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Cloning and Characterization of Genes Controlling Flower Color in *Pharbitis nil* Using AFLP (Amplified Fragment Length Polymorphism) and DDRT (Differential Display Reverse Transcription)

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To analyze molecular traits determining pigmentation between *Pharbitis nil* violet and white, Amplified Fragment Length Polymorphism (AFLP) and Differential Display Reverse Transcription (DDRT) experiments were carried out with either genomic DNAs or total RNAs isolated from both plants. Results of AFLP experiment in combination of 8 *Eco*RI primers with 6 *Mse*I primers showed 41 violet- and 60 white-specific DNA bands. In the subsequent experiment, 22 violet- and 22 white-specific DNA fragments were amplified by PCR with DNAs eluted. The sizes of the fragments range from 200 to 600 bp. DDRT using total RNA produced 19 violet- and 17 white-specific cDNA fragments, ranging from 200 to 600 bp. The fragments obtained by both AFLP and DDRT had been cloned into pGEM T-easy vector, amplified and subjected to the nucleotide sequence analyses. As a result of Blast sequence analysis, most of them sequenced up to date showed no similarity to any known gene, while few has similarity to known animal or plant genes. An AFLP clone V6, for example, has a strong sequence similarity to the human transcription factor LZIP-alpha mRNA and a DDRT clone W19 to *Solanum tuberosum* 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA.

key words: *Pharbitis*, flower color, AFLP clones, DDRT clones, nucleotide sequence analyses

INTRODUCTION

Colors in higher plants are generally determined by three pigments, flavonoid, carotenoid and betalain. Carotenoid confers yellow and orange colors in sunflower and tomato [1], betalain found in Caryophyllales is a nitrogen compounds derived from tyrosin and provides various colors from yellow to red, and flavonoid controls various colors ranging from pale yellow to red, violet and blue [7]. Anthocyanins, a group of flavonoids, are synthesized by phenylpropanoid pathway [13-14], and its chromophore is called anthocyanidin [22].

Phenylalanine is a precursor of all flavonoids and converted to 4-coumaroyl CoA by several enzymatic steps. Chalcone synthase (CHS) can concentrate one molecule of 4-coumaroyl CoA and three malonyl CoA to synthesize the first flavonoid, naringenin chalcone, which is yellow in color [8]. Naringenin chalcone is converted to colorless naringenin by chalcone flavonone isomerase (CHI). The chalcone then is enzymatically substituted in its three flavonoid rings, resulting in the formation of three anthocyanidins, delphinidin, pelagonidin and cyanidin. These anthocyanidins can be converted into hundreds of anthocyanins by modifications such as glycosylation and acylation [19].

Japanese morning glory has intensively been studied over three hundred years with respect to flower color and morphology [25]. Mutations producing white flowers are classified into four groups on the basis of pigmentation on the corollar, flower-tube, stem, and seed coat. The white *Pharbitis* used in the present study will be classified into A group genes that produce flowers with white corollas and tubes, green stems, and pigmented seeds. In addition, the white plants show several distinct characteristics; reduction in fertility, delayed germination, and susceptibility to pathogens and cold.

Two different display techniques for gene cloning, AFLP (amplified fragment length polymorphism) and DDRT (differential display reverse transcription), have adopted in this study. AFLP technique is a method where total genomic DNAs digested with restriction endonucleases and ligated with oligonucleotide adaptors can be amplified by PCR and analyzed by electrophoresis with a sequencing gel [26]. This method becomes very important technique used in various purposes, such as the breeding of animals and plants, diagnostics, DNA fingerprinting for microbial typing and DNA-based marker for gene cloning [2, 15, 23]. DDRT method, that is a technique to identify and isolate differentially expressed genes, includes reverse transcription and PCR using oligo-dT primers [9-11, 20].

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To elucidate color determination at molecular levels, we applied AFLP and DDRT, and described AFLP and DDRT clones specific either to white or violet *Pharbitis nil.*

MATERIALS AND METHODS

Plant materials and culture condition Seeds of *Pharbitis nil* Choisy cv. Violet and mutant with white flower were stirred with concentrated sulfuric acid for 30 min, rinsed and imbibed overnight in running water. The seeds were sown in prewashed vermiculite in 12 cm diameter pots and grown in a culture room with Hoagland nutrient solution at 25±2°C under 16 h light/8h dark photoperiod.

Isolation of genomic DNA Total DNA from *Pharbitis* leaves was isolated by CTAB (cetyltrmithylammonium bromide) method [17]. Leaf tissue was ground in a mortar and pestle in liquid nitrogen, and the powder was transferred into a 50 ml tube, and 10 ml extraction buffer (50mM Tris, pH 8.0, 0.7M NaCl, 50 mM EDTA, 1% CTAB, 0.1% β-mercaptoethanol) was added to each grams of the sample. The mixture was incubated at 60°C for 30-60 min with occasional mixing by gentle swirling, and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged at 5,000 xg for 10 min at room temperature. The chlorofrom extraction was repeated once. An aqueous phase was transferred to a new tube, added 2/3 volume of isopropanol, mixed by gentle inversion and kept at -20°C for at least 1 h. The DNA pellets were collected by centrifugation at 10,000 xg for 10 min, dissolved in 1 mL TE with five microliters of RNase (10 $\mu g/\mu l$) and incubated at 37°C for 30 min. After three times of phenol-chloroform-isoamylalcohol (24:24:1) extraction, DNA was precipitated by addition of 1 M NaCl and 2 volumes of EtOH and 1 h incubation at -20°C. The DNA pellets were collected by centrifugation and washed by 75% EtOH. The pellets were dried and dissolved in TE.

RNA extraction Leaves from violet and white *Pharbitis* plants were ground in liquid nitrogen with a mortar and pestle. Total RNAs were extracted from 5 g of the powder using the single-step method by Chomczynski and Sacchi [3].

Amplified fragment length polymorphism Genomic DNAs isolated from white- and violet-*Pharbitis* plant leaves were completely digested with *Eco*RI and *Mse*I. The digested fragments were purified and ligated with *Eco*RI-adaptor [5′-AATTG-GTACGCAGTC (*Eco*RI-oligo 2), 5′-CTC GTAGACTGCG-TACC (*Eco*RI-oligo 1)] and *Mse*I-adaptor [5′ - TACTCAGGACTCAT (*Mse*I-olig 2), 5′-GACGATGAGTC-CTGAG (*Mse*I-oligo 1)]. The ligation mix was diluted by 10 times and stored at -20°C until use. Preamplification reaction was carried out in the presence of 30 ng of *Eco*RI primer (5′-GACTGCGTACCAATTC+A) and *Mse*I primer (5′ - GATGAGTCCTGAGTAA+C) for 20 cycles of 94°C for 30s, 56°C for 60s, and 72°C for 60s. The reaction products was diluted by 50 times and stored at -20°C. *Eco*RI selective primers for AFLP-PCR, which are extended by 1 to 3 bp at its 3′, were labeled with T_4 polynucleotide kinase in the presence of γ-^{33}P-dATP at 37°C for 1 h. Selective AFLP amplification was carried out with the labeled *Eco*RI primer and unlabeled *Mse*I primer. The PCR performed with 10 files (1 cycle for file 1 through 9, 33 cycles for file 10): file 1; 94°C for 60s, 65°C for 60s, 72°C for 90s, file 2: 94°C for 60s, 64°C for 60s, 72°C for 90s, file 3; 94°C for 60s, 63°C for 60s, 72°C for 90s, file 4; 94°C for 60s, 62°C for 60s, 72°C for 90s, file 5; 94°C for 60s, 61°C for 60s, 72°C for 90s, file 6; 94°C for 60s, 59°C for 60s, 72°C for 90s, file 7; 94°C for 60s, 58°C for 60s, 72°C for 90s, file 8; 94°C for 60s, 57°C for 60s, 72°C for 90s, file 9; 94°C for 60s, 56°C for 60s, 72°C for 90s, and file 10; 94°C for 60s, 64°C for 60s, 72 °C for 90s. The PCR product was separated on a squencing gel and the gel was dried and exposed to X-ray film.

AFLP specific bands were excised from the dried gel. DNA was eluted by "Crush & Soak" method [18] and PCR-amplified using "Universal AFLP Cloning"-primers (U-EcoRI and U-MseI).

Differential display reverse transcription Differential display reverse transcription was performed by using RNA map kit (Gene Hunter) [9]. Reverse transcription was done in 4 independent reactions using H-T_{11}G, H-T_{11}C, and H-T_{11}A primers. Each reaction mixture was composed of 2 μl of total RNA, 4.0 μl of 5X RT buffer, 1.6 μl of dNTP (250 μM), 2.0 μl of H-T_{11}M primer (2 μM), and 9.4 μl of dH2O. The mixture was incubated at 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, and then 37°C for 10 min. After adding 1 μl of MMLV reverse transcriptase, the mixture was incubated at 37°C for 60 min. The reaction was stopped by incubation at 95°C for 5 min. One tenth volume of the mixture was subjected to PCR amplification in the presence α-^{35}S-dATP using *Taq* polymerase (AmpliTaq, TAKARA). Amplification was carried out for 40 cycles of 94 °C for 30s, 40°C for 2 min, 72°C for 30s, and an additional extension period at 72°C for 5 min. The 80 different primer sets used for PCR amplification were the combinations of twenty 5′-AP (arbitarary primer) with the 3′-T_{11}MN primers that were used for initial cDNA synthesis. The amplified cDNA fragments were separated by 6% denaturating polyacrylamide gel electrophoresis in TBE buffer at 60W. The gel was dried and exposed to X-ray film.

The differentially expressed cDNA bands were selected after autoradiography and excised from the dried gel. The gel slices along with the 3MM paper were soaked in 100 μl dH_2O for 10 min, boiled for 15 min and spun for 2 min. The supernatant was transferred to a new microfuge tube, and 10 μl of 3 M sodium acetate, 5 μl of glycogen (10 mg/mL) and 450 μl of 100% EtOH were added. The DNA was then precipitated for 30 min

at -80°C. The cDNA pellets were collected by spinning for 10 min at 4°C. Pellets were rinsed with 200 μl ice-cold 85% EtOH and dissolved in 10 μl dH_2O. Four microliters of each of the pellets were used for reamplification with the same primer set and PCR condition.

Cloning of PCR products and preparation of plasmid DNA PCR amiplified products were inserted into pGem-T-Easy vector (Promega) and transformed into JM109 *E. coli* cells. The recombinant plasmid DNA was isolated by the alkali lysis method [18].

Seqeuncing and analysis of cDNA fragments Nucleotide sequencing of the cDNA fragments was performed using T7 sequenase version 2.0 sequencing kit (Amersham) according to the protocols provided by the manufacturer. The individual seqeunces were analysed with Blast DNA search program through GenBank and EMBL database.

RESULTS AND DISCUSSION

Since white flower *Pharbitis* has exhibited constant phenotypes throughout several generations, it is expected that violet and white *Pharbitis* plants have different genotypes. Therefore, we carried out AFLP experiment with genomic DNAs in order to clone either violet- or white-specific DNA fragments. Figure 1 shows the first PCR products after restriction enzyme digestion and adaptor ligation. With this reaction mix, we performed AFLP-PCR and obtained 41 violet specific bands and 60 white specific bands from 56 primer combinations (8 *Eco*RI primers × 7 *Mse*I primers). One of the results is shown in Fig. 2. We cut out the bands, eluted DNAs and amplified by PCR. Figure 3 shows 23 and 22 AFLP clones, ranging from 200 to 600 bp, obtained from white- and violet-*Pharbitis* plants, respectively. We inserted all clones into pGEM-T-Easy vectors and analyzed their nucleotide sequences by a sequencing. Most of the AFLP clones did not match to any known genes up to date, but some showed somewhat sequence homology to genes cloned from animals and plants (Table 1). Particularly, *V6* and *W2* clones seem to be important because both will be transcription factors to control the gene expression. However, two clones would act in different circumstances. Another clones, *W10* and *W13*, showed high similarities with *Brugia malayi* (clone *Bmcyp-1*) peptidylprolyl isomerase mRNA and *Sparus aurata* activin-b mRNA, partial cDNA, respectively.

To clone cDNAs expressed differentally in violet- and white-*Pharbitis* plants, we performed DDRT experiment using

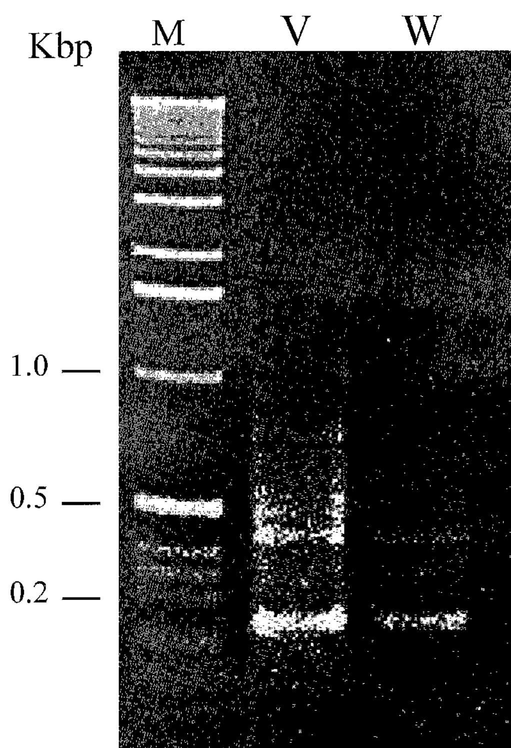

Figure 1. The first PCR products obtained after adaptor ligation. The products were seperated on 1% agarose gel and stained with EtBr. V and W indicate violet- and white-*Pharbitis*, respectively. M, 1 kbp DNA ladder.

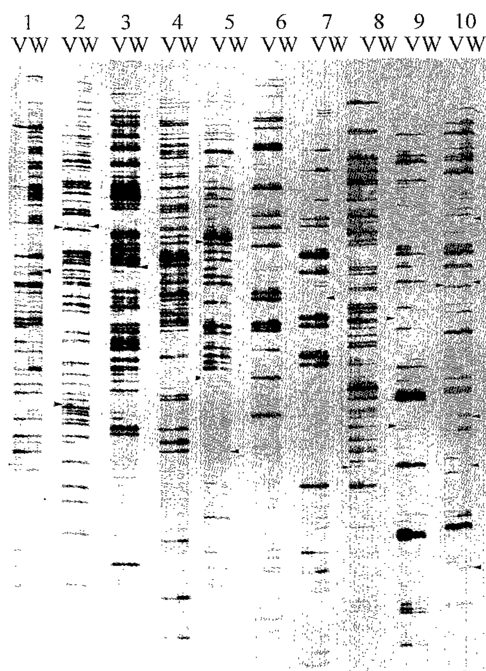

Figure 2. AFLP fingerprints. PCR products were seperated on 6%-sequencing gel, dried and exposed to X-ray film. V and W indicate violet- and white-*Pharbitis*, respectively. Numbers on top indicate different combinations in primers; 1, *Eco*RI+ ACT/*Mse*I +CA; 2, *Eco*RI + ACA/*Mse*I + CA; 3, *Eco*RI + AGG/*Mse*I +CA; 4, *Eco*RI + AGC/*Mse*I+ CA; 5, *Eco*RI + ACT/*Mse*I + CTT; 6, *Eco*RI + ACA/*Mse*I + CTT; 7, *Eco*RI + AGG/*Mse*I + CTT; 8, *Eco*RI + ACT/*Mse*I + CTG; 9, *Eco*RI + ACA/*Mse*I + CTG; 10, *Eco*RI +AGG/MseI + CTG. Arrows indicate bands specific in either violet- or white-*Pharbitis*.

Table 1. Summary of DNA sequence homology analysis and search for AFLP clones.

AFLP Clone		The Most Holomogous Clone
Violet Specific	W30	*S. solfataricus* SsMTAP gene for 5′-methylthioadenosine
	V2	*Lepilemur dorsalis* genomic fragment, RAPD with primer operon OPH4
	V6	Human transcription factor LZIP-alpha mRNA
	V7	*Oligotropha carboxidovorans hoxV, hoxS, hoxL, hoxZ* genes
	V8	*Arabidopsis thaliana* DNA chromosome 4, ESSA I FCA contig fragment
	V9	*Caenorhabditis elegans* cosmid Y48E1B
	V10	*Discorea tokoro* gene, AFLP fragment
	V11	*Botrytis cinerea* strain T4 cDNA library under conditions of nitrogen deprivation
	V14	*Arabidopsis thaliana* BAC F4C21 from chromosome IV, top arm, near 17 cM,
White Specific	W1	*Caenorhabditis elegans* cosmid K022
	W2	(Promoter of) *Homo sapiens* RF-C/activator 1 homolog (RAD17) mRNA
	W4	*Mus musculus* (clone DE-5) mRNA fragment
	W5	*M. musculus* mRNA for estrogen receptor-related protein
	W6	Human DNA sequence from clone 1052M9 on chromosome Xq25
	W8	Hepatitis GB virus C genomic RNA, complete sequence
	W10	*Brugia malayi* (clone Bmcyp-1) peptidylprolyl isomerase mRNA
	W11	*Homo sapiens* Xp22 PAC RPCI1-5G11
	W12	*Arabidopsis thaliana* chromosome II BAC F14B2 genomic sequence
	W13	*Sparus aurata* activin-b mRNA, partial cds
	W16	*S. cerevisiae* gene for Sac2 protein and Mnt1 protein
	W25	*Arabidopsis thaliana* genomic DNA, chromosome 5
	W26	*Arabidopsis thaliana* DNA chromosome 4, BAC clone T22F8
	W39	*Caenorhabditis elegans* cosmid B0416
	W40	*Caenorhabditis elegans* cosmid B0416

total RNA. Reverse transcription was carried out with oligo dT primer having different bases, H-T_{11} G, A or C. After that, cDNA fragments were amplified by PCR with the combinations of twenty 5′-AP (arbitarary primer) with three 3′-T_{11}MN primers (G, A, T) that were used for initial cDNA synthesis. Amplified PCR products were separated on a sequencing gel and exposed to x-ray film. One of the results are shown in Fig. 4. From 15 primer combinations, we identified 65 and 40 DDRT-clones specific each for violet- or white-*Pharbitis*, respectively. Finally, we obtained 19 DDRT-cDNA clones from violet- and 17 clones from white-*Pharbitis* plants (Fig. 5). As shown in AFLP results, most of the DDRT-clones have no similarity to any known genes in animals or plants up to now. We summarized some of the results

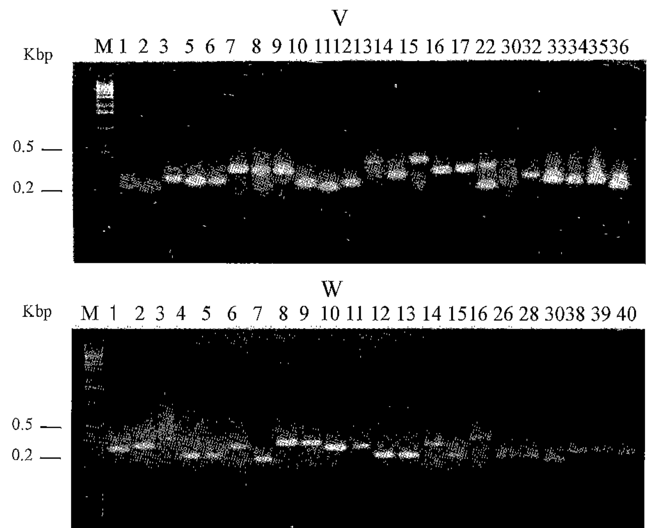

Figure 3. AFLP DNA fragments specific in either violet- (V) or white *Pharbitis* (W). The specific bands were cut from sequencing gels and DNAs were eluted. The eluted DNAs were amplified by PCR and run on the 2% agarose gel. Numbers on top indicate clone numbers. M, 1 kbp DNA ladder.

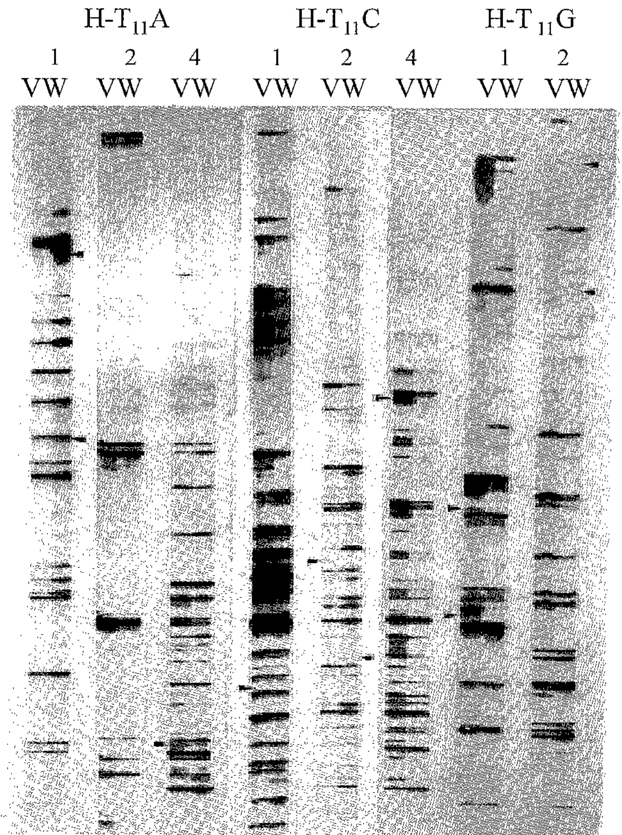

Figure 4. DDRT fingerprints. Total RNAs were isolated from violet- and white-*Pharbitis*. The amplified cDNA subpopulations of 3′ termini of mRNAs were separated on a DNA sequencing gel. Primer sets for PCR comprised H-T_{11} A, H-T_{11} C, H-T_{11} G and arbitrary primers, 1, 2 and 4. Arrowheads indicate cDNA fragments specific in either sample.

Table 2. Summary of DNA seqence homology analysis and search for DDRT clones.

DDRT Clone		The Most Holomogous Clone
Violet Specific	V5	*Arabidopsis thaliana* chromosome II section 64 of 255 of the complete
	V6	*Triakis scyllia* MHC class I antigen UAA (UAA*116 allele) mRNA,
	V7	Human DNA sequence from clone RP1-78F24 on chromosome 22q12.1-12.3 Contains one exon of an Oxysterol-binding protein (OSBP) LIKE gene, STS and GSSs, complete sequence
	V9	*Lycopersicon esculentum* cytosolic Cu,Zn super oxide dismutase (Sod) gene, partial cds; and dehydroquinate dehydratase/shikimate :NADP oxidoreductase gene
	V11	*Arabidopsis thaliana* calcium-dependent protein kinase (CDPK19) gene,
	V17	*psaEa*=photosystem I subunit PSI-E [*Nicotiana sylvestris, leaves*
	V18	Tomato yellow leaf curl virus replication associated protein *Rep (rep)* gene, partial CDs; pre-coat protein gene, complete cds; and coat protein gene, partial cds
	V22	Lepilemur dorsalis genomic fragment, RAPD with primer operon OPH4, clone A10a
White Specific	W 4	*Mus musculus* alpha -4 integrin subunit gene, 3′ UTR
	W 6	*Calonectris diomedea* randomamlifiedpolymorphic DNA
	W19	*Stronglyocentrotus puratus* RNA helicase PRP mRNA partials (100%)
	W29	*S. cerevisiae* OST gene *S. cerevisiae* chrosome Vll reading frame ORF YGL 226w
	W30	*Calonectris diomedea* randomamlified polymorphic DNA Solanum thberosum 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA
	W65	*Lycopersicon esculentum* mRNA for hypothetical protein
	W67	*Glycine max* env pseudogene, partial sequence; uncharacterized long terminal repeat, complete sequence: gag-pol polypritein

in Table 2. W19 has 100% sequence identity to *Stronglyocentrotus puratus* RNA helicase PRP1 mRNA partials and *W30* shows high similarity to *Solanum tuberosum* 3-hydroxy-3-methyl-glutaryl coenzyme A reductase mRNA. *V9* and *V11* clones are also very interesting.

The color of flowers seems to be mostly controlled by anthocyanin pigments, a flavonoid, which are synthesized through a phenylpropanoid pathway [13-14]. People have specially focused on several structural genes in the pathway, such as *CHS*, *DFR* (dihydroflavonol-4-reductase), *F′3,5′H* (flavonol-3′,5′-hydroxylase), *3-GT* (UDP-glucose: flavonoid *3-O*-glucosyltransferase) and *5-GT* (UDP-glucose: anthocyanin 5-*O*-glucosyltransferase) genes. In the case of *Pharbitis* plants,

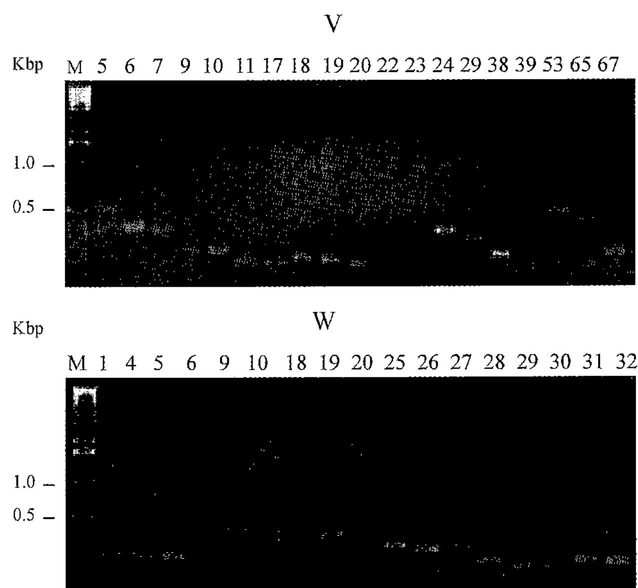

Figure 5. Identification of reamplified PCR products. cDNA fragments specific for either violet- and white-*Pharbitis* designated V and W, respectively. Numbers on top indicate clone numbers. M, 1 kbp DNA ladder.

the flower color could be determined depending on which *CHS* among 13 different genes would be expressed [5, 6]. Researchers also interested in regulatory genes which control above structural genes in the pathway. Maize *R* gene contains bHLH motif, a sort of *myc* transcription factor [4]. *An2* and *jaf13* from *Petunia* are MYB domain protein and bHLH motif proteins, respectively, that activate *DFR* protmoter [16]. In addition, *Petunia an11* gene consists of five WD-40 motifs, implying that it can control genes associated with flower color determination through a protein-protein interaction [24].

To change flower color in regard to genetic engineering perspective, researchers have introduced several genes into different plant species. *Petunia* synthesizing orange-pelagonidin pigments could be produced by introducing maize *DFR* gene [12], and a white flower was obtained by transformation of *CHS* and *DFR* genes from blue flower *Torenia* [21]. Particulary, a violet carnation, Moondust™, has been commercialized by introducing both *Petunia F3′, 5′H* and *DFR* genes into a white carnation plant [22]. Unexpectedly, we could not observed any structural genes or regulatory genes for the anthocyanin biosynthetic pathway. However, several clones are very interesting in regard to their possible involvement directly or indirectly in the pigment synthesis in *Pharbitis*.

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