Molecular cloning of soybean anthocyanin biosynthetic pathway genes and their applications in flower color engineering

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Abstract

Color is an important trait for many beautiful flower species which are lack of specific colors due to germplasm limitation. This Taiwan-US collaborative project was initiated to overcome the genetic barrier in breeding new color for several important ornamental crops including pigment gene cloning, vector construction, genetic transformation, and mass propagation of transgenic plants. First, a total of 30 anthocyanin related genes of CRWD soybean variety with *iRT* genotype and a black seedcoats phenotype were cloned for the metabolic engineering in our interest plant species. Second, constructs of cloned genes for overexpression and gene knockout were conducted. Third, agrobacterium mediated transformation systems have been established in chrysanthemum, poinsettia, phalaenopsis orchid to obtain new color.

Cloning and characterization of the cloned genes suggests that the black seedcoats soybean variety contains most complicated anthocyanin biosynthetic pathway and provides the best genetic resource for the metabolic engineering of this pathway. We have been success in engineering flower color by transformation of soybean pigment genes to the above species and confirmed by phenotype, HPLC and/or Southern hybridization.

Introduction

There are always desires for diversities of flower colors from the point of view of consumer therefore who can create new varieties in a short time would have strong competent in market (Mol *et al*., 1995). From all prospects, the genetic transformation will fit this acquirement properly (Tanaka et al., 1998). Flower colors are decided by many factors and among them pigments of anthocyanin is the major group (Inagaki *et al.*, 1999). Anthocyanin pathway is one of unique and well-studied pathway of plant however most studies were focused in the few model plants like maize, snapdragon and petunia (Mol *et al*., 1989; Oud *et al*., 1995; van Houwelingen *et al*., 1998). To our knowledge, the black seedcoat soybean variety must contain homozygous *iRT* genotype and produce most complicated anthocyanin pigments including pelargonidin (sub-pathway 1), cyanide(sub-pathway 2), and delphinidin(sub-pathway 3) since early stage of seed development providing the best material for studying this pathway (Wang et al. 1994). Due to genetic limitation the rose plants contain pelargonidin and cyanide but lack of sub-pathway 3, therefore, no blue rose is available in nature. The sub-pathway 1 and 2 are presented in the pericarps of maize germplasm, tansformation of a maize *DFR* to the red petunia produced pelargonodin and showed brick-red color which was the world first successful flower color engineering and demonstrated a new trend for developing new flower color through molecular breeding (Mol *et al*., 1989).

Strategy for mloecular breeding of flower color

Traditional breeding methods including intra- and inter- specific hybridization and selection have produced many varieties with novel flower color. Moreover, spontaneous or mutagenesis derived mutants are also good population for breeding of novel flower color. However, the limited gene pool in single species may result in restriction of some flower color. For instance, many flowers such as rose and lily have the beautiful flower but without bluish color due to the lacking of flavonoid 3', 5'-hydroxylase (F3', 5'H) enzyme activity for delphinidin accumulation. The cDNA of F3', 5'H cloned from *Vinca major*, *Campanula medium*, *Eustoma russellianum* were overexpressed in petunia and tobacco, both the transgenic plants accumulated delphinidin and light bluish petals were observed (Mori et al., 2004; Okinaka et al., 2003; Shimada et al., 1999). Moreover, *Torenia hybrida* Summerwaves developed by Suntory Ltd., transformed with F3', 5'H has blue flowers (Suzuki et al., 2000). Recently, bluish roses were generated by introduction of viola *F3', 5'H* gene with iris *DFR* gene and down-regulated endogenous DFR expression simultaneously (Katsumoto et al., 2007). These results showed molecular breeding is a powerful method that provides an opportunity to introducing foreign gene to ornamental crops to creates new flower color.

Several steps are required for the genetic engineering of flower cooler：

1. Choose determine a plant species and/or material producing most abundant anthocyanin pigments. Here we choose the soybean variety with black seedcoats as resource for cloning pigment genes because its pathway has been genetically and biochemically studied.
2. Determine the genetic constrain or defect in the target plant species for producing the aimed-color.
3. Clone and construct the gene necessary for providing new flower color to the target

species or variety through genetic transformation.

4. Transform the gene-construct and regenerate the transgenic plants.
5. Screening and analyzing to obtain the transgenic plant with expected phenotype and genotype.
6. The release a new color variety of target species.

Researches on the metabolic engineering of flower color previously at the Laboratory of Molecular Genetics, TARI, and recently at the Laboratory of Genetics and Genomics, Department of Agronomy, NVHU will be reported.

The black seedcoat soybean is a good resource for pigment genes cloning

Flavonoids and isoflavonoids are plants specific secondary metabolites. These C6-C3-C6 compounds are derived from the flavonoid biosynthetic pathway and play important roles in pigment production, pathogen defense, UV protection, hormone biosynthesis, signal transduction, especially for plant to survive from environmental and ecological stresses. Therefore, study on the anthocyanin biosunthetic pathway producing theses secondary metabolites will do great benefit to human being.

In order to conduct metabolic engineering on the anthocyanin biosynthetic pathway soybean CRWD (Ching-Ren-Wo-Dow) variety developed by C. S. Wang with the homozygous *iRT* genotype and black seedcoat phenotype was choosen as the basic material for RNA extraction, cDNA library construction and anthocyanin biosynthetic related genes cloning. So far, genes from ten groups of this pathway have been cloned by cDNA library screening including: 2 CHS (Chalcone synthase), 3 CHI (Chalcone isomerase), 5 F3H (Flavanone 3-hydroxylase), 3 F3'H (Flavonoid 3'-hydroxylase), 1 F3',5'H (Flavonoid 3',5'-hydroxylase) also called blue gene, 7 DFR (Dihydroflavonol 4-reductase), 2 GST（Glutathione S-transferase,）, three ANS

(anthocyanin synthase), 1 FLS (flavone synthase), 1 UFGT, 1 myb, and 1 myc。The molecular characterization of these genes confirmed and suggested that black seedcoat of soybean provide the most complicated anthocyanin biosynthetic pathway as we known. (Fig. 1)

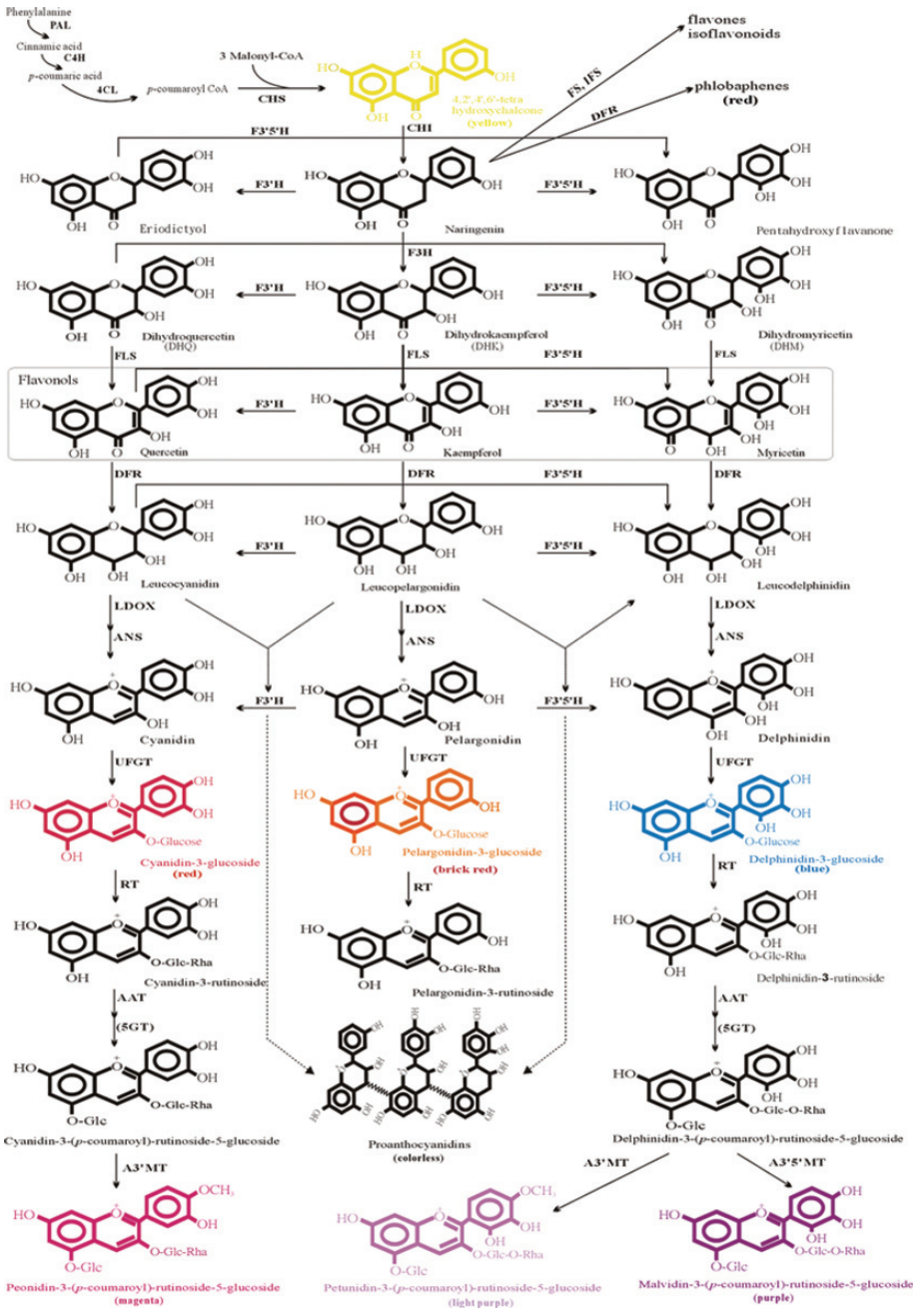
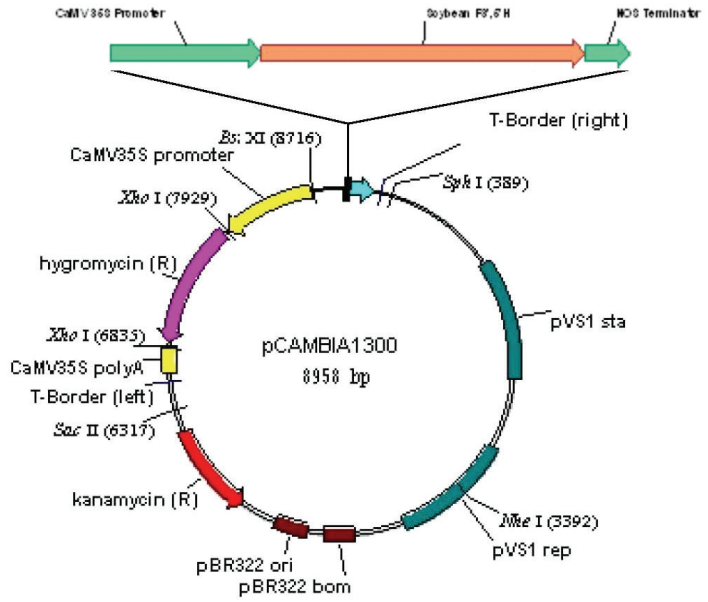


Fig. 1. The anthocyanin biosynthetic pathway in the black seedcoats of soybean.

Construction of transformation vector

In this study, series of pCAMBIA vectors were applied as the backbone for construction of pigment genes (*CHI*, *F3H*, *DFR*, *F3',5'H*) cloned from *Glycine max* 'CRWD' variety for agrobacterium-mediated transformation. For example, F3'5'H driven by 35S promoter (CaMV, cauliflower mosaic virus) was constructed in pCAMBIA1300 as shown in Fig. 2 for transformation. The EHA105 strain of *Agrobacterium tumefaciens* carrying the specific construct was used for transformation.



CaMV 35S-F3,5H-NOS-pCambia 1300

Fig.2. Binary vector used to generate GmF3', 5'H overexpressing tobacco plants.

Establish of transformation system for flower color engineering

In this study, chrysanthemum, phalaenopsis, poinsettia as well as model plant tobacco were chosen as targets for flower color engineering. The agrobacterium-mediated transformation system was established for transformation of the constructs to each plant species.

Gene transformation and regeneration in chrysanthemum

Pigment genes were transferred to all the target plant species by *Agrobacterium*-mediated method, putative transgenic plants were screened in the selection media containing proper antibiotics. An efficient transformation system has been established and as demonstrated in the Fig. 3. Though there exits cultivar specific problems in transformation of chrysanthemum our system can be modified for many commercial variety. All the transformation process can be completed within nine months to obtain flowering plants. After antibiotic screening, they were further screened for both GUS and target genes by PCR. The flower color on petals was analyzed by both PCR and HPLC to detect the integration and expression of the target gene since the early stage of flower development (Figs.4, 5). The transformation of soybean F3H to chrysanthemum Pink diary variety enhanced the pigment synthesis and accumulation at all developmental stages resulted in a darker pink and the color lasting longer duration. The results also supported by the increasing of cyaniding amount the HPLC analysis of pigment amount ranged from 2.03 to 5.75 (Table 1) suggested that transformed with soybean F3H gene may significant enhance follower color and last longer in chrysanthemum.

Transformation of soybean F3H to the Yellow Hsiouphoen variety which contains only carotenoid dramatically turn its color to dark pink and producing cyanidin but no carotenoid (Fig. 5). The result suggested that the carotenoid biosynthetic pathway in the petals of transgenic F3H plant has been shut down and replaced by the a anthocyanin biosynthetic pathway. This was supported by HPLC analysis as shown in Table 2.

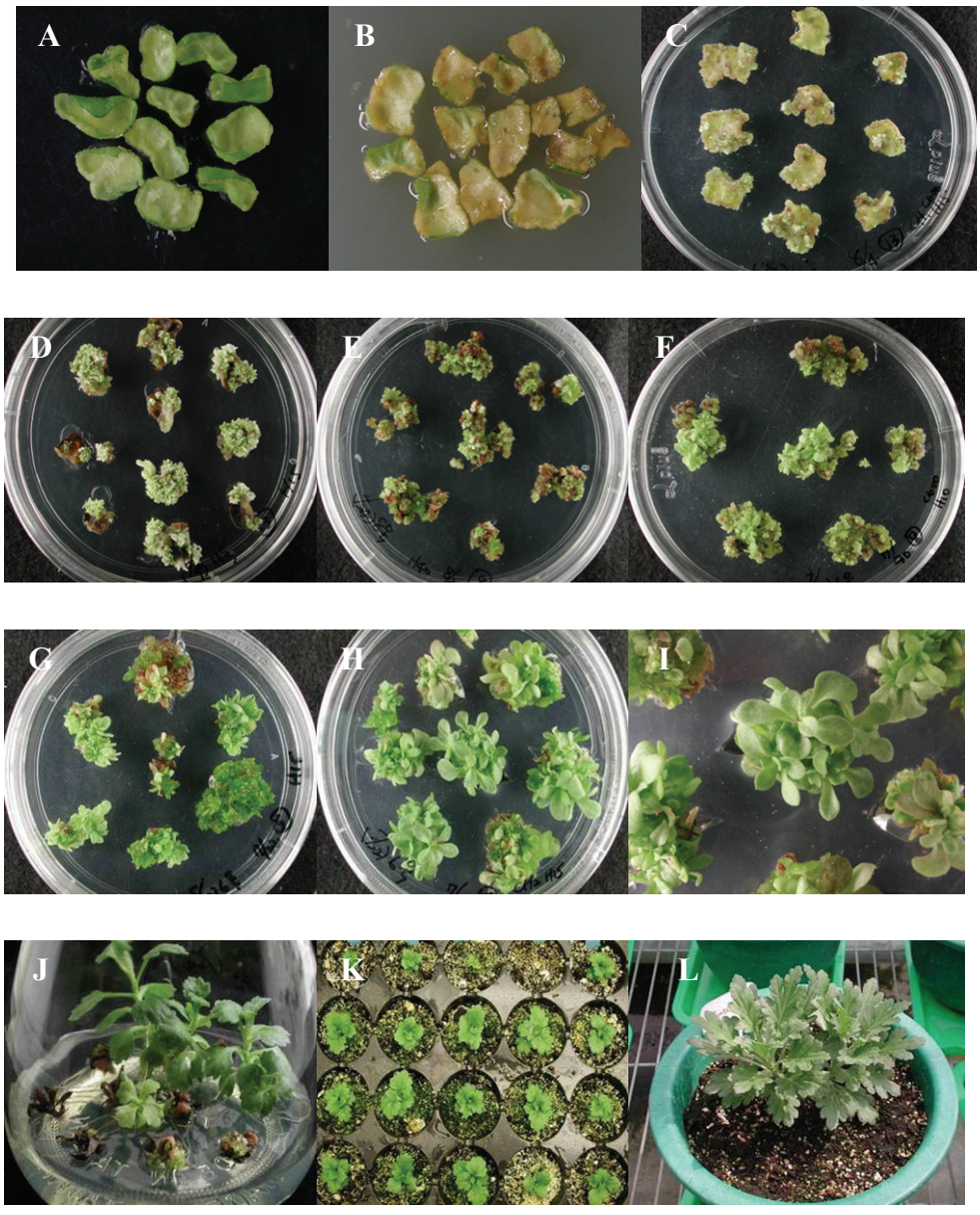


Fig 3. Genetic transformation system for chrysanthemum.
(A) preculture of leaf explant, (B) co-cultivation with agrobacterium, (C) callus formation in selection medium at 1 month after transformation, shoot regeneration at (D) 2 month, (E) 3 month, (F) 4 month, (G) 5 month, and (H) 6 month after transformation, (I)bud elongation, (J) rooting on selection medium, (K) acclimation of PCR screed plants, (L) transgenic plants.



Fig. 4. Pink diary (upper, wild type) transformed with soybean F3H gene (lower) shows enhanced follower color at five stages of flower development.

Table 1. Transgenic Pink Diary produced higher cyaniding amount.

Plant	Flowering Stage（cyanidin, μg/ g FW）				
	1	2	3	4	5
Wild type plant	26.44	46.44	22.77	16.89	10.47
Transgenic plant	82.37	94.3	80.79	62.4	60.16

＊ Not detected



Fig 5. Yellow Hsiouphoen contains carotenoid (upper, wild type), transgenic soybean F3H plant (lower) produces cyanidin and shows pink color.

Table 2. Yellow Hsiouphoen (upper, wild type) transformed with soybean F3H produces cyanidin and shows pink color.

Plant	Flowering Stage（cyanidin, μg/ g FW）		
	1	2	3
Wild type plant	nd*	nd	nd
Transgenic plant	16.7	35.39	11.63

* Not detected

Transformation of pigment genes to poinsettia

A high embryogenesis system has been established for poinsettia in order to introduce new color through genetic transformation (Fig. 6). Two poinsettia varieties, 'Nobel Star' and 'Peter Star-White', were transformed using *Agrobacterium tumefaciens* strain EHA105 carrying soybean pigment genes: F3H, F3',5'H and anthocyanidin synthase (ANS) *via* embryogenesis system. So far, 5 and 7 putative transgenic poinsettia of 'Nobel Star' harboring with F3H or ANS gene, respectively were verified by PCR. Five or 23 putative transgenic poinsettia of 'Peter Star-White' were also verified by PCR to contain F3H or F3',5'H gene, respectively. The phenotypic result will be analyzed in the very near future.

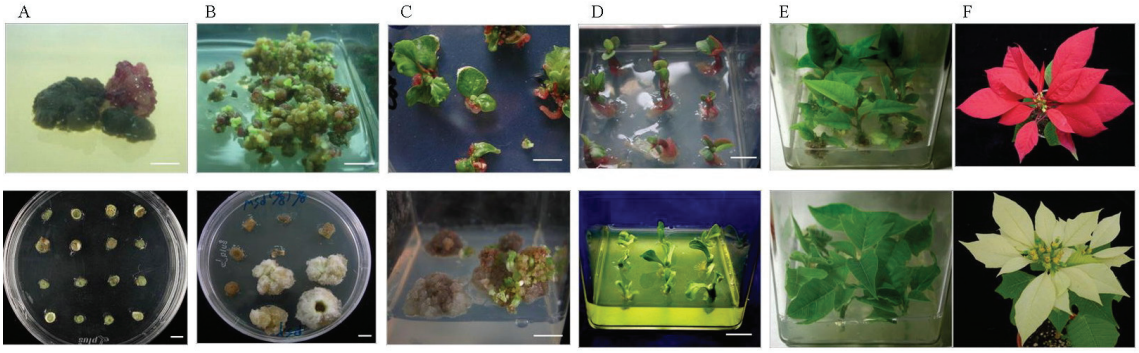


Fig. 6. Process of somatic embryogenesis and transgenic regeneration of poinsettia. 'Nobel Star' (upper) and 'Peter Star-White' (lower). (bar=10 mm)

A. stem explants expansion in the 2nd week (lower), somatic embryos from callus in the 12th week (upper), B. callus induced from stem explant in the 4th week(lower), somatic embryos developed in the 16th week(upper), C. somatic embryos mature and elongate in the 14th week(lower), D. plantlets regenerated from somatic embryos in the 24th week,E. transgenic plants regeneration in the 32nd week, F. plants established and flowering in the greenhouse.

Expression of soybean F3', 5'H gene in tobacco

Tobacco has been a model transformation system for studying gene function. According to our study only sub-pathway 1 is presented in tobacco leading to produces only cyanidin and its derivatives therefore it also serves a good material for studying molecular engineering of flower color. Several F3', 5'H genes were cloned from some plant species, however, only few function were demonstrated in transgenic plants. It was important to investigate the function of *GmF3', 5'H* overexpressed in transgenic plants. Tobacco (*Nicotiana tabacum* cv. Petit Havana) was a good candidate, because it originally lacked F3', 5'H genes and has pink flowers. Binary vector harboring *GmF3', 5'H* driven by the CaMV 35S promoter was transformed into tobacco. In 7 independent transgenic tobacco plants no visible changes other than flower color were observed, apparent changes in flower color intensity were observed in transgenic tobacco plants. The flower color of transformants expressing *GmF3', 5'H* were not blue but magenta and darker than that of control plants. All of them were increased in anthocyanin amount indicated that *GmF3',5'H* hydroxylated B-ring at 3 and 5 positions in transgenic tobacco successfully.

Conclusions

Our results demonstrated that 1. the anthocyanin biosynthetic genes cloned from black seedcoats of soybean can functionally express in many plant species and showed altered metabolites and phenotypes. It's suggested that these genes may provide good resources for metabolic engineering of anthocyanin and its related pathways in plant species. 2. Those unexpected results may due to the complicated and un-identified metabolic network in the target plant species.

Acknowledgements

The authors want to express their sincere thanks to USDA and AIT for their long term financial support on the project “Metabolic engineering of flower color” (91-SA-1.4-IC-01(2)) and travel grant to C. S. Wang. Initiation of this project by Dr. Teh-Yeh Ku and coordinated by Dr. Ming-Jhe Wu are also acknowledged.

Literature cited

Boase, M. R., R. C. Butler, and N. K. Borst. 1998. Chrysanthemum cultivar – *Agrobacterium* interactions revealed by GUS expression time course experiments. Sci. Hort. 77: 89-107.

Chen, Y. M., C. S. Wang, and C. Y. Chu. 2006. Flower bud as explant for Agrobacterium tumefacien-mediated transformation of pigment gene in chrysanthemum. Horticulture NCHU 31: 81-94.

Fukai, S., J. D. Jong, and W. Redemaker. 1995. *Agrobacterium*-mediated Genetic transformation of *chrysanthemum*. Acta Hort. 392: 147-153.

Hooykaas P. J. J. and Schilperoort R. A. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19: 15-38.

Kudo, S., N. Shibata, Y. Kanno, and M. Suzuki. 2002. Transformation of *chrysanthemum* Via *Agrobacterium tumefaciens*. Acta Hort. 572: 139-147.

Ledger, S. E., S. C. Deroles, and N. K. Given. 1991 Regeneration and *Agrobacterium*-mediated transformation of *chrysanthemum*. Plant Cell Reports 10: 195-199.

Li, T. F., C. S. Wang, and C. Y. Chu. 2008.Transformation of Soybean Pigment Genes

to Poinsettia. Hort. NCHU 31: 81-94.

Lu, C. Y., G. Nugent, and T. Wardley. 1990. Efficent, direct plant regeneration from stem segments of *chrysanthemum*. Plant Cell Reports 8: 733-736.

Mol J. N. M., Stuitje A. R. and van der Krol A. 1989. Genetic manipulation of floral pigmentation genes. Plant Mol. Biol. 13: 287-294.

Mol J. N. M., Holton T. A. and Koes R. 1995. Floriculture: genetic engineering of commercial traits. TIBTECH. 13: 350-355.

Sanford J. C., Smith F. D., and Russell J. A. 1993. Optimizing the biolistic process for different biological applications. Meth. Enzymol. 217: 483-509

Sherman, J. M., J. W. Moyer, and M. E. Daub. 1998. A Regeneration and *Agrobacterium*-mediated transformation System for Genetically Diverse *chrysanthemum* Cultivars. J. Amer. Soc. Hort. Sci. 123(2): 189-194.

Smith R. H. and Hood E. E. 1995. *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Sci.35: 301-309.

Tanaka Y., Tsuda S. and Kusumi T. 1998. Metabolic engineering to modified flower color. Plant Cell Physiol. 39: 1119-1126.

Wang C. S and Vodkin LO (1994) Extraction of RNA from tissues containing high levels of procyanidins that bind RNA. *Plant Mol Biol Rep* 12: 132-145.

Wang, C. S., J. J. Todd, and L. O. Vodkin. 1994. Chalcone synthase mRNA and activity are reduced in yellow soybean seed coats with dominant *I* alleles. Plant Physiol. 105: 739-748.

Wang. C. S., C. N. Hsia, J. J. Chen, R. J. Griesbach, and H. T. Hsu. 1997. Developing

new flower colors by transformation of pigment biosynthetic gene(s). Proceeding of the symposium for Reviewing COA-USDA Agricultural Science Cooperative Program, Taichung, Taiwan.

Wang, C. S. 1999. Cloning of anthocyanin biosynthetic genes and molecular genetic studies at TARI. Proceedings Symposium of Scientific Cooperation in Agriculture between INRA (France) and Council of Agriculture (Taiwan, R.O.C.) pp. 285-289. April 19-20, Toulouse, France.

Zheng, Z. L., Z. Yang, J. C. Jang, J. D. Metzger. 2001. Modification of plant architecture in chrysanthemum by ectopic expression of the tobacco phytochrome B1 gene. J Am Soc Hort. Sci. 126: 19– 26.