixtures onto a  $10 \times 12$  cm nylon membrane in 96-well format (**Fig. 2.2**). To perform experimental replication, at least four membranes should be prepared.
6. Soak the membranes in 0.5 M Tris-HCl (pH 7.5) for 3 min and rinse with distilled water for 1 min.
7. Fix the PCR product on a nylon membrane using UV cross-linker (e.g., Stratalinker) at energy of 120,000  $\mu\text{J}$  and dry up the membrane at  $65^\circ\text{C}$ .
8. Put the prepared membranes into a plastic hybridization bag. The membranes can be stored at  $-20^\circ\text{C}$ .

### 3.5.2. Labeling Probes

1. Mix 7.5  $\mu\text{g}$  of each total RNA (adjust to 7  $\mu\text{L}$  with equipped RNase-free water to the kit) from tester and driver samples with 1  $\mu\text{L}$  of an oligo(dT) primer and 2  $\mu\text{L}$  DIG DNA labeling mix.
2. Incubate the mixture at  $65^\circ\text{C}$  for 5 min and chill on ice for 1 min.
3. Add 2  $\mu\text{L}$  of a  $10 \times$  RT buffer, 4  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$ , 2  $\mu\text{L}$  of 0.1 M DTT, and 1  $\mu\text{L}$  of RNase OUT<sup>TM</sup> and incubate at  $42^\circ\text{C}$  for 2 min.
4. Add 1  $\mu\text{L}$  of Superscript II, incubate at  $42^\circ\text{C}$  for 50 min and then at  $72^\circ\text{C}$  for 15 min.
5. Add 1  $\mu\text{L}$  RNase H and incubate at  $37^\circ\text{C}$  for 20 min. Total volume of labeled probe should be 21  $\mu\text{L}$ .

### 3.5.3. Hybridization, Washing, and Detection

1. Add 10 mL of a high SDS hybridization buffer to plastic hybridization bags (from **Section 3.5.1**) and incubate at  $42^\circ\text{C}$  for 1 h.
2. Add 100  $\mu\text{L}$  of high SDS hybridization buffer to 21  $\mu\text{L}$  of each probe solution prepared from tester and driver samples (from **Section 3.5.2**).
3. Boil the probe mixture for 5 min and chill on ice for 1 min.

4. Discard a high SDS hybridization buffer from the plastic hybridization bag.
5. Add 5 mL of a fresh high SDS hybridization buffer and each probe mixture to the plastic hybridization bag. Incubate overnight at 42°C.
6. Wash the membranes twice with 2× SSC, 0.1% SDS for 15 min at room temperature.
7. Wash twice with 0.1× SSC, 0.1% SDS at 68°C for 15 min.
8. Rinse in washing buffer at room temperature for 3 min.
9. Incubate in 100 mL blocking solution at room temperature for 30 min.
10. Incubate in 20 mL antibody solution at room temperature for 30 min.
11. Wash twice in 100 mL washing buffer at room temperature for 5 min.
12. Equilibrate in 20 mL detection buffer at room temperature for 5 min.
13. Incubate in 20 mL freshly prepared CSPD solution in the dark for 5 min.
14. Wrap the hybridized membranes with plastic films and expose these membranes simultaneously to X-ray film for 1 h.
15. Examples of the signals for reverse northern are shown in **Fig. 2.2**. Select the spots which show higher intensity in the tester sample (indicated with arrows). Reverse northern should be conducted with at least two replicates. Proceed with the reproducible spots for further analysis (e.g., sequencing and expression analysis).

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## 4. Notes

1. Sodium borate decahydrate may be hard to dissolve. In that case, dissolve sodium borate decahydrate with continuous heating at 65°C.
2. The sequence of PCR product should not contain *RsaI* site. The example of apple *Ub* primers are as follows: 5'-TCGCTGGAAAGCAGCTTGAAGA-3' and 5'-GCTTTCGGCAAAGATCAGACG-3'.
3. Nested PCR primer 1 (5'-TCGAGCGGCCCGCCGGGCAGGT-3') and nested PCR primer 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') are available through usual

oligonucleotide ordering companies. Desalted purification grade is enough for this analysis.

4. This protocol was developed based on the hot borate method (12) and can be applied to polysaccharide- and polyphenol-rich samples such as fruit and flowers.
5. If the pellet could not be well suspended, increase the volume of 10 mM Tris-HCl. Subsequently, change the volume of solutions used in the later steps at the same ratio.
6. The ratio of poly(A)<sup>+</sup> RNA contained in total RNA varies with species and tissues. For example, 0.25% poly(A)<sup>+</sup> RNA was contained in total RNA from mature apple skin.
7. Adaptors will not be ligated to the driver cDNA in this section.
8. Do not denature the primary hybridization samples at this stage. Also, do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver.
9. All cycling parameters were optimized on a GeneAmp PCR Systems 9700 (Applied Biosystems). Cycling parameters for other thermal cycler machines may require optimization.
10. To prevent the amplification of undesired sequences, PCR cycles should be optimized (13). In many cases, reduction of the PCR cycles gives favorable results. In our previous study (11), the first and second PCR cycles were determined as 25 and 8 cycles, respectively.
11. To check the concentration of the subtracted and unsubtracted secondary PCR products, analyze 8  $\mu$ L of each secondary PCR product on a 2% agarose gel run in 1 $\times$  TAE buffer.
12. If you do not observe a decrease in abundance of PCR product in the subtracted sample, repeat the PCR amplification (**Section 3.3.7**) with reduced PCR cycles (*see Note 10*).
13. Alternatively, PCR-Select differential screening kit (Clontech) can be used for differential screening. For details, please see the PCR-Select differential screening kit user manual.

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