J. Plant Biotechnology (2005) Vol. 7 (1), pp. 45~50

Journal of Plant Biotechnology

Approach for Cloning and Characterization of Blue/White Flower Color Specific cDNA Clones from Two *Commelina* Species

Gunho Lee, Mooshik Yeon, Yoonkang Hur*

Genome Research Institute, Chungnam National Univeristy, Daejon 305-764

Abstract

To clone blue and white flower color specific genes, mRNA differential display was carried out with two different *Commelina* species, *C. communis* Linne for blue color and *C. coreana* Leveille for. *leucantha* Nakai for white color. Fifty two and 100 cDNA clones specific for blue or white flower color, respectively, were ranging from 200 to 700 bp in size. From the reverse northern blot analysis, 12 and 7 positive clones were selected for blue and white flower, respectively. These clones appear to be novel cDNAs for these *Commelina* plants, but not color specific. This finding was supported by the northern blot analysis. However, two clones, B18 and B19, derived from blue flowered *Commelina* were highly expressed than in the white *Commelina* species, implying that further study will be valuable. The results indicated that both mRNA display experiment and dot blot analysis may not sensitive enough to clone color-determining gene from the plant, leading to explore more advanced method, like high-density colony array study (HDCA).

Key words: *Commelina* species, mRNA differential display, flower color

Introduction

Pigments like flavonoids, carotenoids and betalains are main components for flower color in plants. The flower color is mainly determined by structure of anthocyanins, co-pigmentations and the vacuolar pH of petals (Goto and Kondo 1991; Yoshida 1995; Yabuya et al. 1997; Tanaka et al. 1998; Mol et al. 1999). Horticultural plants with various flower colors have been developed by conventional breeding and selection continuously. However, the effort to obtain blue or violet flowers from important floricultural plants, such as rose, carnation, tulip and chrysanthemum, has failed because they do not accumulate delphinidin-type anthocyanins. This, in turn, is due to the absence of flavonoid 3', 5'-hydorxylase (*F3', 5'H*), resulting in no accumulation of 3',5'-hydroxylated anthocyanins in petals (Mol et al. 1999; Fukui et al. 2003; Okinaka et al. 2003; Mori et al. 2004).

F3',5' H, a member of cytochrome P450 family, is a key enzyme in the synthesis of 3',5'-hydroxylated anthocyanins by catalyzing the hydroxylation of dihydroflavonol B-ring and the gene encoding the enzyme was first cloned from *Petunia hybrida* (Holton et al. 1993a). Another *F3', 5'H* clone from *P. hybrida*, designated *cytochrome b5*, enhanced F3',5' H activity (De Vetten et al. 1999). Recently, violet flower carnation varieties (Florigene MoondustTM; FMD and Florigene MoonshadowTM; FMS) have been successfully developed by expressing heterologous *F3', 5'H* (Tanaka et al. 1998; Mol et al. 1999).

Co-pigments such as flavones and flavonols formed co-pigment complexes with anthocyanine, resulting in a bathochromic shift in the visible λmax and making more blue color (Asen et al. 1972). Either introduction of flavonol synthase (*FLS*) gene or antisense suppression of dihydroflavonl 4-reductase (*DFR*) gene caused change in co-pigement contents, thereby the flowers look bluer (Holton et al. 1993b; Adia et al. 2000). On the other hand, the down-regulation of chalcone synthase (*CHS*) genes with RNA interference resulted in removal of blue flower color from *Torenia hybrida* (Fukusaki et al. 2004).

* Corresponding author, E-mail: ykhur@cnu.ac.kr
Received Oct. 18, 2004; Accepted Mar. 10, 2005

Vacuolar pH of petal affects flower color; strong acidic condition leads to red color, neutral pH purple and alkaline pH blue (Yabuya et al. 1997). However, the vacuolar pH of most plant cells is weakly acidic so that most anthocyanins contribute to unstable purple color. According to recent study on hydrangea sepal, the vacuolar pH of blue cells is higher than that of red ones even though all colored sepals contain the same anthocyanin, delphinidin 3-glucoside (Yoshida et al. 2003).

Efforts to isolate gene conferring blue flowers color have a long history, but none has been cloned so far. Researchers involved in isolation and characterization of gene for blue flower have focused on torenia (Adia et al. 2000; Fukusaki et al. 2004) and *Gentiana triflora* (Fujiwara et al. 1997; Fujiwara et al. 1998; Yoshida et al. 2000). However, none of these possesses bright blue color as much as that of *Commelina communis*. This study was undertaken to isolate the blue color determination genes by differential display reverse transcription PCR (DDRT-PCR) using mRNA populations from different-colored, blue and white flowered, *Commelina* plants.

Materials and Methods

Plant materials

Two *Commelina* species, blue flowered (*C. communis* Linne) and white flowered (*C. coreana* Leveille for. *leucantha* Nakai), were collected from the field and grown at the campus of the Chungnam National University, Korea. Floral buds and flowers (Fig. 1) were sampled during summer season and stored at -70℃ until use.

mRNA Differential display

Total RNA was isolated from floral buds of the two *Commelina* species by modified TRIzol reagent solution (Invitrogen). Differential display was performed by using the RNAimage kit (Gene Hunter) according to the Manufacturer's protocol. Reverse transcription was done in four independent reactions using three types of oligo-dT primers. The reaction mixture was incubated at 65℃ for 5 min, 37℃ for 60 min, 75℃ for 5 min, and then, 37℃ for 10 min. After adding MMLV RT reverse transcriptase, the mixture was incubated at 37℃ for 60 min. The reaction was stopped by incubating the mixture at 95℃ for 5 min. One tenth of the volume of the mixture was subjected to PCR amplification in the presence of α-^{33}P-dATP. PCR was carried out for 40 cycles of 94℃ for 30 sec, 40℃ for 2 min, and 72℃ for 30 sec, and an additional extension was carried out at 72℃ for 5 min. Eighty different primer sets used for the PCR amplification were organized in the combination of twenty 5'-AP (arbitrary primer) with the 3'-T_{11}MN primers that were used for the initial cDNA synthesis. The amplified cDNA fragments were separated on 6% denaturating polyacrylamide gel electrophoresis in TBE buffer at 60 watts constant power. The gel was dried and exposed to a X-ray film.

The differentially expressed cDNA bands were selected after autoradiography and excised from the dried gel. The gel slices, along with the 3 MM 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. Then, the DNA was precipitated for 30 min at -70℃. The cDNA pellets were collected by spinning for 10 min at 4℃. The pellet was washed and dissolved in 10 μl of dH_2O. Four microliters of each DNA was used for reamplification with the same primer set and the same PCR conditions.

Dot blot analysis

To ensure specificity of the reamplified PCR products, dot blot analysis was carried out. Equal amounts (200 ng) of the amplified and denatured DNA were spotted onto the nytran supercharged membrane (Schleicher & Schuell) using Bio-Dot Microfiltration apparatus (Bio-Rad). The membrane was dried and incubated in UV cross-linker for 150 sec. Probes were prepared by reverse transcription of RNA samples as follows. Total RNAs (5 μg), freshly extracted from floral buds of *C. communis* and *C. coreana*, were subjected to reverse transcription in the presence of radio-labeled α-^{32}P-dCTP with oligo-dT primers as described as in the manufacturer's manual (Super Script RT-System, GIBCO BRL). The probe was purified using a QIAquick spin column (Qiagen). Hybridization was carried out by incubating the membrane in a

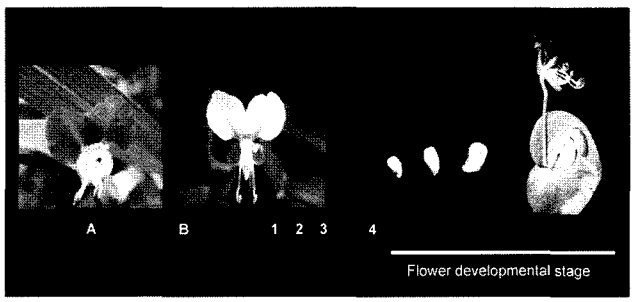

Figure 1. Blue and white flowered *Commelina* species, and floral bud development. A, blue flowered *Commelina* (*C. communis* Linne); B, white flowered *Commelina* (*C. coreana* Leveille for. *leucantha* Nakai). Right panel shows developmental stages of the floral buds from *C. communis* : stage 1-4.

solution (5×SSC, 5×Denhardt's reagent, 0.1% SDS, 50% formamide and 100 μg/mL denatured fragmented salmon sperm DNA) for overnight at 42℃. After hybridization, the membrane was washed twice at room temperature with 6× SSPE/0.5% SDS for 15 min, twice at 37℃ with 1×SSPE/ 0.5% SDS for 15 min and once at 65℃ with 0.1×SSPE/ 0.1% SDS for 30 min. The signal was detected with Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One software.

Cloning and sequencing analysis

The selectively specific PCR products were ligated to pGem-T-Easy vector (Promega), and transformed into *E. coli JM109 cells. The recombinant plasmid* DNA was sequenced using the ABI Prism 3700 automated sequencer (Applied Biosystem Division of Perkin Elmer). Sequence data were analyzed with known sequences in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) with BLAST search program.

Northern blot analysis

Northern hybridization with total RNAs, isolated using the Trizol reagent, was performed following the standard protocol (Sambrook and Russell 2001). Twenty micrograms of total RNA were subjected to electrophoresis on 1% formaldehyde-agarose gel and transferred to a nylon membrane filter (Schleicher & Schuell). The RNAs were bound to the membrane by cross-linking with UV. In order to ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. The RNA on the filter was hybridized with ^{32}P-labeled putative color specific clones in a hybridization solution containing 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS and 100 μg/mL salmon sperm DNA at 42℃ for 16 h. The filter was washed twice with 2×SSPE and 0.1% SDS for 5 min at room temperature and then twice for 30 min at 42℃. The signal was detected with Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One software.

Results and Discussion

Flower structure of *Commelina* species is peculiar which bears both an open flower and bract. The bract possesses three developing floral buds; stage 1 and 2 without blue color, and stage 3 with pale blue color (Figure 1). For mRNA differential display experiment, total RNAs were extracted from floral buds of stage 1 and 2 from blue as well as white flowered *Commelina* species. We expect that flower color-determining genes will be expressed in these buds, but not in the stage-3 bud where pigments for blue color have already been synthesized and deposited.

Electrophoretic separation revealed hundreds of differential bands from 16 primer combinations (two H-T_{11} primers and eight AP primers). These DNA bands were eluted and amplified by PCR. Finally, 52 and 100 cDNA clones specific for blue or white flowered *Commelina* species, respectively, were obtained (Figures 2, 3). These bands ranged from 200 to 700 bp in size.

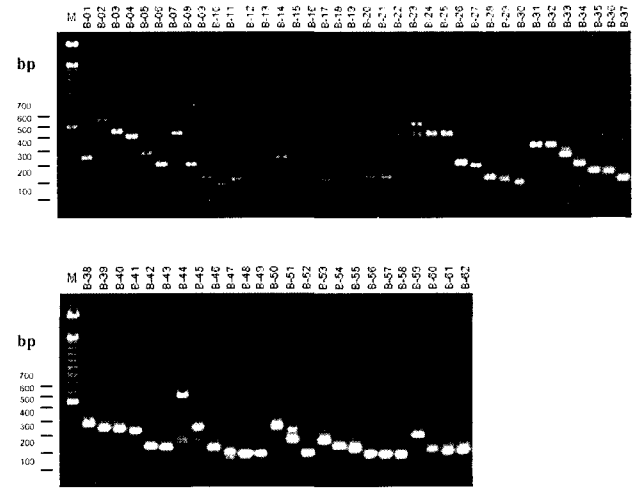

Figure 2. PCR-amplified 52-putative blue-color specific bands. Putative blue-color specific clones were eluted from the gel of mRNA differential display experiment, amplified by PCR, electrophoresed on 1.5% agarose gel and stained with EtBr. Size markers (100 bp DNA ladder) are indicated to the left in base pair (bp).

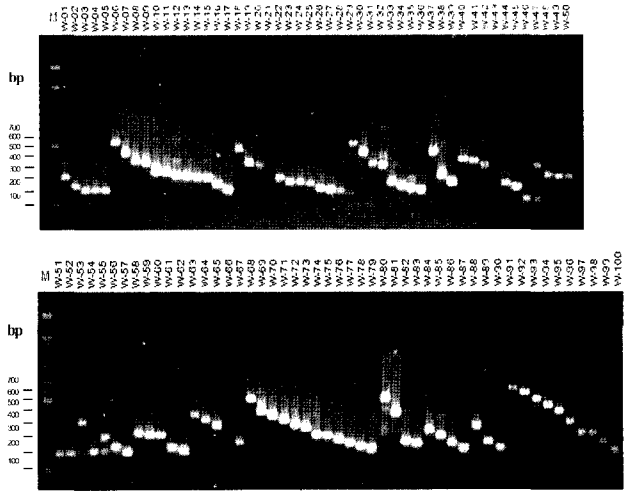

Figure 3. PCR-amplified 100-putative white-color specific bands. Putative white-color specific clones were eluted from gel of mRNA differential display experiment, amplified by PCR, electrophoresed on 1.5% agarose gel and stained with EtBr. Size markers (100bp DNA ladder) are indicated to the left in base pair (bp).

To confirm the specificity of amplified cDNAs, the DNAs were spotted onto a nytran supercharged membrane using Bio-Dot Microfilteration Apparatus and hybridized with labeled probes that were prepared from mRNA (data not shown). From the reverse northern blot analysis, 12 and 7 positive clones were selected for blue and white flowered species, respectively. These cDNAs were subcloned into pGem-T-Easy vector and subjected to sequence analysis. All information for putative blue and white flowered clones is summarized in Table 1. As shown in Table 1, most of the clones appear to be novel for *Commelina* plants because no similar sequences were found in the NCBI database. Only three clones have similarities with known DNAs: B3, B27 and W94. B3 clone has high identity to *Zea mays* calcium-dependent protein kinase (ZmCPK11: GenBank Accession No, AY301062). Ca^{2+}-dependent or calmodulin-like domain protein kinases (CDPKs), which are found only in plants, green algae, and certain protists, are an important group of sensor-responder proteins that function through intramolecular interactions to decode Ca^{2+} signals (Hrabak et al. 2003). Therefore, B3 may not to be associated with blue pigmentation in the flowers of *Commelina* plants. B27 clone shows 100% identity to human chromosome 12 DNA in 266 bp. However, the function of this DNA sequence is unknown. W94 clone has relatively high identity to an unknown protein of rice at the amino acid sequence level. Also, the function of this rice protein is still unknown. It would be interesting to identify full-length sequences for B27 and W94 and to examine the expression characteristics.

We expected that some of the genes involved in flavonoid biosynthesis (Shirley 1996) would be cloned in this experiment, particularly, genes encoding key enzymes of flavonoid biosynthesis, such as *CHS*, *DFR* and *F3',5'H*. However, none of those genes were detected. Perhaps, the expression level of such genes was too low in the material used.

Specificity of the selected cDNA clones was determined by northern blot analysis (Figure 4). All transcripts (5 for

Table 1. Summary of putative blue and putative white color specific clones selected by reverse northern blot analysis

Clone			Result of BlastN search	Result of BlastX search
Origin	Designation	Size (bp)		
Blue	B1	155	NGMF	NSSF
	B3	402	*Zea mays* calcium-dependent protein kinase ZmCPK11 mRNA (172/200) 86%)	Calcium-dependent protein kinase ZmCPK11 of *Zea mays* (77/98) 78%)
	B10	407	NGMF	NGMF
	B11	340	NGMF	NSSF
	B12	272	NGMF	NSSF
	B16	233	NGNF	NSSF
	B18	406	NGMF	NSSF
	B19	211	NGMF	NSSF
	B20	221	NGMF	NSSF
	B21	468	NGMF	NSSF
	B27	318	*Homo sapiens* 12 BAC RP11-473N11 (266/266: 100%)	NSSF
	B60	244	NGMF	NSSF
White	W27	205	NGMF	NSSF
	W35	218	NGMF	NSSF
	W70	468	NGMF	NGMF
	W75	300	NGMF	NSSF
	W77	244	NGMF	NSSF
	W79	205	NGMF	NSSF
	W94	531	NGMF	Unknown protein of *Oryza sativa* (42/79: 53%)

Blue: *C. communis* Linne
White: *C. coreana* Leveille for. *leucantha* Nakai
MGMF: no good match found (less than 40% identity)
NSSF: no significant similarly found (no sequence existed).

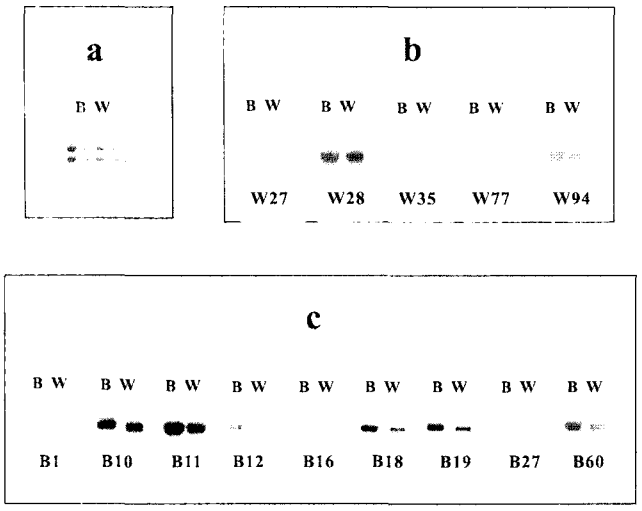

Figure 4. Northern blot analysis of putative blue- or white-color specific clones. Twenty micrograms of total RNA from either blue flowered *Commelina* (B) or white flowered *Commelina* (W) were fractionated on 1% formaldehyde-agarose gel, transferred to a nylon membrane filter and hybridized with PCR-labeled probe. **a**, EtBr-stained gel showing equal loading; **b**, result of RNA gel hybridization for putative white-specific clones; **c**, result of RNA gel hybridization for putative blue-specific clones.

putative blue and 9 for putative white color) were detected in both species, implying that none of them is color specific. Some clones, such as B18 and B19, seemed to be highly expressed in blue colored *Commelina*, but not specific. These results also indicate that both mRNA display experiment and dot blot analysis would not be the first choice of gene cloning without fortune. It will be better one uses high-throughput method for the initial screening. It is proposed to undertake alternate strategy, like a high-density colony array study (HDCA) (Chen et al. 2003) in order to clone gene for blue flower color.

Acknowledgements

We thank to Dr. Prikshit Plaha for critical reading of this manuscript.

References

Adia R, Yoshida K, Kondo T, Kishimoto S, Shibata M (2000) Copigmentation gives bluer flower on transgenic torenia plants with the antisense dihydroflavonol-4-reductase gene. Plant Sci 160: 49-56

Chen IP, Haehnel U, Altschmied L, Schubert I, Puchta H (2003) The transcriptional response of *Arabidopsis* to genotoxic stress - a high-density colony array study (HDCA). Plant J 35: 771-786

De Vetten N, ter Horst J, van Schaik HP, de Boer A, Mol J, Koes R (1999) A cytochrome b5 is required for full activity of flavonoid 3',5'-hydroxylase, a cytochrome P450 involved in the formation of blue flower colors. Proc Natl Acad Sci 96: 778-783

Fujiwara H, Tanaka Y, Fukui Y, Nakao M, Ashikari T, Kusumi T (1997) Anthocyanin 5-aromatic acyltrasferase from *Gentiana triflora*, purification, characterization and its role in anthocyanin biosynthesis. Eur J Biochem 249: 45-51

Fujiwara H, Tanaka Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Nakao M, Fukui Y, Yamaguchi M, Ashikari T, Kusumi T (1998) cDNA cloning, gene expression and subcellular localization of anthocyanin 5-aromatic acyltransferase from *Genetiana triflora*. Plant J 16: 421-431

Fukui Y, Tanaka Y, Kusumi T, Iwashita T, Nomoto K (2003) A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3',5'-hydroxylase gene. Phytochemistry 63: 15-23

Fukusaki E, Kawasaki K, Kajiyama S, An CI, Suzuki K, Tanaka Y, Kobayashi A (2004) Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference. J Biotechnol 111: 229-240

Goto T Kondo T (1991) Structure and molecular stacking of anthocyanins-flower color variation. Angrew Chem Int Ed Engl 30: 17-33

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu CY, Farcy E Stevenson TW, Cornish EC (1993a) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366: 276-279

Holton TA, Brugllera F, Tanaka Y (1993b) Cloning and expression of flavonol synthases from *Petunia hybrida*. Plant J 4: 1003-1010

Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford H, Kudla J, Luan S, Nimmo HG, Sussman MR, Thomas M, Walker-Simmons K, Zhu JK, Harmon AC (2003) The *Arabidopsis* CDPK-SnRK superfamily of protein kinases. Plant Physiol 132: 666-680

Mol J, Cornish E, Mason J, Koes R (1999) Novel coloured flowers. Curr Opin Biotechnol 10: 198-201

Mori S, Kobayashi H, Hoshi Y, Kondo M, Nakano M (2004) Heterologous expression of the flavonoid 3', 5'-hydroxylase gene of *Vinca major* alters flower color in transgenic *Petunia hybirida*. Plant Cell Physiol 22: 415-421

Okinaka Y, Shimada Y, Nakano-Shimada R, Ohbayashi M, Kiyokawa S, Kikuchi Y (2003) Selective accumulation of delphinidin derivatives in tobacco using a putative flavonoid 3',5'-hydroxylase cDNA from *Campanula medium*. Biosci Biotechnol Biochem 67: 161-165

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, New York

Shirley BW (1996) Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci 1: 377-382

Tanaka Y, Tsuda S, Kusumi T (1998) Metabolic engineering to modify flower color. Plant Cell Physiol I 37: 1119-1126

Yabuya T, Nakamura M, Iwashita T, Yamaguchi M, Takehara T (1997) Anthocyanin-flavone copigmentation in bluish purple flowers of Japanese garden iris (*Iris ensata* Thunb.). Euphytica 98: 163-167

Yoshida K (1995) Cause of blue petal color. Nature 373: 291

Yoshida K, Toyama-Kato Y, Kameda K, Kondo T (2003) Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode Plant Cell Physiol 44: 262-268

Yoshida K, Toyama Y, Kameda K, Kondo T (2000) Contribution of each caffeoyl residue of the pigment molecule of gentiodelphin to blue color development. Phytochemistry 54: 85-92