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Gibberellic Acid Regulates Flavonoid 3',5'-Hydroxylase Gene Transcription in the Corolla of *Gentiana scabra*

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The flavonoid 3',5'-hydroxylase (F3',5'H) gene is in the NADPH-Cytochrome P450 reductase family. A partial F3',5'H cDNA was cloned by RT-PCR from corollas of *Gentiana scabra*. Activity of F3',5'H was observed during flower development, and activity was highest at the stage prior to flower opening. The enzyme activity was increased with the addition of 50 μM gibberellic acid (GA_3) immediately prior to flower opening. Western blot analysis showed that purified F3',5'H protein from *G. scabra* was cross-reacted with rabbit anti-*Gentiana triflora* NADPH-Cyt P450 reductase antibodies. Only one 80 to 82 kDa protein was detected by western blot analysis of fresh microsomal extracts. The enzyme activity was not distinctly changed by adding 50 μM GA_3 to corollas. Therefore, we conclude that GA_3 controls the expression of the F3',5'H gene at the transcriptional level, not at the translational level.

Keywords: GA_3, *Gentiana scabra*, NADPH-Cyt P450 reductase, RT-PCR, western blot analysis

Flavonoids are secondary metabolites found in higher plants, which have numerous important functions. They serve as signaling molecules in plant-microbe interactions (Nam and Kang, 1995), provide pigmentation to attract pollinators, and act as phytoalexins (Dooner et al., 1991; Koes et al., 1993). Flavonoids are precursors of the anthocyanins, which confer colour (pink, red, mauve, or blue) to different plant parts, such as petals and seeds. The anthocyanin biosynthetic pathway has been well established (Martin and Gerats, 1992; Holton and Cornish, 1995).

Because flower or seed colour is easily observed, the anthocyanin biosynthetic pathway has been studied as a genetic model system, particularly in petunia, snapdragon, and maize (Holton et al., 1993). Blue and violet flowers generally contain derivatives of delphinidin, whereas red and pink flowers contain derivatives of cyanidin or pelargonidin. Differences in hydroxylation patterns of these three major classes of anthocyanidins are controlled by the cytochrome P450 enzymes: flavonoid 3'-hydroxylase (F3H) and flavonoid 3',5'-hydroxylase (F3',5'H) (Martin et al., 1991; Holton et al., 1993; Holton and Cornish 1995; Toguri et al., 1993; Menting et al., 1994b). F3',5'H leads to the formation of dihydromyricetin flavonol by hydroxylation of flavonones (Holton et al., 1993). Dihydromyricetin is an important intermediate flavonol that converts to delphinidin. In particular, hydroxyl-group incorporation at the 3'- and 5'- positions in the flavonoid B-ring gives rise to flavonoid, which, with further enzymatic modifications, forms delphinidin anthocyanidins (Holton et al., 1993). Theses key enzymes determine flower colour because they hydroxylate the B-ring of the dihydroflavonols. This hydroxylation eventually determines the structure of the anthocyanidin and, therefore, flower colour (Holton and Tanaka, 1994). *Gentiana scabra* produces mostly blue flowers, and is one of major cut flowers in Japan. The main anthocyanin in this species is gentiodelphin (Hosokawa et al., 1996).

During the development of petunia flowers, the anthers produce gibberellins (Barendse et al., 1970), which are transported into the corolla where they promote growth and pigmentation of the tissue. The role of GA_3 in the synthesis of anthocyanin has been studied in an in-vitro culture system. When young green corollas were detached and grown in a sucrose medium, GA_3 induced pigmentation and the expression of flavonoid genes, such as chalcone synthase, chalcone isomerase, and dihydroflavonol reductase (Weiss and Halevy, 1989; Weiss, 1990).

Genes encoding for enzymes of the anthocyanin biosynthetic pathway have been cloned and characterized from a variety of plant species. However, only a small number of genes are known to be regulated. Little is known about GA_3 as it relates to the molecular mechanisms of the F3',5'H gene. In this study we investigated GA_3-controlled expression of the F3',5'H gene in *G. scabra* corollas. Here, we also report the cloning of the F3',5'H partial cDNA, and the biochemical characterization of the F3',5'H enzyme from *G. scabra*.

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MATERIALS AND METHODS

Data Base Gene Bank Screening

Key-word searches in the F3',5'H data base Gene Bank at the NCBI web-server (http://www.ncbi.nih.gov) identified the flavonoid 3',5'-hydroxylase gene of *Petunia hybrida* and *Gentiana triflora* flowers.

Plant Materials

Fresh and pigmented corollas were collected from commercially available *G. scabra* cut flowers at different developmental stages (Fig. 1).

Reverse Transcription and PCR Cloning

Total RNA was extracted from frozen tissues by the rapid guanidine thiocyanate purification method, according to Turpen and Griffith (1986). The quality of the samples was evaluated by gel electrophoresis (Sambrook et al., 1989), and the RNA was stored at −70°C. Multiple transcript analysis with the first-strand cDNA synthesis kit for RT-PCR (AMV) was performed, according to kit protocol (Boehringer Mannheim) and a modified procedure by Nam et al. (1999). Total RNA (1 μg) was reverse-transcribed for 10 min at 25 (pre-annealing), then for 1 h at 42°C transcription by 20 units AMV reverse transcriptase (BM) in a 20-μL reaction mixture containing 1.6 μg oligo-$(dT)_{15}$ primer, 1X RT buffer (BM), 5 mM $MgCl_2$, 1 mM dNTP, and 50 units of RNase inhibitor. The RT mix was then heated (to 99°C) to inactivate all enzymes. An equal amount of cDNA was used for amplification in 100 μL of the corresponding buffer, which was complemented with 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 0.2 μM of each primer with 2.5 units of *Taq* polymerase. The the rmocycler program was at 94°C for 2 min, 35 cycles at 94°C for 30 s; annealing temperature of 60°C for 30 s, then 72°C for 90 s : 72°C for 5 min. Reaction products (10 μL) were analyzed by gel electrophoresis.

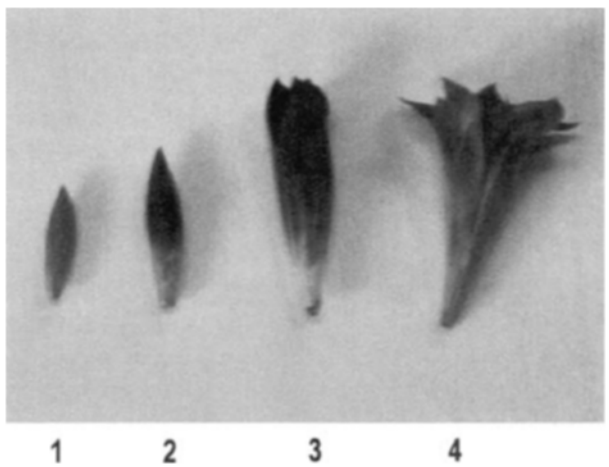

Figure 1. The four stages of flower development in *Gentiana scabra*. Stage 1: early flower (2.2 cm); Stage 2: complete, unopened flower (3 cm); Stage 3: prior to flower opening (3.8 cm); stage 4: fully open flowers (3.8 cm).

The primers, i.e., F3'5'H-1 (5'-ATGGTTTTTGCACATTATGGT-3') and F3'5'H-2 (5'-TCCTGCACAAATTCTTCGTCC-3'), were used to amplify about 0.9 Kb product from the cDNA. PCR fragments were cloned into a pGEM-T easy vector (Promega), according to the manufacturer's instructions, and sequenced by the Korean Basic Science Institute (Taejon).

Isolation of Solubilized Microsomal Proteins, Including F3',5'H

All steps were carried out on ice or at 4°C, except where stated otherwise. Flower bud limbs were ground with 1 mL of buffer A [(100 mM potassium phosphate, 0.1% (w/v) BSA, 0.1 μM pepstatin, 0.1 μM leupeptin, 0.1 mg mL^{-1} PMSF, 0.25 M sucrose, 0.25 M mannitol, 20 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM EGTA, 5 μM FMN, and 1 μM FAD; pH 7.5)] per gram of tissue (fresh weight), and containing 10 mg mL^{-1} polyclar AT (BDH, Poole, UK). The PMSF, a proteinase inhibitor, was added to the extraction medium before grinding, to avoid solubilization of the hydrophilic form of the NADPH-Cyt P450 reductase by endogenous proteases (Menting et al., 1994a, 1994b; Lee and Kim, 1995). Homogenization continued until the homogenate had a smooth consistency. The homogenate was centrifuged at 12,000*g* for 30 min (10°C), and the supernatant was then recentrifuged at 12,000*g* for 30 min (10°C).

The supernatant was filtered through Miracloth and centrifuged for 1 h at 105,000*g* (10°C). Pellets were then suspended in buffer B (100 mM MOPS-NaOH, 0.1% (w/v) BSA, 0.1 μM pepstatin A, 0.1 μM leupeptin, 20% (v/v) glycerol, 1 mM EDTA; pH 7.0) and stored at −70°C. Proteins, including the F3',5'H enzyme, were solubilized by adding 0.75 mg solid CHAPS per mg microsomal protein (final concentration 0.75% CHAPS). The mixture was incubated for 45 min, at 4°C, with stirring. After centrifugation at 105,000*g* for 1 h at 10°C, the supernatant was stored at −70°C.

Western Blotting and Immunodetection

Polyacrylamide-slab-gel electrophoresis was performed in the presence of 0.1% (w/v) SDS, with 12.5% (w/v) polyacrylamide for the separating gel and 5% (w/v) polyacrylamide for the stacking gel. The 100-µg samples of solubilized proteins, in 4x sample buffer [(10 mL of 0.5 M Tris-HCl (pH 6.8), 2 g SDS, 1.25 mL 2-mercaptoethanol 1.25, 10 mL 100% (v/v) glycerol, 3.75 mL dH_2O, and 0.3% amount bromophenol blue)], were boiled for 10 min before being loaded onto the gel. Electrophoresis was carried out at room temperature and electrotransferred on to a nitrocellulose membrane (Bio-Rad, 0.45 µm pore size) under ≤500 milliamp for 5 h. The remaining protein-binding sites on the nitrocellulose were then blocked by 4% (w/v) non-fat dry milk in a solution of PBS (10 mM sodium phosphate buffer, pH 7.4, 0.8% NaCl), with 0.05% (v/v) Tween 20. The anti-*G. triflora* antibodies (10 µg of IgG per 10 mL of PBS-TM) were reacted with the antigens overnight at room temperature, with gentle agitation. After careful washing, the nitrocellulose membrane was incubated with a goat anti-rabbit antibody alkaline phosphatase (Sigma) conjugate in PBS-TM at room temperature for 4 h, with gentle agitation. The secondary antibody was then washed off with PBS-T and stained at room temperature with 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.33 mg mL^{-1} nitroblue tetrazolium chloride (NBT), and 0.17 mg mL^{-1} 5-bromo-4-chloro-3-indoylphosphate (BCIP).

RESULTS AND DISCUSSION

Isolation of F3',5'H cDNA

To isolate F3',5'H cDNA from *G. scabra*, two nested pairs of degenerate oligonucleotide primers were designed and synthesized. These primers corresponded to identical amino acids in F3',5'H cDNAs from several plant species, and were used to amplify a 900-bp product from first-strand cDNA of *G. scabra*. PCR fragments were sequenced and compared with other F3',5'H genes. The *G. scabra* F3',5'H sequence obtained here (data not shown) shared conserved residues with the NADPH binding region of F3',5'H (Tanaka et al., 1996), and showed sequence homology to mammalian, yeast, and bacterial NADPH-Cyt P450 reductase enzymes. Comparison of the deduced amino acid sequence of the *G. scabra* F3',5'H gene to other known F3',5'H sequences revealed a high degree of identity (Table 1). Its affinity to *G. triflora* (Tanaka et al., 1996) was 92%. Affinities to the F3',5'Hs of *Eustoma russellianum* (lisianthus; Kikuchi et al., 1993), petunias *Hf1* and *Hf2* (Holton et al., 1993), *Solanum melongena* (egg plant; Toguri et al., 1993), and *Campanula medium* (Kikuchi et al., 1993) were 73%, 71%, 68%, 63%, and 63%, respectively. Because *Gentiana* and *Eustoma* belong to the same family, Gentianaceae, their affinities were higher. The identities to other species' Cytochrome P-450s were less than 40%. These results support the idea that the F3',5'Hs form a subsubset in the Cyt P-450 superfamily (Toguri et al., 1993).

Table 1. Comparison of the deduced amino acid sequence of *G. scabra* to known F3',5'H sequences in various plant species.

Plant	Identity (%)
Gentiana scabra	100
Gentiana triflora	92
Eustoma grandiflorum	73
Eustoma russellianum (Lisianthus)	71
Petunia hybrida Hf1	68
Petunia hybrida Hf2	63
Solanum melongena (Egg plant)	63

Developmental Gene Expression of Flavonoid 3',5'-Hydroxylase

The developmental process in *G. scabra* corollas

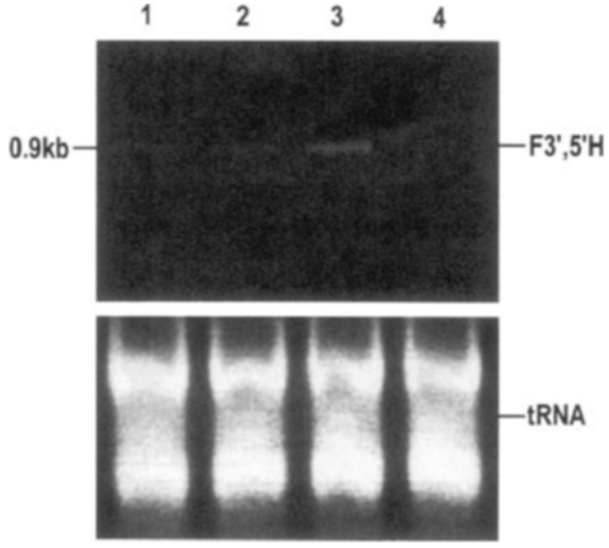

Figure 2. Expression analysis of the F3',5'H gene at four flower development stages by RT-PCR. Lane H: HindIII-digested λDNA as size marker; Lanes 1 to 4 correspond to Stages 1 to 4 in flower development. Ethidium bromide-stained total RNA bands are at the bottom.

was divided into 4 stages (Fig. 1). Because expression of the F3′,5′H gene was so low, it had to be confirmed through RT-PCR (Park, 1997). To define the relationship between F3′,5′H activity and development, four stages, from flower buds to fully open flowers, were analyzed (Fig. 2). F3′,5′H gene expression was observed in three stages of the corolla, from flower buds to partially open flowers (Stages 1, 2, and 3 in Fig. 2). The amount of transcript was greatest at Stage 3, just prior to flower opening. In mature, fully open flowers (Stage 4), the transcript was undetectable. The expression of the *G. scabra* F3′,5′H gene parallels that found in other plant and mammalian monooxygenase systems (Menting et al., 1994b).

We also compared the steady-state F3′,5′H mRNA levels of corollas that were in sucrose solution in the presence or absence of GA_3, a plant hormone. GA_3 plays an important role in the development of petunia flowers and regulation of anthocyanin pigment synthesis in corollas (Weiss and Halevy, 1989). An increased level of F3′,5′H mRNA transcript in corollas was found at all three concentrations: 10 μM, 50 μM, and 100 μM of GA_3 hormone solution, whereas a low level of transcript was detected in the GA_3 hormone-free solution (Fig. 3). F3′,5′H gene activity was greatest in the 50-μM GA_3 solution. This indicates that the accumulation of F3′,5′H mRNA in the corollas may be controlled by gibberellin.

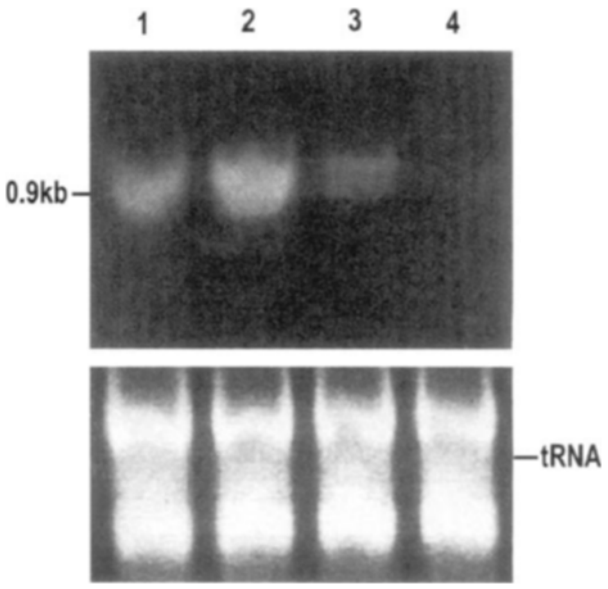

Figure 3. Regulation of F3′,5′H by GA_3 treatment during flower development. Total RNA was isolated from corolla tissues at Stage 3 (just prior to flower opening), which were grown in a 0.15 M sucrose medium for 24 h with GA_3 concentrations of 10 μM (lane 1), 50 μM (lane 2), 100 μM (lane 3), and without GA_3 (lane 4). Expression level was determined by RT-PCR. Ethidium bromide-stained total RNA bands are at the bottom.

Whereas sucrose had a general metabolic effect on gene expression by acting as an energy source, the stimulatory role of GA_3 was specific. The promoting effect of GA_3 on accumulation of the F3′,5′H gene could have been caused by an increased transcription rate or because of increased stability of the transcript. These results parallel the expression level of other anthocyanin genes, flavanone 3-hydroxylase, chalcone synthase, and chalcone isomerase with GA_3 (Weiss et al., 1990, 1992, 1993). Gibberellin promoted pigmentation and the accumulation of F3′,5′H mRNA in the detached corollas. This shows that gibberellin may be necessary for the continuous expression of the gene during the early stages of corolla development. Here, we provide evidence that gibberellin is required for the regulation of F3′,5′H-gene transcription.

Immunological Analysis of the F3′,5′H Enzyme

Two polyclonal antibodies were produced for experimental use by probing western blots of purified antigen from *G. triflora* (Kim and Kim, 1999). The antibodies differed, with one showing strong response on western blots of purified antigen, the other having a weak response.

To determine whether the stimulatory effect of gibberellin was also at the translational level, we preformed immunological assays with isolated F3′,5′H enzymes. The F3′,5′H enzyme levels of corollas in sucrose solutions were compared in the presence or absence of GA_3. We isolated the soluble microsomal proteins, including F3′,5′H, from 5 g of corollas just prior to flower opening, at four different concentrations of GA_3. Western blot analysis was performed as shown in Figure 4. The *G. triflora* antibody cross-reacted with *G. scabra* F3′,5′H, thereby indicating that the enzyme has epitopic homology to *G. triflora* NADPH-Cyt P450 reductase. Only one 80 to 82-kDa protein was detected in fresh microsomal extracts. The enzymes were found at all four GA_3 concentrations (0 μM, 10 μM, 50 μM, and 100 μM) in 0.15 M sucrose solutions (Fig. 4). Although enzymatic activity was greatest with 50 μM GA_3 in corollas, the enzyme level was not distinctly different, regardless of the condition of the corollas, compared with that of the previously mentioned mRNA activities. F3′,5′H gene expression, therefore, may be not regulated at the translational level. However, expression of the F3′,5′H gene is regulated by gibberellin, as

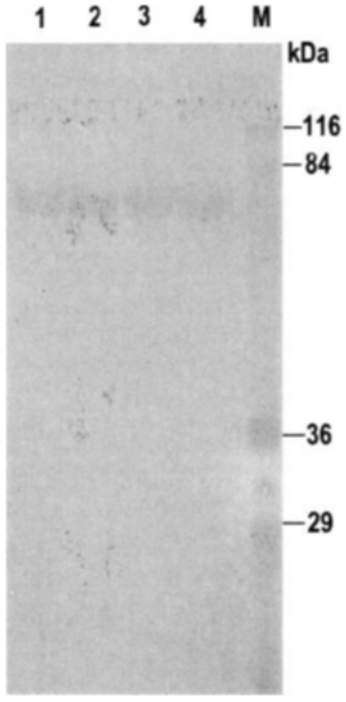

Figure 4. Western blot analysis of the F3',5'H enzyme during flower development in *G. scabra*. The soluble microsomal proteins, including F3',5'H protein, were extracted from corolla tissues (stage 3), and grown in a 0.15 M sucrose medium for 24 h, without (lane 1) or with GA_3: 10 μM (lane 2); 50 μM (lane 3); 100 μM (lane 4). Lane M: Standard protein molecular marker (Bio-Rad). The soluble microsomal proteins, including F3',5'H, were isolated from 5 g of corollas at the stage just prior to flower opening, and at four concentrations of GA_3. One hundred micrograms of total protein in a 75 μL volume were loaded onto each lane.

are other genes of the flavonoid pathway, including chalcone synthase, chalcone isomerase, and dehydroflavonol (Weiss and Harvey,1989; Weiss et al., 1992). This may indicate that the hormone regulates all flavonoid genes in the corolla in a similar manner, which would be consistent with the coordinated expression of those genes during corolla development. In the case of the development and pigmentation of *G. scabra* flowers, GA_3 may induce the synthesis of a trans-regulatory protein or a receptor that plays a direct role in the induction and prolonged transcription of the flavonoid genes. It should now be possible to further dissect the regulatory pathway that leads to gene expression during the addition of GA_3, and to biochemically characterize the proteins involved using the in-vitro system. We conclude, therefore, that gibberellin controls the expression of F3',5'H at the transcription level, rather than at the translational level.

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LITERATURE CITED

Barendse GWM, Pereira RAS, Barkers PA, Driessen FM, van Eyden EA, Linskens HF (1970) Growth hormones in pollen, styles and ovaries of *Petunia hybrida* and *Lilium* species. Acta Bot Neerl 19: 175-186

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25: 173-199

Holton TA, Brugilera F, Leste DR, Tanaka Y, Hyland CD, John GTM, Chin-Yi Lu, Farcy E, Stevenson TW, Cornish EC (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366: 276-279

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell 7: 1071-1083

Holton TA, Tanaka Y (1994) Blue roses - A pigment of our imagination? Trends Biotechnol 12: 41-42

Hosokawa K, Fukushi E, Kawabata J, Fujii C, Ito T, Yamamura S (1996) Studies on flower pigments in *Gentiana sp.* III. Identification of anthocyanin in blue cultivar Albireo. Ikushugakkaishi 45 supp 1: 196

Lee D-H, Kim Y-S (1995) Purification and Characterization of Alkaline Invertase from Hypocotyls of Mung Bean (*Phaseolus radiatus* L.). J Plant Biol 38: 349-358

Kikuchi Y, Kiyokawa S, Shimada Y, Shimada R, Ohbayashi M, Okinaka Y (1993) Novel plant gene. International patent publication number WO 93/18155

Kim MJ, Kim Y (1999) Purification and characterization of NADPH-Cytochrome P450 reductase from *Gentiana triflora* flower. Mol Cells 9: 470-475

Koes RE, Quattrocchio F, Mol JNM (1993) The flavonoid biosynthetic pathway in plant: function and evolution. BioEssays 16: 123-132

Martin C, Gerats T (1992) The control of flower coloration, *In* B Jordan, ed, Molecular Biology of Flowering, CIB international, Wallingford, pp 219-255

Martin C, Prescott A, Machay S, Bartlett J, Vrijklandt E (1991) The control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J 1: 185-197

Menting JGT, Cornish E, Scopes RK (1994a) Purification and partial characterization of NADPH-cytochrome c reductase from *Petunia hybrida* flowers. Plant Physiol 106: 643-650

Menting JGT, Scopes RK, Stevenson TW (1994b) Characterization of flavonoid 3',5'-hydroxylase in microsomal

membrane of *Petunia hybrida* flowers. Plant Physiol 106: 633-642

Nam J, Lee KY, Oak JH, An G, Lee IK (1999) Identification of MADS genes from a brown alga, *Sargassum fulvellum*. J Plant Biol 42: 81-84

Nam MH, Kang BG (1995) Impairment of Polar Auxin Transport by Protein Kinase Inhibitors in Etiolated Pea Seedlings. J Plant Biol 38: 343-348

Park SY (1997) Molecular characterization and identification of anthocyanin biosynthetic genes from higher plants. Master's thesis. Sangmyung University, Chonan

Sambrook K, Fritsch EF, Maniatis T (1989) Molecular cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Tanaka Y, Yonekura K, Fukuchi-Mizutani M, Fukui Y, Fujiwara H, Ashikari T, Kusumi T (1996) Molecular and biochemical characterization of three anthocyanin synthetic enzymes from *Gentiana triflora*. Plant Cell Physiol 37: 711-716

Toguri T, Umemoto N, Kobayashi O, Ohtani T (1993) Activation of anthocyanin synthesis genes by white light in eggplant hypocotyl tissues, and identification of an inducible P-450 cDNA. Plant Mol Biol 23: 933-946

Turpen TH, Griffith OM (1986) Rapid isolation of RNA by a guanidine thiocyanate/cesium chloride gradient method. Bio Techniques 4: 11-15

Weiss D (1990) Regulation of corolla pigmentation and development in petunia flower. Ph.D. thesis. The Hebrew University of Jerusalem, Israel

Weiss D, Halevy AH (1989) Stamens and gibberellin in the regulation of corolla pigmentation and growth in *Petunia hybrida*. Planta 179: 89-96

Weiss D, van Blockland R, Kooter JM, Mol JNM, van Tunen AJ (1992) Gibberellic acid regulates chalcone synthase gene transcription in the corolla of *Petunia hybrida*. Plant Physiol 98: 191-197

Weiss D, van der Luit, Kroon JTM, Mol JNM, Kooter JM (1993) The petunia homologue of the *Antirrhinum majus candi* and *Zea mays A2* flavonoid genes; homology to flavanone 3-hydroxylase and ethylene-forming enzyme. Plant Mol Biol 22: 893-897

Weiss D, van Tunen AJ, Halevy AH, Mol JNM, Gerats AGM (1990) Stamens and gibberellic acid in the regulation of flavonoid gene expression in the corolla of *Petunia hybrida*. Plant Physiol 94: 511-515