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エキナセアの花色生合成に関する生化学・分子生物学解析

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Study on flower coloration in echinacea

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Chapter 3

Insertion of *ANS* gene caused the formation of white-flowered mutation in *Echinacea purpurea*

Chapter 1

General introduction

Flower color is one of the most important characteristics of ornamental plants, which is affected by the different kinds of plant pigments, such as flavonoids, carotenoids and betalains (Tanaka *et al*., 2008; Tripathi *et al*., 2018). Flavonoids are plant secondary metabolites. There are more than 8,000 derivatives (Veitch and Grayer, 2011), which function in roles like ultraviolet protection and pigmentation in plants (Shirley, 1996; Fini *et al*., 2011). The most widespread group of flavonoids is the anthocyanins, which is tightly linked to flower development (Weiss et al., 2000) and responsible for most of the red, pink, purple, and blue colors in plants (Field *et al*., 2001; Honda and Saito, 2002; Miyagawa *et al*.,2015)

Anthocyanins are synthesized along with flavonoid biosynthesis through a series of enzymatic reactions that convert chalcone into three major anthocyanidin types: cyanidin (red to magenta), pelargonidin (brick red to scarlet) and delphinidin (purple to violet) (Tanaka *et al*., 2008). Structural genes are the key control for the biosynthesis process. Anthocyanin biosynthesis requires enzymes (Fig1.1) including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoid glycosyltransferase. Additionally, flavone synthase (FNS), and flavonol synthase (FLS), are used to synthesize flavone and flavonol (Holton and Cornish, 1995; Martens and Mithöfer, 2005).

Durbin *et al*. (2003) showed that color phenotypes in plants were closely associated with changes in the regulation of gene expression. For example due to the lack of F3′5′H, rose, carnation, chrysanthemum, lily, and many other plant species are short of blue flowers.(Katsumoto *et al*., 2007; Azadi *et al*., 2016; Xue *et al*., 2016; Noda *et al*., 2017).The mutations in *f3'h*, *dfr* or *ans* caused an accumulation of colorless flavonoids in the white petals of *A. majus* (Martin *et al*., 1985; 1991), *Pharbitis nil*

(Saito *et al*., 1994 ; Hoshino *et al*., 1997), *Dianthus caryophyllus* (Stich *et al*., 1992; Mato *et al*., 2000) and *Eustoma grandiflorum* (Davies *et al*., 1993). The DFR and ANS enzymes were late in the anthocyanin biosynthesis pathway. Whittal *et al* (2006) observed that the co-regulation of the *DFR* and *ANS* genes eliminated floral pigmentation in *Aquilegia*.

Echinacea purpurea belongs to the genus *Echinacea* (Asteracea). The genus *Echinacea* comprises a small number of species that are hardy, herbaceous perennial plants, native to parts of North America (Miller and Yu, 2004). *E. purpurea* has been developed various drugs to cure many diseases. In Yorii-town of Japan, *E. purpurea* has also been cultivated and developed many new products. For example, it was used as a raw material for herbal tea and had a high immune effect. Recently, *E. purpurea* is also widely used for garden landscape, cut flowers and potted plants based on its unique flower shape and outstanding flower color, and is one of the most important commercial flower in the floricultural industry. A wide array of coneflower colors can be produced by selection-based breeding. 'Virgin', 'Green Jewel', 'Hot papaya' and 'Orange passion' etc. have been planted in Japan (Fig. 1.2). But no ‘special species for Japan. Therefore, I want to cultivate new species with novel flower color and higher nutritional value.

Flower color has been regarded as an important character in deciding the horticultural value. Enriching flower colors is one of the long-term goals of flower breeders, hence, getting information of *E. purpurea* pigment components, formation mechanism of flower color and molecular basis underlying the color change are important for carrying out molecular breeding programs towards flower colors, and for intensive study, exploitation, and utilization of *Echinacea* in the future. In this study, *E. purpurea* and 'Virgin' were investigated for their pigment components and molecular basis underlying their color change. To the best of our knowledge, this is the first report documenting molecular analysis of flower color mutations in *Echinacea*.

References

Azadi P, Bagheri H, Nalousi AM, Nazari F, Chandler SF. Current status and biotechnological advances in genetic engineering of ornamental plants. Biotechnol. Adv.,34:1073–1090,2016.

Davies KM, Bradley JM, Schwinn KE, Markham KR, Podivinsky E. Flavonoid biosynthesis in flower petals of five lines of lisianthus (Eustoma grandiflorum Grise.). Plant Sci., 95:67–77, 1993.

Durbin ML, Lundy KE, Morrell PL, Torres-Martinez CL, Clegg MT.Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. Mol. Phylogenetics and Evol., 29(3):507–518, 2003.

Field TS, Lee DW, Holbrook NM.Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol., 127: 566–574, 2001.

Fini A, Brunetti C, Di Ferdinando M, Ferrini F, TattiniM. Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. Plant Signal Behav., 6: 709–711, 2011.

Holton, T. A., Cornish, E. C. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell, 7: 1071–1083, 1995.

Honda T, Saito N. Recent progress in the chemistry of polyacylated antho-cyanins as flower color pigment. Heterocycles, 56: 633–692, 2002.

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, Tao GQ, Nehra NS, Lu CY, Dyson BK, Tsuda S, Ashikari T, Kusumi T, Mason JG, Tanaka Y.Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol., 48: 1589–1600, 2007.

Martens, S, Mithöfer, A. Flavones and flavone synthases. Phytochemistry. 66: 2399–2407, 2005.

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES. Molecular analysis of

instability in flower pigmentation of Antirrhinum majus, following isolation of the pallida locus by transposon tagging. EMBO J., 4: 1625–1630, 1985.

Martin C, Gerats T. The control of flower coloration, in: BR Jordan (Ed.). The Molecular Biology of Flowering. CAB International,Wallingford Oxford. 219–255, 1991.

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E..Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J., 1: 37–49,1 991.

Mato M, Onozaki T, Ozeki Y, Higeta D, Itoh Y, Yoshimoto Y, Ikeda H, Yoshida H, Shibata M. Flavonoid biosynthesis in white flowered Sim carnation (*Dianthus caryophyllus*). Sci. Hort., 84: 333–347, 2000.

Miyagawa N, Miyahara T, Okamoto M, Hirose Y, Sakaguchi K, Hatano S, Ozeki Y. Dihydroflavonol 4-reductase activity is associated with the intensity of flower colors in delphinium. Plant Biotechnol., 32: 249–255, 2015.

Miller SC, Yu HC. Echinacea: the Genus *Echinacea*, CRC Press, Washington D.C., 2004.

Noda N, Yoshioka S, Kishimoto S, Nakayama M, Douzono M, Tanaka Y, Aida R. Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism. Sci. Adv., 3: e1602785, 2017.

Saito N, Cheng J, Ichimura M, Yokoi M, Abe Y, Honda T. Flavonoid in the acyanic flowers of *Pharbitis nil*. Phytochemistry. 35: 687–691, 1994.

Shirley BW. Flavonoid biosynthesis: ‘new’ functions for an ‘old’ pathway. Trends Plant Sci., 1: 377–382, 1996.

Tanaka Y, Sasaki N, Ohmiya A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J., 54: 733–49, 2008.

Tripathi AM, Niranjan A, Roy S. Global gene expression and pigment analysis of two contrasting flower color cultivars of Canna. Plant Physiol. Biochem., 127: 1–10, 2018.

Veitch NC, Grayer RJ. Flavonoids and their glycosides, including anthocyanins. Nat. Prod. Rep., 21: 539–573, 2004.

Weiss D. Regulation of flower pigmentation and growth: multiple signaling pathways

control anthocyanin synthesis in expanding petals. Physiologia Plantarum. 110: 152–157, 2000.

Whittall JB, Voelckel C, Kliebenstein DJ, Hodges SA. Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in Aquilegia. Mol. Ecol., 15: 4645–4657, 2006.

Xue L, Wang ZG, Zhang W, Li YX, Wang J, Lei JJ. Flower pigment inheritance and anthocyanin characterization of hybrids from pink-flowered and white-flowered strawberry. Sci. Hortic., 200: 143–150, 2016.

Table and Figure

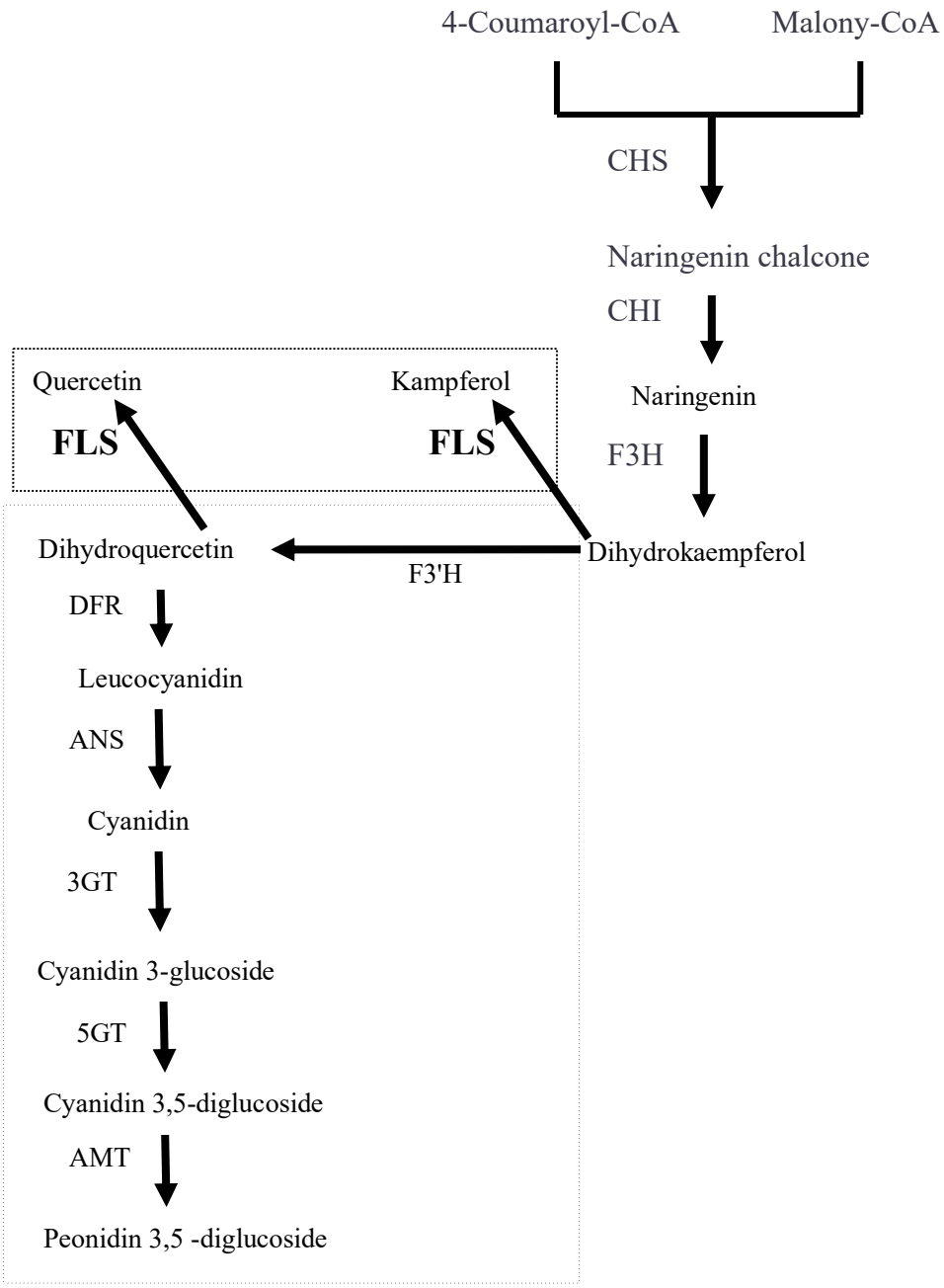


Fig. 1.1. Illustration of the flavonoid biosynthetic pathway in *E. purpurea*. Two pathways were enclosed in a dashed square. CHS, chalcone synthase; CHI, chalcone isomerase; 2′GT, chalcone 2′-glucosyltransferase; F3H, flavone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydro-avonol 4-reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; 5GT, anthocyanin 5-glucosyltransferase; AMT, anthocyanin methyltransferase

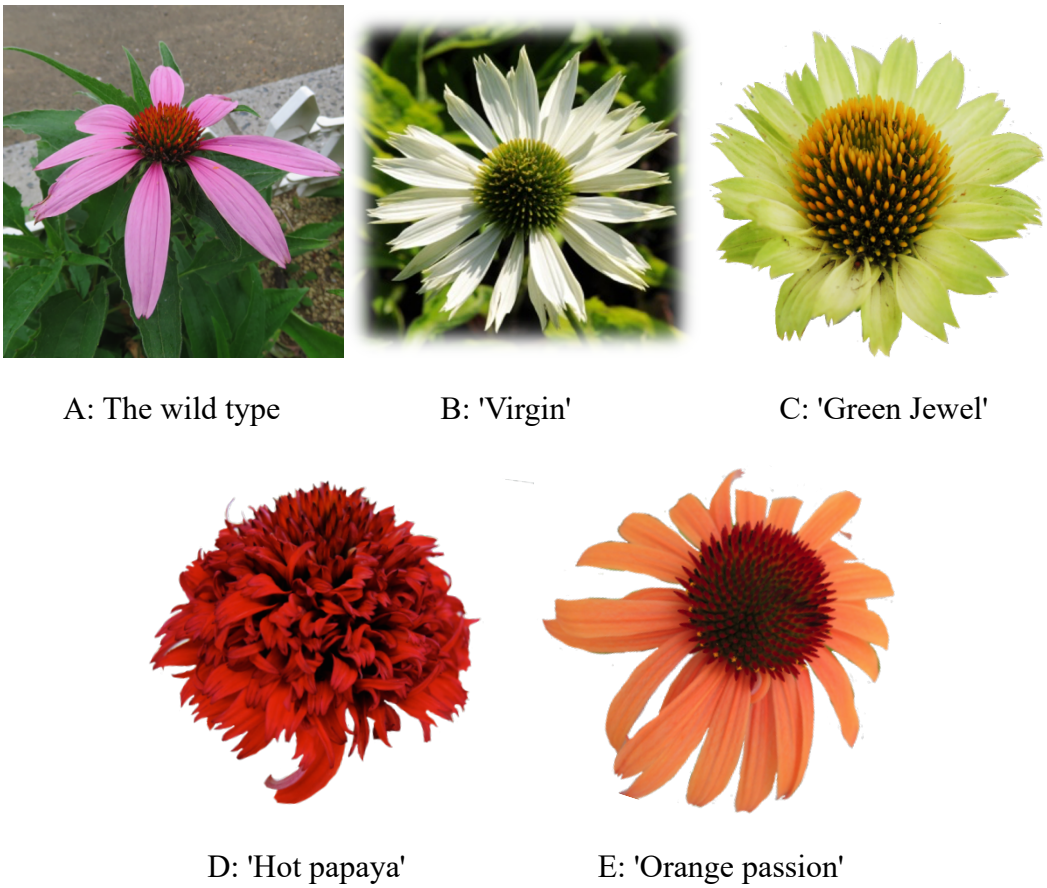


Fig. 1.2 *E. purpurea* varieties grown in Japan.

A: *E. purpurea* (Wild type), B: 'Virgin', C: 'Green Jewel', D: 'Hot papaya', E: 'Orange passion'

Chapter 2

Molecular cloning and characterization of a flavonoid 3'-hydroxylase gene from *Echinacea purpurea*

Abstract: *Echinacea purpurea* (*E. purpurea*) is one of the most important medicinal and ornamental plants and is widely grown as flowering potted or garden plants in many countries. However, variation in flower color is somewhat limited in the cultivars, and therefore understanding the genetic networks of flower coloration in *E. purpurea* is required. Anthocyanin is responsible for producing many floral colors in the visible spectrum. To broad our understanding of the biosynthetic pathway of anthocyanin, in this study, I firstly isolated the complete open reading frames (ORF) of *flavonoid 3'-hydroxylase* (*F3'H)* gene of *E. purpurea* and named *EpF3'H*. Expression patterns in different organs of *E. purpurea* were also determined. The results strongly suggest that the *EpF3'H* genes participate in anthocyanin synthesis. This would lay a foundation for understanding the anthocyanin biosynthesis pathways in *E. purpurea*.

2.1 Introduction:

E. purpurea (Fig. 1.2) is one of the most important and well-known medicinal plants in the world, belonging to the Asteraceae family. It is native to eastern North America and popularly called purple cone flower and eastern purple cone flower. For their showy flowers, many species are cultivated in gardens and become ornamental plants. At present, the demand for *E. purpurea* is greater than the wild supply, so it is widely cultivated all over the world, including Japan. In the past, *Echinacea* has been used against infectious diseases and snakebite (Miller et al., 2004 ; Barrett, 2003). Nowadays, It is believed to have immune system stimulating properties (Di Carlo *et al*., 2003), anti-microbial (Binns *et al*., 2002; Merali *et al*., 2003; Stojicevic *et al*.,2009), anti-inflammatory (Zili *et al*., 2009, Sharma *et al*., 2010, Vazirian *et al*.,2014) and anti-oxidant properties (Manayi *et al.,*2012, 2013; Tsai *et al*.,2012). Various compounds belonging to different classes of secondary metabolites have been isolated and identified in *E. purpurea* extract. Flavonoids (Barnes *et al*., 2005; Bohlmann *et al*., 1983),

alkamides (Barnes *et al*., 2005), caffeic acid derivatives (Tsai *et al*., 2012), polysaccharides (Classen *et al*., 2000), alkaloids, and amides are believed to be biologically and pharmacologically active. Molecular genetic studies of *E. purpurea* have tended to focus on its genetic diversity (Kapteyn *et al*., 2002; Aziz and Sauve, 2008). However, few have examined the anthocyanin-related genes.

Flower color is one of the most outstanding, important traits of flower. Variation of flower coloration is mainly derived from the end-products of secondary metabolism, particularly flavonoids. The most abundant and predominant flavonoid pigments are the anthocyanins, which produce a wide range of colors, such as orange, pink, red, magenta, purple, violet, and blue (Schwinn and Davies, 2004). Modification of different aglycones, such as hydroxylation, glycosylation, methylation and acylation, leads to a wide variety of anthocyanins. These modifications are considered involved in creating all kinds of flower colors. Among these modifcations, a related P450s, F3'H catalyse hydroxylation of the B-ring is a common process in plant secondary metabolism. F3'H has broad substrate specificity and catalyse hydroxylation of flavanones, dihydroflavonols, flavonols and flavones. Because flavanones and dihydroflavonols are precursors of anthocyanins, the enzyme determine the hydroxylation pattern of the B-ring and thus flower color, correspondingly. Presence of F3 'H leads to 3'- position hydroxylated cyanidin-based anthocyanins that tend to have red color. (Ayabe and Akashi, 2006; Tanaka, 2006). The *F3'H* gene was first isolated and characterized from *petunia* (*Petunia hybrida*) by Brugliera *et al*., 1999. And subsequently have been isolated from many plant species such as *Perilla frutescens* (Kitada *et al*.,2001), *Torenia* (Ueyama *et al*., 2002), *Asteraceae* (Seitz *et al*., 2006), and *Brassica napus* (Xu *et al*., 2007). Its biochemical functions are usually identified by measuring the color changes in plants. Such as in soybean a single-base deletion in the *F3'H* gene resulted in deletion of the heme-binding domain of the *F3'H* and color change of pubescence color from brown to grey (Toda et al. 2002). A *f3'h* mutant would result in the production of pelargonidin-based anthocyanin (Hoshino *et al*., 2003). These studies suggested that some F3 'Hs were responsible for hydroxylation of anthocyanin.

In the study, I successfully isolated the *F3'H* cDNA from the wild type flowers of *E.*

purpurea (Fig. 1.2). This is the first report of molecular basis underlying the color in *E. pururea*, and the results will not only provide new insights into the flavonoid biosynthesis in dicot plants but also contribute to carry out molecular breeding programs towards flower colors, and for intensive study, exploitation, and utilization of *E. pururea* in the future.

2.2 Materials and methods

2.2.1 Plant materials

The wild type and Virgin' of *E. purpurea* were purchased from Sakata Seed Co. (Japan) and cultivated outdoor. Petals were collected from flowers at 4 different stages (Fig. 2.1): S1-petal is green, S2-the upper petal is green, the lower part is grey reddish-purple, S3-petal is grey reddish-purple, S4-petal is reddish-purple. I used at least three pots for biological repeats. All samples were frozen in liquid nitrogen upon collection and stored at −80 ℃ prior to RNA extraction.

2.2.2 Cloning of the full-length cDNA of EpF3'H

Total RNA was extracted from petals of developmental stage (S1 - S4), and leaves of *E. purpurea*. 2 μg RNA was reverse-transcribed in a total volume of 20 μL using the M-MLV reverse transcriptase and treated with DNase I (Takara, Japan) according to the manufacturer's instructions. To amplify full-length cDNA fragments, 3'-rapid amplification of cDNA ends (3'RACE) and 5'RACE was performed using the 3'-full RACE core set and the 5'-full RACE core set (Roche, Germany), respectively. Primers used for isolation of cDNA clones are listed in Table 2.1.

A full-length cDNA sequence of *F3'H* gene in *E. purpurea* was deduced by assembling the products of the 3'RACE, 5'RACE and named *EpF3'H*. A pair of primers was designed in Table1, and PCR amplification was performed using the cDNA of *E. purpurea* as the template. The PCR amplification procedure was as follows: predenature at 95 °C for 5 min, then 95°C 30 s, 60 °C 30 s, 72 °C 90 s, for 30 cycles, finally

extended at 72 °C for 5min. The PCR product was separated by 2% agarose gel electrophoresis, isolated by NucleoSpin® Gel and PCR Clean-up (TaKaRa, Japan), then cloned into the JM109 vector and sequenced. DNA sequencing was performed using the automated sequencer (ABI PRISM® 3700 Genetic Analyzer; Applied Biosystems) according to the manufacturer's protocol.

2.2.3 Phylogenetic analysis

Phylogenetic trees were constructed using Genetyx-ver12 software with the neighbor-joining method based on ClustalW multiple alignments (Saitou and Nei 1987). Accession numbers of genes used for the phylogenetic analysis are listed in Fig. 2.2. The reliability of the trees was evaluated using the bootstrap resampling method (Felsenstein 1985) with 1,000 replicates.

2.2.4 Comparative and bioinformatic analysis

Comparative and bioinformatic analysis of *EpF3'H* were performed online using the following websites: http://web.expasy.org/protparam/; and http://smart.embl-heidelberg.de/.

2.2.5 Expression profile analyses

Real-time quantitative PCR was carried out to investigate the expression profiles of *EpF3'H* in leaves and different stages of petals in *E. pururea*, using the SYBR green method in QuantStudio™ 1 Real-Time PCR System (Thermo Fisher Scientific, USA). Each PCR reaction (20 μL) contained the following components: 12 nM primers, 1μl cDNA, 1 X PowerUPSYBR® Green PCR Master Mix (Agilent Technologies, USA). The real-time quantitative PCR is divided into three stages, the first is hot stage: 50 ℃ 2 min, 95 ℃ 10 min. The second is PCR stage: 95 ℃ 15 s, 62 ℃ 1 min 40 Cycles. The last is the melting curve analysis: 95 ℃ 15 s, 60 ℃ 1 min, 95 ℃ 1s. The primers were showed in Table 2.1. The amplified fragment of *EpF3'H* was about 600 bp. Quantitation of the gene expression was done with comparative Ct method (Bogs *et al*., 2006; Muller,

et al., 2002). *Actin* gene (*EpACT*1) was used as the reference gene for comparison of expression level. The assay was repeated four times.

2.3 Results

2.3.1. Isolation of the Full-Length cDNA of EpF3'H *Gene*

Isolation of the full-Length cDNA of *EpF3'H* Gene Based on the alignment of the F3'Hs sequences from the other plants. Using the conserved sequence of amino acids, degenerate PCR was performed. A pair of primers EpF3'H-FP1 and EpF3'H-RP1 was designed (Table 2.1) to amplify a core fragment of the *F3'H* gene. This fragment was used to design the primers for amplifying the 3 ' and 5 'ends of cDNA by 3'-RACE and 5'-RACE.Thus, an integrity cDNA sequence of *EpF3'H* was obtained (Fig. 2.2). It contains an ORF of 1,533 bp, which encodes 510 amino acid residues. It is predicted that the molecular weight of the protein is 56.49 kD and the pI is 8.46.

2.3.2. Homology and structural characters of EpF3'H

A phylogenetic tree of F3'Hs (Fig. 2.3a) revealed that EpF3'H and the F3'Hs of *Gerbera* hybrid cultivar, *Chrysanthemum morifolium* , clustered together and formed a subgroup. And separated from other groups, including *Tulipa fosteriana*, and *Vitis vinifera*. Protein multiple sequence alignment analysis (Fig. 2.3b) showed a high homology of *EpF3'H* to *Gerbera* hybrid cultivar (DQ218417), *Chrysanthemum morifolium* (AB523844). The similarities were 83.52%, 82.67% respectively. These high identities and the dendrogram suggested that the *EpF3'H* protein have close phylogenetic relationships with the composite family and imply that *EpF3'H* is a member of the F3'H family and might have the same catalytic function as the other F3'Hs.

EpF3'H has four cytochrome P450-specific conserved motifs and three F3'H-specific conserved motifs (Fig. 2.4) according to NCBI conserved domain search (Marchler-Bauer and Bryant, 2004). $P_{32}PGPTPWP_{39}$ was the proline-rich "hinge"

region, which is essential to optimal orientation and activity of the enzyme (Yamazaki *et al*., 1993; Murakami *et al*.,1994). F_{437}GAGRRICVG$_{446}$ is the heme domain and necessary for heme iron binding. The binding pocket for the oxygen molecule motif A_{301}GTDTS$_{306}$ is necessary for catalytic activity (Chapple, 1998). A folding motif (an E_{358}-R_{361}-R_{400} triad) is the pocket locking motif acts and can lock the Cys-pocket in the right position (Hasemann *et al*.,1995). V_{75}VVASS$_{80}$, G_{419}GEK$_{422}$ and V_{425}DVKG$_{429}$ of EpF3'H were three F3'H-specific conserved motifs and similar to many other species (Brugliera *et al*., 1999; Xu et al.,2007). The presence of all these F3'H-specific conserved motifs indicated that *EpF3'H* is a typical F3'H protein.

2.3.3 Comparison of EpF3'H sequences

Alignment of deduced amino acid sequences of F3'H proteins in *E. purpurea* and 'Virgin' showed that the two sequences shared 100% identities.

2.3.4 Gene expression of EpF3'H

Real-time quantitative PCR analysis (Fig. 2.5) showed that *EpF3'H* were both detected in the leaves and flowers in the wild type and 'Virgin' with different levels of expression. *EpF3'H* was most abundantly expressed in both S4, followed by, S2, S1, S3 and the leaf in the wild type, followed by, S3, S1 and S2 in 'Virgin'. The expression pattern of *EpF3'H* was approximately corresponds to the anthocyanin accumulation pattern in the wild type and 'Virgin'.

2.4 Discussion

In the wild type of *E. purpurea*, two anthocyanin derivatives Cyanidin 3-glucoside (Cy3G), and Cyanidin 3-malonyl glucoside (Cy3MalG) were reported to be the main pigmentation component (Cheminat *et al*., 1989). The difference between Cyanidin- and other derived anthocyanins is due to the different number of substituents on the B ring of the flavonoid backbone, and their color characteristics are affected. Hydroxylation is the first step of cyanidin biosynthesis in *E. purpurea*. F3'H can introduce hydroxyl

groups at the 3'- position and catalyzes dihydrokaempferol to dihydroquercetin. Therefore, identification of a functional *F3'H* gene is an important step to understand anthocyanin hydroxylation and flower coloration in *E. purpurea*.

In this study, I sought to identify a F3'H homologue that function in anthocyanin hydroxylation and flower coloration. A *F3'H* genes were isolated by degenerate PCR from mRNAs in petals of *E. purpurea* and analyzed its nucleotide sequence. The *EpF3'H* gene was found to be a 1,533 bp long ORF encoding 510 amino acid protein. Putative molecular mass is 56.49 kDa. Phylogenetic analysis of F3'H amino acid sequences showed that the EpF3'H isolated in this study clustered with the plants of the composite family of *Gerbera* hybrid cultivar, *Chrysanthemum morifolium* in taxonomic system. Nucleotide BLAST-n revealed that the cloned cDNA sequence and the deduced protein of *EpF3'H* were showed high identity to F3'Hs from other plant species via multialignments.

F3'H is cytochrome P450-dependent mono-oxygenases that require NADPH as a co-factor (Tanaka and Brugliera, 2013). Like other members of the super family, *EpF3'H* contains four cytochrome P450-specific conserved motifs and three F3'H-specific conserved motifs. Some studies of F3'Hs from several plants have demonstrated that F3'H compete for substrate recruitment and hydroxylate 3' position of dihydroflavonols to synthesise the precursors of reddish-purple pigments (Tanaka *et al*., 2009; Tanaka and Brugliera, 2013). The sequence similarities between EpF3'H and the other F3'Hs imply close functions.

Real-time quantitative PCR analysis showed that the expression of *EpF3'H* were detected in petals and leaves. Chen et al. (2011) also reported that *CgF3'H* expressed in all organs of *Chrysanthemum*. The expression level of *EpF3'H* was higher in the pigmented petals than in lightly pigmented petals. This was similar to the expression levels of *LvF3'H* in the lily cultivar 'Vivian' which were higher in the pigmented petals than in un-pigmented petals (Yin *et al*., 2020). The expression of *EpF3'H* was about consistent with the formation and accumulation trend of anthocyanin during flower development. This pattern may synergistically promote the synthesis and accumulation of anthocyanins in *E. purpurea* petals. However, *EpF3'H* expression in S3 did not

appear to be associated with anthocyanin accumulation. The possible explanation for this anomaly could be *EpF3'H* was probably regulated by post-transcriptional regulation, such as its activation was inhibited, or like CHS in the *petunia* (Saito et al., 2006) having different splicing forms. It may be due to the other flavonoid biosynthesis genes, such as *DFR* and *ANS* are not abundantly expressed in S3. Or *EpF3'H* has function(s) other than anthocyanin biosynthesis that remains to be explored. For instance, F3'H is supposed to be involved not only in anthocyanin biosynthesis but also in flavone biosynthesis (Kitada *et al*., 2001).

EpF3'H was expressed lower in leaf than in flower. In contrast, Huang et al. (2007) found that the expression of *F3'H* was lower in the petals of crofton weed than in leaves, and the other studies also found high expression of F3'H in leaves (Schoenbohm *et al*., 2000; Kitada *et al*., 2001; Schlangen *et al*., 2010). The results showed that the *F3'H* gene has different temporal and spatial expression patterns in different varieties, tissues and growth stages (Brugliera *et al*.,1999; Huang *et al*., 2007). Their tissue-specific accumulation maybe controlled by the operation of regulatory genes, such as the most significant transcription factors, bHLH, MYB, and WD40, which can activate the operation of structural genes for the biosynthesis of anthocyanins (Gonzalez *et al*., 2008). To test this would require analysis of the effect of these transcription factors on hydroxylation activity toward anthocyanins, using methods such as overexpression, RNAi constructs targeting transcription factors. Resolution of this mystery will contribute to an improved understanding of the mechanism of anthocyanin hydroxylation. In short, the results suggested that *EpF3'H* are functional components of the coloration and may be decisive for anthocyanins B-ring hydroxylation pattern in *E. purpurea.*

Previous studies have confirmed that *F3'H* gene of *I. quamoclit* can alter flower color (Zufall and Rausher, 2004; Ueyama *et al*.,2002). These results indicate that the F3'Hs could enhance color intensity of petals of plants (cyanidin-type) anthocyanins (Ueyama *et al*., 2002; Nakatsuka *et al*., 2006; Han *et al*., 2010). From these reports and our results, it is reasonable to speculate that F3'H function in anthocyanin hydroxylation and is involved in determining flower coloration of *E. purpurea*, similar to the function of

F3'Hs in morning glory species (Hoshino *et al.*, 2003), *Arabidopsis thaliana* (Schoenbohm *et al*., 2000), *G.triflora* (Nakatsuka *et al*., 2005), *Antirrhinum* (Ishiguro *et al.*, 2012).

Genes for flavonoid biosynthesis enzymes can be separated into an 'early gene' group including *CHS*, *CHI*, *F3H*, *F3'H* and *3GT*, and a 'late gene 'group including *DFR*, *ANS* and *GST* (Yonekura-Sakakibara et al.,2007). The wild type and 'Virgin' had the same amino acid sequences of F3'H proteins and expressed similar in different stages, which correspond to the same expression levels of *F3H* gene in blue and white series cineraria (Li *et al*., 2011). This suggests that there was not a direct correlation between *EpF3'H* homologues gene expression and different flower color. The possible explanations for this may be the unavailability of the relevant substrates in specific tissues, or the high transcriptional activity of *EpF3'H* in 'Virgin' have exceeded the threshold level that triggers post-transcriptional silencing mechanisms (Wassenegger and Pélissier, 1998) . Or the difference between 'Virgin' and the wild type may be emerge at the next steps of *F3'H* in biosynthesis pathway, such as *DFR*, *ANS* and *GST* were not abundantly expressed.

2.5 References

Ayabe S, Akashi T. Cytochrome P450s in flavonoid metabolism. Phytochem. Rev., 5: 271–282, 2006.

Aziz AN, Sauve RJ. Genetic mapping of *Echinacea purpurea* via individual pollen DNA fingerprinting. Mol Breed., 21: 227–232, 2008.

Barnes J, Anderson LA, Gibbons S, Phillipson JD. *Echinacea species (Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): A review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol., 57: 929–954, 2005.

Barrett B. Medicinal properties of Echinacea: a critical review. Phytomedicine, 10: 66–86, 2003.

Binns SE, Hudson J, Merali S, Arnason JT. Antiviral activity of characterized extracts

from Echinacea spp. (Heliantheae: Asteraceae) against herpes simplex virus (HSV-I). Planta Med., 68: 780–783, 2002.

Bogs J, Ebadi A, McDavid D, Robinson SP. Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. Plant Physiol., 40: 279–291, 2006.

Bohlmann F, Hoffmann H. Further amides from *Echinacea purpurea*. Phytochemistry, 22: 1173–1175, 1983.

Brugliera F, Barri-Rewell G, Holton TA, Mason JG. Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of *Petunia hybrida.* Plant J., 19: 441–451, 1999.

Carla S, Stefan M, Christian E, Gert F, Bernd W. Identification of the *Arabidopsis thaliana Flavonoid 3'-Hydroxylase* gene and functional expression of the encoded P450 enzyme. Biol. Chem., 381: 749–753, 2000.

Chapple C. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu. Rev. Plant Biol., 49: 311–343, 1998.

Cheminat A, Brouillard R, Guerne P, Bergmann P, Rether B. Cyanidin 3-malonylglucoside in two *Echinacea* species. Phytochemistry, 28: 3246–3247, 1989.

Chen SM, Li CH, Zhu XR, Deng YM, Sun W, Wang LS, Chen FD, Zhang Z. The identification of flavonoids and the expression of genes of anthocyanin biosynthesis in the chrysanthemum flowers. Biol. Plant, 56: 458–464, 2012.

Chen SM, Lv GS, Zhu XR, Liu ZL, Guan ZY, Zhan Z. Cloning and expression profiles of *CgF3'H* in *Chrysanthemum*. Mol. Plant Breed., 9: 623–628, 2011.

Classen B, Witthohn K, Blaschek W. Characterization of an arabinogalactan protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the beta glucosyl Yariv reagent. Carbohydr. Res., 327: 497–504, 2000.

Di Carlo G, Nuzzo I, Capasso R, Sanges MR, Galdiero E, Capasso F, Carratelli CR. Modulation of apoptosis in mice treated with *Echinacea* and St. John's wort. Pharmacol. Res., 48: 273–277,2003.

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex

in Arabidopsis seedlings. Plant J., 53: 814–827, 2008.

Grotewold, E. The genetics and biochemistry of floral pigments. Annu. Rev. Plant Biol., 57: 761–780, 2006.

Han Y, Vimolmangkang S, Soria-Guerra RE, Rosales-Mendoza S, Zheng D, Lygin AV, Korban SS. Ectopic expression of apple *F3'H* genes contributes to anthocyanin accumulation in the Arabidopsis *tt7* mutant grown under nitrogen stress. Plant Physiol., 153: 806–820, 2010.

Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, Deisenhofer J. Structure and function of cytochrome P450: a comparative analysis of three crystal structures. Structure, 3: 41–62, 1995.

Hoshino A, Morita Y, Choi JD, Saito N, Toki K, Tanaka Y, Iida S. Spontaneous mutations of the flavonoid 3'-hydroxylase gene conferring reddish flowers in the three morning glory species. Plant Cell Physiol., 44: 990-1001, 2003.

Huang WK, Cheng HM, Guo JY, Gao BD, Wang FH. Cloning, sequence analysis and expression in Escherichia coli of *flavonoid 3'-hydroxylase* gene of Eupatorium adenophorum sprengel. Plant Physiol. Commun., 43, 821–826, 2007.

Ishiguro K, Taniguchi M, Tanaka Y. Functional analysis of *Antirrhinum kelloggii* flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase genes; critical role in flower color and evolution in the genus *Antirrhinum*. J. Plant Res., 125: 451–456, 2012.

Joseph F. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 783–791, 1985.

Kapteyn J, Goldsbrough P, Simon J. Genetic relationships and diversity of commercially relevant *Echinacea* species. Theor. Appl. Genet., 105: 369–376, 2002.

Kitada C, Gong Z, Tanaka Y, Yamazaki M, Saito K. Differential expression of two cytochrome P450s involved in the biosynthesis of flavones and anthocyanins in chemo-varietal forms of *Perilla frutescens*. Plant Cell Physiol., 42: 1338–1344, 2001.

Koes R, Verweij W, Quattrocchio F. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci., 10: 236–242, 2005.

Li Y, Meng L, Hu K, Dai S. Correlation analysis between expression of a Cineraria F3H homologus gene and its anthocyanin biosynthesis. Mol. plant Breed., 9: 1497–1504, 2011.

Manayi A, Khanavi M, Saiednia S, Azizi E, Mahmoodpour MR, Vafi F, Malmir M, Siavash F, Hadjiakhoondi A. Biological activity and microscopic characterization of Lythrum salicaria L. Daru J. Pharm. Sci., 21: 61, 2013.

Manayi A, Mirnezami T, Saeidnia S, Ajani Y. Pharmacognostical evaluation, phytochemical analysis and antioxidant activity of the roots of *Achillea tenuifolia* LAM. Pharmacognosy J., 4: 14–9,2012.

Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. Nucleic Acids Res., 32: 327–33, 2004.

Merali S, Binns S, Paulin-Levasseur M, Ficker C, Smith M, Baum B, Brovelli E, Arnason JT. Antifungal and anti-inflammatory activity of the genus *Echinacea*. Pharmaceutical Biol., 41: 412– 420,2003.

Miller SC, Yu HC. Echinacea: the genus *Echinacea*: London: CRC Press, 2004.

Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques, 32: 1372–1378, 2002.

Murakami K, Mihara K, Omura T. The transmembrane region of microsomal cytochrome P450 identified as the endoplasmic reticulum retention signal. J. Biochem., 116: 164–175, 1994.

Nakatsuka T, Nishihara M, Mishiba K, Yamamura S. Heterologous expression of two gentian cytochrome P450 genes can modulate the intensity of flower pigmentation in transgenic tobacco plants. Mol. Breed. 17: 91–99, 2006.

Nakatsuka T, Nishihara M, Mishiba K, Yamamura S. Temporal expression of flavonoid biosynthesis-related genes regulates flower pigmentation in gentian plants. Plant Sci.,168: 1309–1318, 2005.

Saito R, Fukuta N, Ohmiya A, Itoh Y, Ozeki Y, Kuchitsua K, Nakayama M. Regulation of anthocyanin biosynthesis involved in the formation of marginal picotee petals in

Petunia. Plant Sci., 170: 828–834, 2006.

Saitou N , Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406–425, 1987.

Schlangen K, Miosic S, Thill J, Halbwirth H. Cloning, functional expression, and characterization of a chalcone 3-hydroxylase from Cosmos sulphureus. J. Exp. Bot. 61: 3451–3459, 2010.

Schoenbohm C, Martens S, Eder C, Forkmann G, Weisshaar B. Identification of the Arabidopsis thaliana flavonoid 3'hydroxylase gene and functional expression of the encoded P450 enzyme. Biol. Chem., 81: 749–753, 2000.

Seitz C, Eder C, Deiml B, Kellner S, Martens S, Forkmann G. Cloning, functional identification and sequence analysis of flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase cDNA reveals independent evolution of flavonoid 3',5'-hydroxylase in the *Asteraceae* family. Plant Mol. Biol., 61: 365–381, 2006.

Sharma SM, Anderson M, Schoop SR, Hudson JB. Bactericidal and anti‐inflammatory properties of a standardized Echinacea extract (*Echinaforce*): Dual actions against respiratory bacteria. Phytomedicine,17: 563–568, 2010.

Stojicevic S, Stanisavljevic A, Velickovic D, Veljkovic V, Lazic M. Antioxidant and antimicrobial activities of Echinacea (*Echinacea purpurea* L.) extracts obtained by classical and ulterasound extraction. Chi. J. Chem. Eng., 17: 478–483, 2009.

Tanaka Y, Brugliera F, Chandler S. Recent progress of flower color modification by biotechnology. Int. J. Mol. Sci. , 10: 5350–5369, 2009.

Tanaka Y, Brugliera F. Flower color and cytochromes P450. Philosophical Transactions of The Royal Society B 368: 20120432, 2013.

Tanaka, Y. Flower color and cytochromes P450. Phytochem. Rev. 5: 283–291, 2006.

Toda K, Yang D, Yamanaka N, Watanabe S, Harada K, Takahashi R. A single-base deletion in soybean flavonoid 3'-hydroxylase gene is associated with gray pubescence color. Plant Mol. Biol. 50: 187–196, 2002.

Tsai YL, Chiou SY, Chan KC, Sung JM, Lin SD. Caffeic acid derivatives, total phenols, antioxidant and antimutagenic activities of *Echinacea purpurea* flower extracts. LWT‐Food Sci. Technol., 46: 169–76, 2012.

Ueyama Y, Suzuki K, Fukuchi-Mizutani M, Fukui Y, Miyazaki K, Ohkawa H, Kusumi T, Tanaka Y. Molecular and biochemical characterization of torenia flavonoid 3'-hydroxylase and flavone synthase II and modification of flower color by modulating the expression of these genes. Plant Sci., 163: 253–263, 2002.

Vazirian M, Dianat S, Manayi A, Ziari R, Mousazadeh A, Habibi E, Saeidnia S, Amanzadeh Y. Anti‐inflammatory effect, total polysaccharide, total phenolics content and antioxidant activity of the aqueous extract of three basidiomycetes. Res. J. Pharmacogn., 1: 15–21, 2014.

Wassenegger M, Pélissier T. A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol., 37: 349–362, 1998.

Xu BB, Li JN, Zhang XK, Wang R, Xie LL, Chai YR. Cloning and molecular characterization of a functional flavonoid 3'-hydroxylase gene from Brassica napus. J. Plant Physiol., 164: 350–363, 2007.

Yamazaki S, Sato K, Suhara K, Sakaguchi M, Mihara K, Omura T. Importance of the proline-rich region following signalanchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. J. Biochem., 114: 652–657, 1993.

Yin XJ, Lin XY, Liu YX, Irfan M, Chen LJ, Zhang L. Integrated metabolic profiling and transcriptome analysis of pigment accumulation in diverse petal tissues in the lily cultivar 'Vivian'. BMC Plant Biol., 20: 446, 2020.

Yonekura-Sakakibara K, Tohge T, Niida R,Saito K. Identification of a flavonol 7-O-rhamnosyltransferase gene determining flavonoid pattern in Arabidopsis by transcriptome coexpression analysis and reverse genetics. J. Biol. Chem., 282: 14932–14941, 2007.

Zili Z, Avery S, Lankun W, Eve SW, Marian LK, Patricia AM, Joan EC. Echinacea increases arginase activity and has anti-inflammatory properties in RAW 264.7 macrophage cells indicative of alternative macrophage activation. J. Ethnopharmacol., 122: 76–85, 2009.

Zufall RA, Rausher MD. Genetic changes associated with floral adaptation restrict

future evolutionary potential. Nature, 428: 847-850, 2004.

Table and Figure

S1 S2 S3 S4

Fig. 2.1 *E. purpurea* were harvested at developmental stages defined as follows:

stage 1; closed bud (less than 15 mm in length)

stage 2; enlarged closed bud with slight pigmented (about 20 mm)

stage 3; bud just opening (clearly pigmented)

stage 4; fully opened flower stage(more than 30 mm).

Table 2.1 Primers used in this study

Primer	Sequence (5–3')
For 3'RACE	
EpF3'H-FP1	AAGGCGTTGGATGACTTTCG
EpF3'H-FP2	CATTGTCGAGGAGCACAAGGCC
EpF3'H-FP3	ATATCGCTCGAGGATCATGG
EpF3'H-FP4	TAACGTGTGTACTGCCAACG
For 5' RACE	
EpF3'H-RP1	ACATTGGGCTTTTCACCTCC
EpF3'H-RP2	TCTCCTTTACGATGGCTTGG
EpF3'H-RP3	TTATCCATCTCTTCCTGGGC
EpF3'H-RP4	ATGTGTCTGTTCCTGCAACG
EpF3'H-RP5	TCAGCATCATCCTTGAGTGC
EpF3'H-RP6	CACCGATGTTGAATTCTCCG
EpF3'H-RP7	TCCAGTTCTGAACACTCTCCG
EpF3'H-RP8	GTACTTTGTGGCTAACGCCG
EpF3'H-RP9	TTATACGCCATATGCTTCGC
For isolation of ORFs	
EpF3'H-FP	ATGACTATTCTAACCCTACTATCATACACC
EpF3'H-RP	TTAACCACTTTCATATACTTGAGG
For RT-PCR	
EpF3'H-FP1	ACAGTGGAATGGGCAATAGC
EpF3'H-RP	TTAACCACTTTCATATACTTGAGG
EpACT-FP1	TATGTTGCTATTCAGGCCGTG
EpACT-RP1	GTGATAACTTGTCCATCAGGC
For Heterologous expression	
SalI - -EpF3'H1-FP	GTCGACATGACTATTCTAACCCTACTATC
KpnI -EpF3'H1-RP	GGTACCTTAACCACTTTCATATACTTGAGG

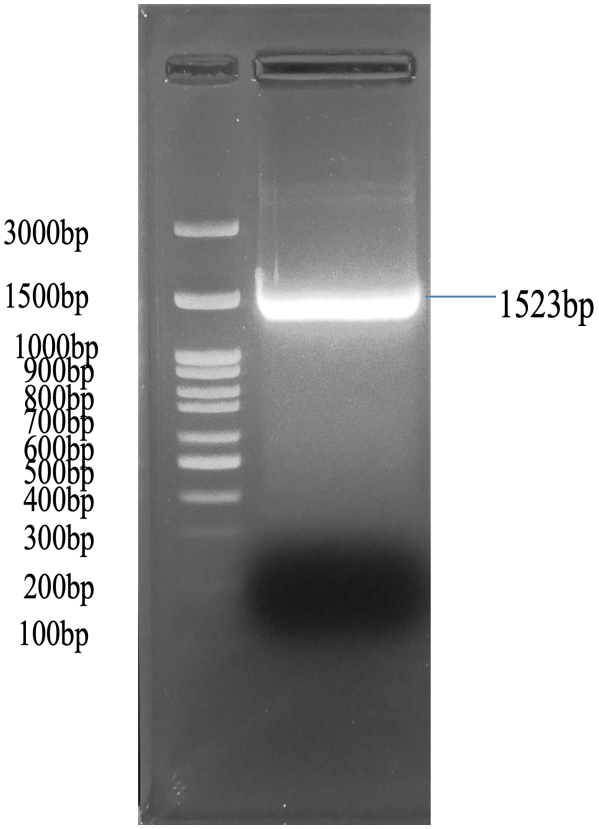
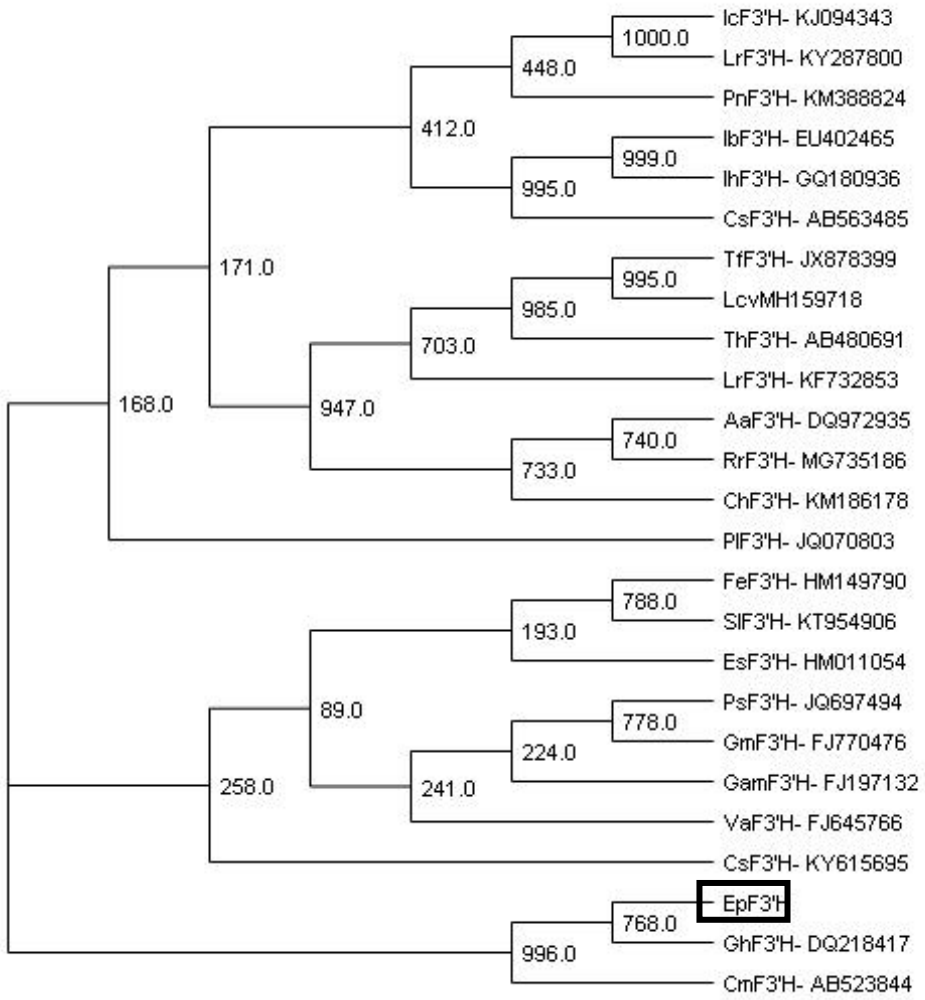
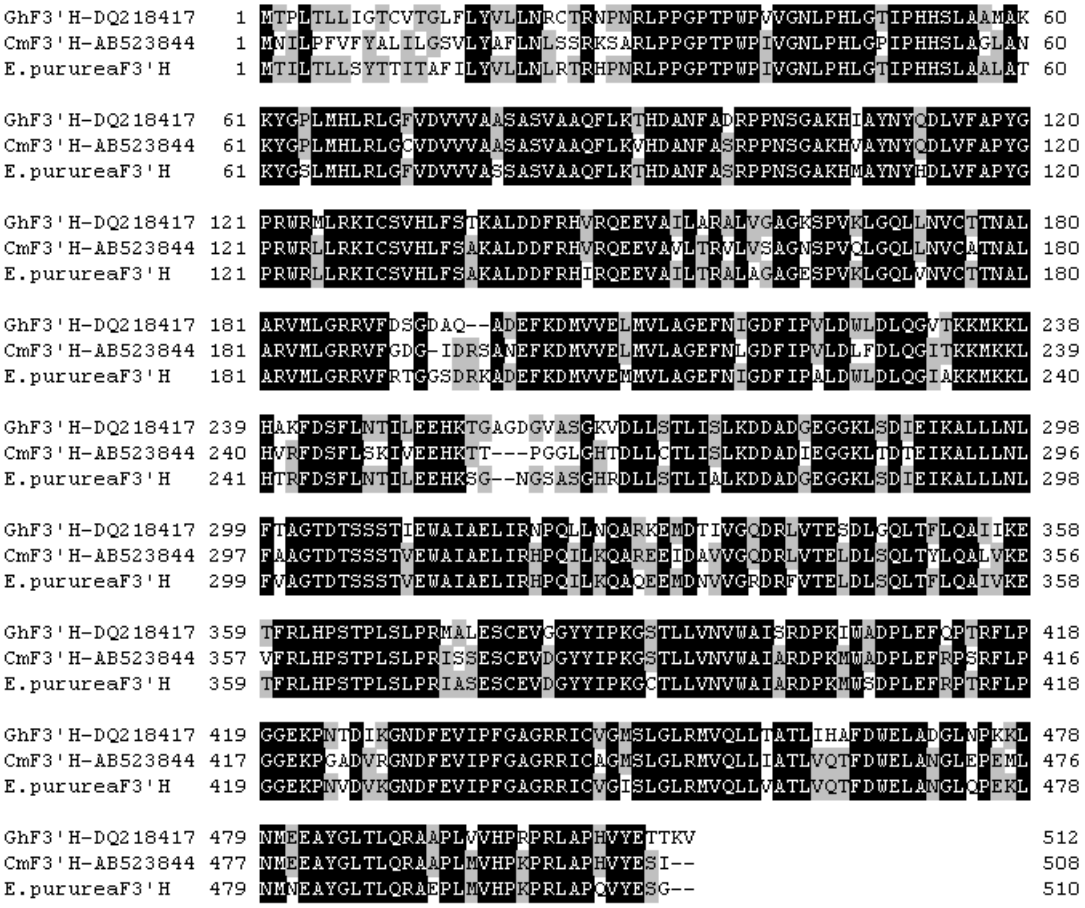


Fig. 2.2. Agarose gel electrophoresis of RT-PCR products of *EpF3'H* in the wild type.



a

Fig. 2.3a Phylogenetic tree of F3'H sequences, constructed using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The black box indicates F3'H isolated from *E. purpurea* in this study. EMBL/DDBJ/GenBank DNA database accession numbers and species names (except *Ep F3'H*) are shown.



b

Fig. 2.3b Multi-alignment of deduced amino acid sequences of EpF3'Hs in *E. purpurea*. *Gerbera hybrid cultivar* (DQ218417), *Chrysanthemum morifolium* (AB523844).

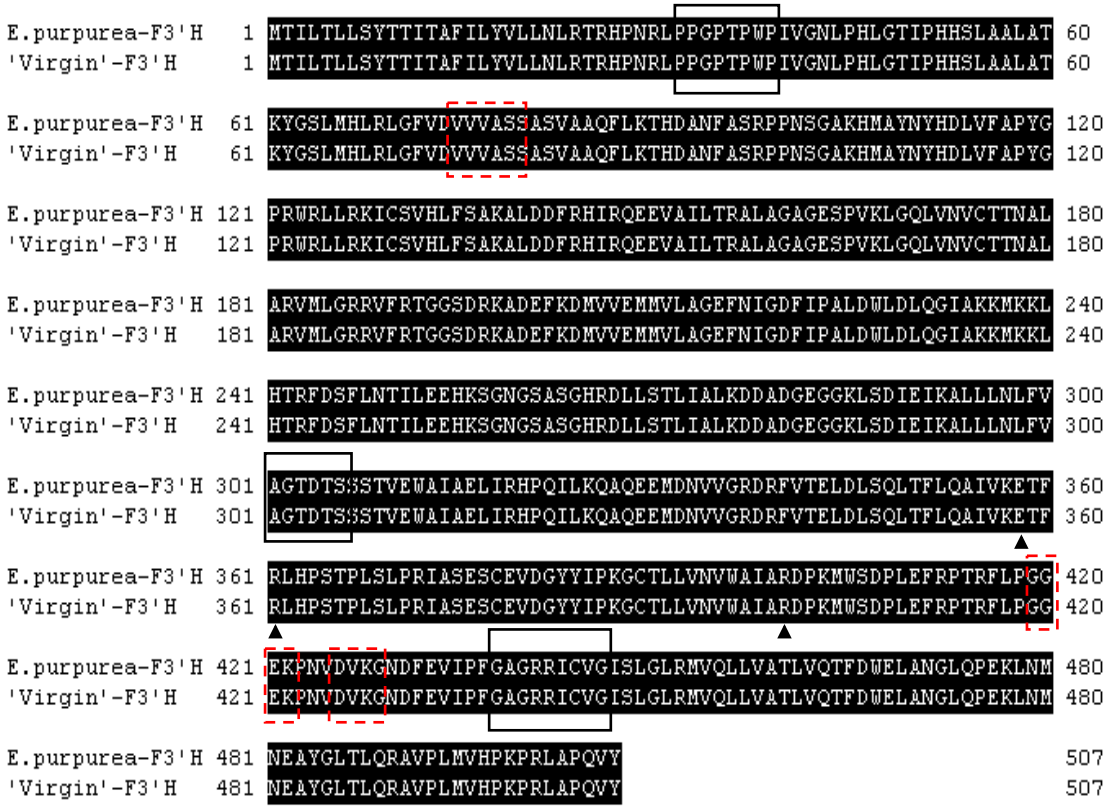
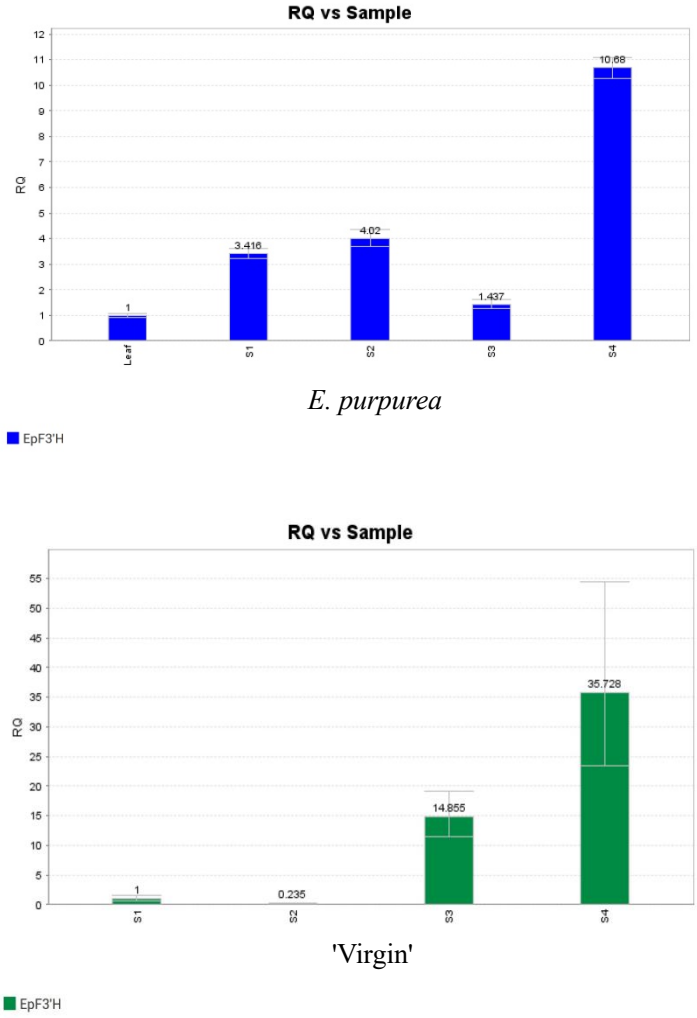


Fig. 2.4 Multi-alignment of deduced amino acid sequences of F3'H in *E. pururea* (Wild type), and 'Virgin'. Identical amino acids are indicated in white foreground and black background; Blocks of similar amino acids are indicated in white foreground and gray background. Black line boxes are cytochrom P450-specific conserved motifs (P32PGPTPWP39, A301GTDTS306, F437GAGRRICVG446). Black triangles indicate the E_{358}-R_{361}-R_{400} triad residues. Red dashed-line boxes are the F3'H-specific motifs (V75VVASS80, G419GEK422 and V425DVKG429) respectively. One mutational loci was showed.



E. purpurea

'Virgin'

Fig. 2.5 Expression analysis of the *EpF3'H* gene of the wild type and 'Virgin'. Both images show RT-PCR products obtained after 40-cycles. S1, S2, S3 and S4 show different stages of flower. The *ACT* gene was used as an internal control.

Chapter 3

Insertion of *ANS* gene caused the formation of white-flowered mutation in *Echinacea purpurea*

Abstract: *Echinacea purpurea* (*E. purpurea*) is naturally reddish- purple flowers. Ultra-performance liquid chromatography (UPLC) analyses showed that cyaniding 3-glucoside (Cy3G) and cyanidin 3-malonylglucoside (Cy3MalG) were the main anthocyanins in petals of *E. purpurea.* 'Virgin', a cultivar with white petals, originates from the wild type. 'Virgin' has excluded anthocyanin contents, but quercetin and kaempferol derivatives been detected. To determine the molecular basis of white coloration in 'Virgin', two genes encoding DFR and ANS enzymes in the anthocyanin biosynthesis pathway were involved in anthocyanin production in *E. purpurea*. The structural analysis showed that *EpDFR* did not change amino acid sequence and was not linked with the white phenotype. An unactive and active ANS allele were identified. The mutant nonfunctional ANS allele containing a 349-bp insertion at the ORF was identified from 'Virgin'. The insertion caused a frame-shift mutation and resulted in creation of a delayed stop codon, and resulted in premature polyadenylation of *EpANS* transcripts. RT-PCR results showed that transcripts of the unactive ANS allele were longer in 'Virgin' than in the wild type, implying the involvement of nonsense-mediated mRNA decay. The insertion has sequence characteristics of transposable elements, but it couldn't transpose. Maybe the insertion is a defective member of a new family of transposable elements. Sequence alignment of the *EpANS* gene from different plant species indicated that only 'Virgin' contained the transposable element. Taken together, the results indicate that the transposable element was maybe responsible for the non-anthocyanin phenotype.

3.1 Introduction

The genus *Echinacea* comprises a small number of species that are hardy, herbaceous perennial plants, native to parts of North America (Wichtl,2004). *E. purpurea* is one of the most valuable medicinal and ornamental plants in the composite family ,with its extra-large, rosy-purple flowers(Fig1.2). Recently, A wide array of coneflower colours can be produced by selection-based breeding. 'Virgin' is one of the distinctive selections of it in the Netherlands by plantsman Piet Oudolf in 2006. Which has frilly-white petals (Fig1.2). To *E. purpurea,* numerous studies on the immune system stimulating properties (Stimpel et al., 1984; Di et al., 2003), anti-microbial (Wacker et al., 1995;Binns et al., 2000; Binns et al., 2002;Merali et al., 2003), anti-inflammatory (Matthias et al.,2008; Sharma et al., 2010; Vazirian et al., 2014) and anti-oxidant properties (Pietta et al., 1998; Orhan et al., 2004; Thygesen et al., 2007; Becker et al., 2009; Manayi et al., 2012; Manayi et al., 2013) have been reported. However, there are few molecular studies on the biosynthesis of anthocyanins in *E. purpurea*.

Flower color is one of the most important characteristics of ornamental plants, which is affected by the different kinds of plant pigments, such as flavonoids, carotenoids and betalains (Tanaka et al., 2008; Tripathi et al., 2018). Flavonoids are plant secondary metabolites. There are more than 8,000 flavonoids derivatives (Veitch and Grayer, 2011), which function in roles like ultraviolet protection and pigmentation in plants (Shirley, 1996; Fini et al., 2011). The most widespread group of flavonoids is the anthocyanins, which is tightly linked to flower development (Weiss et al., 2000) and responsible for most of the red, pink, purple, and blue colors in plants (Field et al., 2001; Honda and Saito, 2002; Miyagawa et al., 2015). Many structural genes encoding enzymes in the anthocyanin biosynthesis pathway (Fig 1.1, Holton and Cornish, 1995; Kim et al., 2004; Kobayashi et al., 2004; Ferrer et al., 2008; Ma et al., 2009; Vogt, 2010; Ben-Simhon et al., 2015) have extensively been studied and characterized in many plant species.

The blockage in the early steps of flavonoid biosynthesis results in formation of white flowers through accumulation of colorless pigments, while the later steps are blocked by mutations results in formation of different colored flowers through accumulation of a

particular anthocyanin. Durbin et al.(2003) showed that color phenotypes in plants were closely associated with changes in the regulation of gene expression. For example due to the lack of F3′5′H, rose, carnation, chrysanthemum, lily, and many other plant species are short of blue flowers. (Katsumoto et al., 2007; Azadi et al., 2016; Xue et al., 2016; Noda et al., 2017). The mutations in the genes encoding flavanone 3'-hydroxylase (F3'H), DFR or ANS caused an accumulation of colorless flavonoids in the white flower phenotypes' petals of *A. majus* (Martin et al., 1985; 1991), *Pharbitis nil* (Saito et al., 1994 ; Hoshino et al., 1997), *Dianthus caryophyllus* (Stich et al., 1992; Mato et al., 2000) and *Eustoma grandiflorum* (Davies et al., 1993). The DFR and ANS enzymes were late in the anthocyanin biosynthesis pathway. Whittal et al (2006) observed that the co-regulation of the DFR and ANS genes eliminated floral pigmentation in *Aquilegia* (Whittal et al.,2006).

In this study, I aimed to investigate the cause of white flower coloration in 'Virgin', and revealed their different functions involved in the flavonoid biosynthetic pathway. To the best of my knowledge, this is the first report documenting molecular analysis of flower color mutations between *E. purpurea* and 'Virgin'.

3.2 Materials and methods

3.*2.1 Plant materials*

E. purpurea seedlings were provided by Kenko-kassei-nojo (Yorii, Japan) and cultivated outdoor. The white 'Virgin' was also bought from Ogihara-shokubutsuen (Ueda, Japan). Petals of *E. purpurea* were collected from flowers at 4 different stages (Fig 2.1); S1-petal is green, S2-the upper petal is green, the lower part is grey reddish-purple, S3-petal is grey reddish-purple, S4-petal is reddish-purple. I used at least three pots for biological repeats. All samples were frozen in liquid nitrogen upon collection and stored at −80°Cprior to RNA extraction.

3.*2.2 UPLC analysis of flavonoid*

Anthocyanins and flavonols were extracted from 100 mg fresh weight of floral tissue

using 1 mL of 10% (v/v) acetic acid aqueous solution, overnight at room temperature (22 °C–25 °C). The extract was passed through a filter with a 0.22-μm pore size membrane (Millipore, Billerica, MA, USA) prior to analysis. The analysis was performed according to a previously reported method using an ACQUITY UPLC with an ACQUITY TQD tandem mass spectrometer (Waters, MA, USA, Noda et al., 2017). MS/MS fragmentation spectra in positive mode were obtained at a collision energy of 30 eV and a cone voltage of 15 V

3.*2.3 Total RNA Extraction*

Using a mortar and pestle, in liquid nitrogen the frozen tissue samples (100 mg) were ground to a powder. Total RNA was extracted from petals of developmental stage (S1 - S4), leaves of *E. purpurea* and 'Virgin' by the cetyltrimethylammonium bromide method (Chang et al., 1993), then eliminated the contamination of genomic DNA by DNase I (TakaRa, Japan) treatment at 37℃ for 1hr.

3.*2.4 Cloning of the full-length cDNA of EpDFRs and EpANSs*

Aliquots of 2μg total RNA extracted from the samples were reverse-transcribed to produce cDNA by oligo(dT)21 primer using the First-Strand cDNA Synthesis Kit according to the manufacturer's protocol (TakaRa, Japan). To amplify full-length cDNA fragments, 3'-rapid amplification of cDNA ends (3'RACE) and 5'RACE was performed using the 3'-full RACE core set and the 5'-full RACE core set (Roche, Germany), respectively. Primers used for isolation of cDNA clones are listed in Table1.DNA sequencing was performed using the automated sequencer (ABI 3700 Genetic Analyzer; Applied Biosystems) according to the manufacturer's protocol.

A full-length cDNA sequence of *EpDFR* and *EpANS* was deduced by assembling the products of the 3'-RACE,5'-RACE. A pair of primers was designed in Table1 and PCR amplification was performed using the cDNA of *E. purpurea* as the template. The *EpDFR* PCR amplification procedure was as follows: predenature at 95 °C for 5 min, 95°C 30s, 64 °C 30 s, for 30 cycles, then 72°C for 7min. The *EpANS* PCR was as follows: 95 °C 5 min, 95°C 30s, 60 °C 30 s, for 30 cycles, then 72°C 5 min. The PCR

product was separated by 2% agarose gel electrophoresis, isolated by a Agarose Gel DNA Extraction Kit (TaKaRa, Japan), then cloned into the JM109 vector and sequenced.

3.*2.5 Semi-quantitative RT-PCR*

The expression levels of the structural genes *EpDFR* and *EpANS* were analyzed by semi-quantitative RT-PCR. The specific primers used for amplification of the anthocyanin biosynthesis genes and their corresponding PCR primers are listed in Table1. The *EpDFR* PCR conditions was as follows: 95 °C 5 min, 95°C 30s, 63°C 30s, for 30 cycles, then 72°C 5min. The *EpANS* PCR conditions was: 95 °C 5 min, 95°C 30s, 62°C 30s, for 30 cycles, then 72°C 5min.And determined their band densities on 2% agarose gels.

3.*2.6 Quantitative RT-PCR analysis*

Real-time quantitative PCR was carried out to investigate the expression profiles of *EpDFR*, *EpANS* in leaves and different stages of petals in *E. purpurea* . RT-PCR used the SYBR green method in CFX384TM Real-Time System (BIO-RAD, USA). Each PCR reaction (20 μL) contained the following components: 12 nM primers, cDNA (2ng), Power-Up TM SYBR TM Green Master mix (Lithuania). The real-time quantitative PCR thermal cycling conditions were 95 °C for 30s, 95°C 1s, 63 °C 30 s, for 39 cycles. After a denaturation step at 95°C for 30s the melting curve analysis was done increasing the temperature of 0.5 °C, from 65 to 95 °C, each 10 sec. The primers *EpDFR*-FP2, *EpDFR*-RP2, *EpANS*-FP1 and *EpANS*-RP (Table 1) were used for real-time PCR to amplify about 400 bp fragments of *EpDFR* and *EpANS*. The PCR products were confirmed using agarose gel electrophoresis and sequencing. Quantitation of the gene expression was done with comparative CT ($\Delta\Delta$CT) method (Muller et al., 2002; Bogs et al., 2006). The expression levels of different genes were normalized to the constitutive expression of *EpACT*

3.*2.7 Bioinformatics Analysis*

The nucleotide sequence and deduced amino acid sequence of *EpDFR* and *EpANS* were analyzed online (http://www. ncbi.nlm.nih.gov; http://web.expasy.org/protparam/; and http://smart.embl-heidelberg.de/.). A phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987) and the MEGA version 3.1 software (Kumar et al., 2001). The reliability of the tree was measured by bootstrap analysis with 1,000 replicates (Felsenstein,1992).

3.3. Results

3.3.1 Flavonoid analysis of E. purpurea and 'Virgin'

To confirm the anthocyanin accumulation, UPLC was performed to detect and quantify the anthocyanin in petals of *E. purpurea* and 'Virgin'. Two peaks present in *E. purpurea* (Fig. 3.1a) were identified as cyanidin derivatives based on mass spectrometry data, which shared the same aglycone ion. Peak 1 was identified as cyanidin 3-O-glucoside (Cy3G). The other compound was identified as cyanidin 3-malonylglucoside (Cy3MalG) as referred to in (Qiu et al., 2013). No anthocyanin accumulation was detected in 'Virgin' (Fig. 3.1b). These results suggested that the difference in their respective colors was due to the different in the levels of the anthocyanins.

UPLC was performed to detect the flavonols accumulation in the wild type and 'Virgin'. Compared with the wild type, the individual and total flavonols content was dramatically increased in 'Virgin' (Fig. 3.1c and d). The UPLC chromatograms showed five same peaks present in the wild type (Fig. 4c) and "Virgin", but two distinct peaks were observed in 'Virgin' (Fig. 3.1d). One peak was identified as quercetin derivatives at m/z 303 (Fig. 3.1e). The another was kaempferol derivatives at m/z 287 (Fig 3.1f). They were designated as FL1 and FL2, respectively (Table 1). Flavonols are the products of lateral steps in the anthocyanin biosynthetic pathway. The metabolic balance between the synthesis of anthocyanins and flavonols of 'Virgin' was probably disrupted by the absence of detectable levels of anthocyanin derivatives.

3.3.2 Gene expression of EpDFR and EpANS

Real-time quantitative PCR analysis (Fig. 3.2) showed that the *EpDFR* expression pattern was consistent with that of *EpANS*. They were both detected in all the stages of petals, but expression levels were significantly different (Fig. 3.2). The highest level was in S4, and then S2, S3, S1 and leaf in the wild type. The *EpDFR* level in S4 had almost twenty times the level found in S1. That in S1 was approximately 20-fold higher than that in the leaves. The highest *EpDFR* activity was in fully opened flowers(S4), and there was almost no expression in the leaves. The *EpANS* expression level in S4 was approximately 35-fold higher than that in S1. These showed that the *EpDFR* and *EpANS* had different temporal and spatial expression patterns in the wild type.

In 'Virgin', The expression patterns of *EpDFR* and *EpANS* were approximately same. The highest level was in S4, and then S3, S2 , S1 .Which parallel increased in anthocyanin pigmentation. In 'Virgin', RT-PCR PCR analysis (Fig. 3.2) showed that the expression pattern of *EpDFR* corresponds to the anthocyanin accumulation pattern in petal. But the *EpDFR* expression level in S4 was approximately 60-fold higher than that in S1. *EpANS* transcript expression of S2 was the lowest. So may be predict the highest *EpDFR* and *EpANS* activities were both in fully opened flowers (S4), the activity of *EpANS*-S2 probably be significantly altered.

For further analysis the expression paterns of *EpDFR* and *EpANS* between the wild type and 'Virgin', the S2 of petals were subjected to semi-quantitative RT-PCR analysis (Fig. 3.3). The PCR showed that there was none of the deduced transcripts were differentially expressed between the *EpDFRs* in the wild type and 'Virgin'. Strong *EpANS* signals were observed and expressed abundantly in the wild type petals. There was a less signal was also detected in 'Virgin', while it was longer than that in *E. purpurea*. Brugliera et al. (1994) also detected longer RT-transcripts and assumed that these transcripts were generated by read-through transcription of the dTph3 sequence and terminated close to, or at, the original site of the RT gene. It corresponds to that the RNA sequences were generated by incorrect termination of transcription around *EpANS*, indicating that the longer transcripts were generated by *EpANS*-mediated aberrant processing and contained *EpANS* sequences.

3.3.3 DFR gene analysis

The degenerate PCR was used to amplify a core fragment of the *EpDFR* gene. BLAST sequence analysis of the product showed that it was highly homologous to DFRs from the other plant species. Thus, this sequence was used to design the gene-specific primer for amplifying the 5'and 3' ends of cDNA by 5'-RACE and 3'-RACE. The isolated ORFs were cloned and named EpDFR1 (accession number: LC593110), which is1068bp and encodes 355 amino acid residues. The molecular mass of this protein was 40.00 kD, and pI was 6.15.

To verify the gene, the *EpDFR* cDNA was amplified using PCR and cloned into a pTAC-2 vector. Ten clones were sequenced, and the results showed that the gene sequences of 'Virgin' were identical to *E. purpurea.* Amino acid sequence of *EpDFR* with that from other plant species was shown in Fig.3.4. The *EpDFR* showed 85.1% and 80.6% similarity with *Saussurea mesusa* and *Chrysanthemun morifolium* respectively. *E. purpurea* had the highest similarity with *Saussurea mesusa.* Phylogenetic analysis was performed based on the deduced amino acid sequences of DFR (Fig. 3.4). The phylogenetic tree indicated that the *EpDFR* proteins of *E. purpurea* clustered in a clade with the DFR proteins from both dicots and monocots.

Some reports have demonstrated a substrate specificity-related region in DFR enzymes (Beld et al., 1989; Johnson et al., 2001). I compared the predicted amino acid sequences of this region in *E. purpurea* and 'Virgin' and found that *EpDFR* showed no amino acid differences in the region proposed to determine substrate specificity.

3.3.4 Characterization of the EpANS sequence

The core fragment of the *EpANS* gene from *E. purpurea* was amplified by the degenerate PCR. 5'-RACE and 3'-RACE were used to get the 5'and 3' ends of cDNA . A 1,077-bp full-length cDNA sequence were cloned and named EpANS1 (accession number: LC593111). It encodes 358 amino acid residues and the molecular mass was 40.83 kD, pI was 5.94. The *EpANS* showed 83.1% and 79.2% similarity with *Chrysanthemun morifolium*,*Saussurea mesusa* respectively. The phylogenetic tree (Fig. 3.4) indicated that the *EpANS* proteins of *E. purpurea* clustered in a clade with the

ANSs from the other plants.

The *EpANS* gene were amplified from *E. purpurea* and 'Virgin'. The size of the alleles were compared. A 349-bp transposon insertion was mapped between positions 923–924bp in exon 2nd downstream of the ATG initiation codon (Fig. 3.5).Comparison with the wild-type, the insertion element with sequence features of a transposable element has been identified in 'Virgin'. The inserted fragment did not contain any encoding transposase sequence but had characteristics of a tourist-like miniature-inverted repeat (MITE), such as 8 bp terminal inverted repeats flanked by a 5bp target site duplication. I thought this insertion sequence as a transposable element.The insertion sequence was a non-autonomous transposable elements since it was too small to encode full-length transposons,which was possibly like the insertion position of *dTph3*s in the *RT* gene (Kroon et al, 1994; Nakajima et al., 2005). The insertion presently at 'Virgin' can no longer transpose because of the structural defect in the terminal inverted region. The inverted repeat sequences of the left were not perfectly identical to the right. There was a mismatch (Fig. 3.6). Kroon et al. (1994) also reported that because of structural defects in the terminal inverted region, the *dTph3* copy presently at the Rt locus can't be transposed. So it may be a defective member of a new family of transposable elements.

I also cloned the *EpANS* gene from cDNAs of other cultivars ('Green jewel 'and 'Green twist') petals. The results showed that their ANS sequences were identical to the wild type. The insertion was only detected in 'Virgin'.

3.4. Discussion

The color of *E. purpurea* is related to the composition and content of flavonoids, such as anthocyanin, flavonols. Flavonoid compounds display a wide variety of biological activities and are regarded as important active components of medicinal plants (Buer et al., 2010; Brunetti et al., 2013). The distribution of flavonoids in *E. purpurea* has been well documented (Bohlmann et al., 1983; Barnes et al.,2005.), but has never been identified the constituents of anthocyanin and flavonols in *E. purpurea* and 'Virgin',

based on UPLC analysis. High levels of anthocyanin compounds were detected in extracts from *E. purpurea*, as compared to nothing in 'Virgin'. The main pigments of reddish-purple petals in *E. purpurea* are Cyanidin-3-malonyglucoside (Cy3MalG) and Cyanidin-3-O- glycosides (Cy3G). The resulted indicated that flower color change was related to anthocyanins contents. The deficiency of anthocyanins was the main reason for the transformation from red to white. Our findings are consistent with previous studies. Zhong et al. (2012) noted that changes to floral color in *P. lactiflora* were related to the composition of pigments and the reduction in anthocyanins. Yang et al. (2015) investigated flower color change in peony cultivars, indicated that a sharp decrease in anthocyanins could be the main contributing factor for the change in color from red to orange and yellow. Cyanidin was detected in *E. purpurea*,which agreed with the description of the cyanidin-red flowers (Brewbaker et al. ,1962; Yin et al., 2014). Meng et al. (2020) hypothesized that altered flavanone and flavone accumulation may lead to pigment elimination in white petal, and the limited flux in cyanidin biosynthesis pathway seems to be the most likely reason for the colorless petal

Flavonols play an important role in yellow coloration (Xue et al., 2016). The phenomenon of changing colors characterizes many ornamental plants, such as *Lonicera japonica, Brunfelsia calycina* (Fu et al., 2013; Zipor et al., 2015) have been reported. Consequently, anthocyanins content was not found whereas quercetin and kaempferol derivatives were identified only in 'Virgin'. Quercetin and kaempferol are flavonols which are among the most abundant flavonoids in plants and are usually found in the form of mono-, di- or tri-glycosides (Winkel-Shirley 2001; Buer et al., 2010; Stracke et al., 2010). The increased levels of flavonols would change the floral color is still elusive, but one possibility is evident that the biosynthesis of flavonols could compete with the common substrates for anthocyanin production. The higher levels of quercetin and kaempferol derivatives suggested the flavonol glucosyl transferase maybe played critical roles in this regulation. Flavonols also usually act as co-pigments to affect the flower color (Aida et al., 2000), which also have been shown to be responsible for the yellow petal of Lathyrus chrysanthus (Markham and Hammett, 1994).

DFR is a key enzyme, which uses NADPH as a cofactor to catalyzes the reduction of dihydroflavonols (Li et al., 2012; Zhou et al., 2008). To our knowledge, various *DFR* genes have been isolated from a wide range of plant species, such as *Malus domestica* (Fischer et al., 2003), *Pyrus communis* (Fischer et al., 2003), *Lotus japonicus* (Shimada et al., 2005), *Medicago truncatula* (Xie et al., 2004), *Citrus sinensis* (Piero et al., 2006), *Camellia sinensis* (Singh et al., 2009), *Populus trichocarpa* (Huang et al., 2012), *Ginkgo biloba* (Cheng et al., 2013), and *Ipomoea batatas Lam* (Wang et al., 2013). In addition, flower color alteration by genetic engineering of the DFR gene has been reported for *Rosa hybrida* (Katsumoto et al., 2007), *Dianthus caryophyllus* (see review; Tanaka et al., 1998), *T. fournieri* (Aida et al., 2000a; Ono et al., 2006), *P. hybrida* (Meyer et al., 1987), *Osteospermum hybrida* (Seitz et al.,2007).

The PCR analysis (Fig. 3.2) showed that *EpDFR* was detected in the petals of the wild type and 'Virgin', and the amino acid sequences of them were completely identical. RT-PCR showed that the highest level of *EpDFR* expression was in the S4, then S2, S3, S1 ,leaf were gradually lowering in the wild type, and then S2, S3, S1 ,leaf in ' 'Virgin' ' were gradually lowering. Nakatsuka et al. (2003) showed that the DFR expression patterns parallel increases in anthocyanin pigmentation, which is approximately correspond to the expression of *EpDFR*. To the expression of S3 in the wild type, the possible explanation for this anomaly could be *EpDFR* was probably regulated by post-transcriptional regulation, such as its activation was inhibited, or like CHS in the *petunia* (Saito et al., 2006) having different splicing forms. It might be due to during flower development some homologous genes of *DFR* may be silent or may produce DFR-related transcripts with different catalytic properties, in different tissues, and at different times (Beld et al., 1989).

ANS is a 2-oxoglutarate (2OG) irondependent oxygenase, which catalyzes the stepwise conversion of leucocyanidins to anthocyanins (Fig. 1.1), complete the transformation of colorless to color compounds (Gong et al. 1997; Saito et al. 1999; Shimada et al. 2005). In the anthocyanin biosynthetic pathway, ANS is located downstream of the DFR (Fig 1.1), affect pathway efficiency and influence flower color depending on the species (Smith et al., 2012). RT-PCR showed that no matter in the

wild type or ‘Virgin', the expression patterns of *EpDFR* and *EpANS* were same, and *EpANS* expression patterns approximately parallel increases in anthocyanin pigmentation. Several studies also showed that the expression pattern of the *ANS* gene corresponds with the accumulation of anthocyanins (Gong et al., 1997; Saito et al., 1999; Shimada et al., 2005).

Transposable elements can be divided into two classes. According to whether their transposition intermediate is RNA or DNA, designated as retrotransposon (class 1) or DNA transposon (class 2, Feschotte et al., 2002; Graig, 2002). Based on both the sequence similarity of the homologue of TIR and the specific number of nucleotides comprising TSD caused by insertion of the element into the host genomic DNA, DNA transposable elements can be grouped into families (Kunze and Weil, 2002). One of the members called MITEs, which are a heterogeneous group of small non-autonomous elements, and include a few dozen to a few hundred base pairs in size. MITEs are frequently found in or close to genes and are flanked by TIRs.

Many non-autonomous elements have been found in most plant genomes (Han et al., 2013). The transposable element of *EpANS* in 'Virgin', a non-autonomous DNA transposon, does not contain any ORFs encoding a transposase, it contains 8-bp terminal inverted repeats and 5-bp target site duplication as typical features of DNA transposons (Wicker et al., 2007), which maybe belong to MITEs . The ransposable element of *EpANS* in 'Virgin' is probably dependent on other transposable elements having mobility components. Lazarow et al. (2013) showed that with the help of the transposases produced by active partners, non-autonomous elements without genes encoding active transposases can still transpose. Insertion of the transposable element of *EpANS* in 'Virgin' inhibited normal *EpANS* transcriptions (Fig. 3.3) and caused low levels of incomplete *EpANS* transcripts (Fig. 3.3). Kim et al. (2004) reported a non-autonomous DNA transposon insertion caused significant reduction in the transcription of the *ANS* gene, resulting in a pink color phenotype in onion. Phadungsawata et al. (2020) found a 226-bp insertion of a non-autonomous transposable element in the coding region of CCD4 resulted in the lack of CCD4 expression and more carotenoid was accumulated in wild-type yellow-flowered petunia.

The nucleotide sequences of the terminal ends of the transposable element of *EpANS* were imperfect inverted repeats, namely one nucleotide difference was present between TIR sequences. Such an imperfect TIR sequence could not be recognized by the transposase of the transposable element, so that the insertion could not be excised anymore. 'Virgin' rose to a stable white phenotype. Hoshino et al (2003) also reported that a nonsense codon generated by the point mutations of *F3'H* in *Ipomoe*a has been reported to cause nonsense-mediated decay (NMD), affecting the stability of mature mRNA. Further studies will be needed to elucidate the possible factor involved in the transposable element mobility and to identify autonomous the transposable element with complete transposase controlling its translocation.

There were few polymorphisms in exons of *EpANS* alleles, as expected, PCR products tagging the transposable element were not detected in any other cultivars tested. Which might be arisen by different regulatory genes. Since no molecular genetic information about the *E. purpurea* is available now, testcrosses between the white 'Virgin' and red wild type or other breeding lines should be carried out to identify the function of the transposable element in the white 'Virgin'.

In addition, different gene(s) other than the transposable element might be causal gene(s) for the white 'Virgin', although complementation to produce anthocyanin in F_1 hybrids between the white and the wild type has not been reported yet. Further studies are required to elucidate the complete mechanism causing the white color in 'Virgin'.

3.6 References

Aida R, Kishimoto S, Tanaka Y, Shibata M. Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. Plant Sci., 153: 33–42, 2000.

Azadi P, Bagheri H, Nalousi AM, Nazari F, Chandler SF. Current status and biotechnological advances in genetic engineering of ornamental plants. Biotechnol. Adv., 34: 1073–1090, 2016.

Barnes J, Anderson LA, Gibbons S, Phillipson JD. Echinacea species (*Echinacea angustifolia* (DC.) *Hell., Echinacea pallida* (Nutt.) *Nutt., Echinacea purpurea* (L.) Moench): A review of their chemistry, pharmacology and clinical properties. J. Pharm. Pharmacol., 57: 929–954, 2005.

Becker EM, Nissen L, Skibsted LH. Antioxidant evaluation protocols: Food quality or health effects. Europe. Food Res. Technol., 219: 561–571, 2004.

Beld M, Martin C, Huits H, Stuitje AR, Gerats AGM. Flavonoid synthesis in Petunia hybrida: partial characterization of dihydroflavonol-4-reductase genes. Plant Mol. Biol., 13: 491–502, 1989.

Ben-Simhon Z, Judeinstein S, Trainin T, Harel-Beja R, Bar-Ya'akov I, Borochov-Neori H. A "White" anthocyanin-less pomegranate (*Punica granatum* L.) caused by an insertion in the coding region of the leucoanthocyanidin dioxygenase (*LDOX*; *ANS*) gene. PLoS ONE, 10: e0142777, 2015.

Binns SE, Hudson J, Merali S, Arnason JT. Antiviral activity of characterized extracts from Echinacea spp. (Heliantheae: Asteraceae) against herpes simplex virus (HSV-I). Planta Med., 68: 780–783, 2002.

Binns SE, Purgina B, Bergeron C, Smith ML, Ball L, Baum BR, Arnason JT. Light-mediated antifungal activity of Echinacea extracts. Planta Med., 66: 241-244, 2000.

Bogs J, Ebadi A, McDavid D, Robinson SP. Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. Plant Physiol., 40: 279–291, 2006.

Bohlmann F, Hoffmann H. Further amides from *Echinacea purpurea*. Phytochemistry.

22: 1173–175, 1983.

Brewbaker JL. Cyanidin-red white clover: a duplicate recessive mutant in Trifolhtm repens L. J. Hered., 53: 163–167, 1962.

Brugliera F, Holton TA, Stevenson TW, Farcy E, Lu C, Cornish EC. Isolation and characterization of a cDNA clone corresponding to the Rt locus of *Petunia hybrida*. Plant J., 5: 81–92, 1994.

Brunetti C, Di Ferdinando M, Fini A, Pollastri S, Tattini M. Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. Int. J. Mol. Sci., 14: 3540–3555, 2013.

Buer CS, Imin N, Djordjevic MA. Flavonoids: new roles for old molecules. J. Integr. Plant Biol., 52: 98–111, 2010.

Cheng H, Li L, Cheng S, Cao F, Xu F, Yuan H, Wu C. Molecular cloning and characterization of three genes encoding dihydroflavonol-4-reductase from Ginkgo biloba in anthocyanin biosynthetic pathway. PLoS ONE, 8, e72017, 2013.

Davies KM, Bradley JM, Schwinn KE, Markham KR, Podivinsky E. Flavonoid biosynthesis in flower petals of five lines of lisianthus (*Eustoma grandiflorum Grise*.). Plant Sci., 95: 67–77, 1993.

Di Carlo G, Nuzzo I, Capasso R, Sanges MR, Galdiero E, Capasso F, Carratelli CR. Modulation of apoptosis in mice treated with Echinacea and St. John's wort. Pharmacol. Res., 48: 273–277, 2003.

Durbin ML, Lundy KE, Morrell PL, Torres-Martinez CL, Clegg MT. Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. Mol. Phylogenet. Evol., 29: 507–518, 2003.

Felsenstein J. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. Genet. Res., 60: 209–220, 1992.

Ferrer JL, Austin MB, Stewart CJ, Noel JP. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol. Biochem., 46: 356–370, 2008.

Feschotte C, Jiang N, Wessler SR. Plant transposable elements: where genetics meets genomics. Nat. Rev. Genet., 3: 329–341, 2002.

Field TS, Lee DW, Holbrook NM. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol., 127: 566–574, 2001.

Fini A, Brunetti C, Di Ferdinando M, Ferrini F, Tattini M. Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. Plant Signal. Behav., 6: 709–711, 2011.

Fischer TC, Halbwirth H, Meisel B, Stich K, Forkmann G. Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from Malus domestica and Pyrus communis cultivars and the consequences for flavonoid metabolism. Arch. Biochem. Biophys., 412: 223–230, 2003.

Fu L, Li H, Li L, Yu H, Wang L. Reason of flower color change in *Lonicera japonica*. Sci. Silvae Sin., 49: 155–161, 2013 (in Chinese).

Gong Z, Yamazaki M, Sugiyama M, Tanaka Y, Saito K. Cloning and molecular analysis of structural genes involved in anthocyanin biosynthesis and expressed in a forma-specific manner in *Perilla frutescens*. Plant Mol. Biol., 35: 915–927, 1997.

Graig NJ. Mobile DNA. In: Craig NJ, Craigle R, Gellert M, Lambowitz AM (eds) Mobile DNA II. American Society for Microbiology Press, Washington DC: 3–11, 2002.

Han Y, Qin S, Wessler SR. Comparison of class 2 transposable elements at superfamily resolution reveals conserved and distinct features in cereal grass genomes. BMC Genom., 14: 71, 2013.

Holton TA, Cornish EC. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell, 7: 1070–1083, 1995.

Honda T, Saito N. Recent progress in the chemistry of polyacylated anthocyanins as flower color pigment. Heterocycles, 56: 633–692, 2002.

Hoshino A, Abe Y, Saito N, Inagaki Y, Iida S. The gene encoding flavanone 3-hydroxylase is expressed normally in the pale yellow flowers of the Japanese morning glory carrying the speckled mutation which produce neither flavonol nor anthocyanin but accumulate chalcone, aurone and flavanone. Plant Cell Physiol., 38: 970–974, 1997.

Hoshino A, Morita Y, Choi JD, Saito N, Toki K, Tanaka Y, Iida S. Spontaneous mutations of the flavonoid 3'-hydroxylase gene conferring reddish flowers in the three morning glory species. Plant Cell Physiol., 44: 990–1001, 2003.

Huang, Y, Gou J, Jia Z, Yang L, Sun Y, Xiao X, Song F, Luo K. Molecular cloning and characterization of two genes encoding dihydroflavonol 4-reductase from *Populus trichocarpa*. PLoS ONE, 7: e30364, 2012.

Johnson ET, Ryu S, Yi H, Shin B, Cheng H, Choi G. Alternation of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Plant J., 25: 325–333, 2001.

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, Tao GQ, Nehra NS, Lu CY, Dyson BK, Tsuda S, Ashikari T, Kusumi T, Mason JG, Tanaka Y. Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol., 48: 1589–1600, 2007.

Kim S, Binzel ML, Yoo KS, Park S, Pike LM. Pink (P), a new locus responsible for a pink trait in onions (*Allium cepa*) resulting from natural mutations of anthocyanidin synthase. Mol. Genet. Genomics., 272: 18–27, 2004.

Kobayashi S, Goto-Yamamoto N, Hirochika H. Retrotransposon- induced mutations in grape skin color. Science, 304: 982, 2004.

Kroon J, Souer E, de Graaff A, Xue Y, Mol J, Koes R. Cloning and structural analysis of the anthocyanin pigmentation locus *Rt* of *Petunia hybrida*: characterization of insertion sequences in two mutant alleles. Plant J., 5: 69–80,1994.

Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, 17: 1244–1245, 2001.

Kunze R, Weil CF. The hAT and CACTA superfamilies of plant transposons. In: Craig NJ, Craigle R, Gellert M, Lambowitz AM (eds) Mobile DNA II. American Society for Microbiology Press. Washington DC: 565–610, 2002.

Lazarow K, Doll M, Kunze R. Molecular biology of maize *Ac*/*Ds* elements: an overview. Plant transposable elements (Part of the Method. Mol. Biol., 1057):

59–82, 2013.

Li HH, Qiu J, Chen FD, Lv XF, Fu CX, Zhao DX, Hua XJ, Zhao Q. Molecular characterization and expression analysis of dihydroflavonol 4-reductase (DFR) gene in *Saussurea medusa*. Mol. Biol. Rep., 39: 2991-2999, 2012.

Ma H, Pooler M, Griesbach R. Anthocyanin regulatory/structural gene expression in *Phalaenopsis*. J. Am. Soc. Hortic. Sci., 134: 88–96, 2009.

Manayi A, Khanavi M, Saiednia S, Azizi E, Mahmoodpour MR, Vafi F, Malmir M., Siavashi F., Hadjiakhoondi A. Biological activity and microscopic characterization of *Lythrum salicaria* L. Daru J. Pharm. Sci., 21: 61, 2013.

Manayi A, Mirnezami T, Saeidnia S, Ajani Y. Pharmacognostical evaluation, phytochemical analysis and antioxidant activity of the roots of *Achillea tenuifolia* LAM. Pharmacogn J., 4: 14-19, 2012.

Markham KR, Hammett KRW. The basis of yellow coloration in Lathyrus-Aphaca flowers. Phytochemistry., 37: 163–165, 1994.

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES. Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the pallida locus by transposon tagging. EMBO J., 4: 1625–1630, 1985.

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E. Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J., 1: 37–49, 1991.

Mato M, Onozaki T, Ozeki Y, Higeta D, Itoh Y, Yoshimoto Y, Ikeda H, Yoshida H, Shibata M. Flavonoid biosynthesis in white flowered Sim carnation (*Dianthus caryophyllus*). Sci. Hort., 84: 333–347, 2000.

Matthias A, Banbury L, Bone KM, Leach DN, Lehmann RP. *Echinacea* alkylamides modulate induced immune responses in T-cells. Fitoterapia, 79: 53-58, 2008.

Meng XQ, Li G, Gu LY, Sun Y, Li ZY, Liu JR, Wu XQ, Dong TT, Zhu MK. Comparative Metabolomic and Transcriptome Analysis Reveal Distinct Flavonoid Biosynthesis Regulation Between Petals of White and Purple *Phalaenopsis amabilis*. J. Plant Growth Regul., 39: 823–840, 2020.

Merali S, Binns S, Paulin-Levasseur M, Ficker C, Smith M, Baum B, Brovelli E, Arnason JT. Antifungal and Anti-inflammatory Activity of the Genus Echinacea.

Pharm. Biol., 41: 412-420, 2008.

Meyer P, Heidmann I, Forkmann G, Saedler H. A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature, 330: 677–678, 1987.

Miyagawa N, Miyahara T, Okamoto M, Hirose Y, Sakaguchi K, Hatano S, Ozeki Y. Dihydroflavonol 4-reductase activity is associated with the intensity of flower colors in delphinium. Plant Biotechnol., 32: 249–255, 2015.

Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques, 32: 1372–1378, 2002.

Nakajima T, Matsubara K, Kodama H, Kokubun H, Watanabe H, Ando T. Insertion and excision of a transposable element governs the red floral phenotype in commercial petunias. Theor. Appl. Genet., 110: 1038–1043, 2005.

Nakatsuka A, Izumi Y, Yamagishi M. Spatial and temporal expression of chalcone synthase and dihydroflavonol 4-reductase genes in the Asiatic hybrid lily. Plant Sci., 165: 759-767, 2003.

Noda N, Yoshioka S, Kishimoto S, Nakayama M, Douzono M, Tanaka Y, Aida R. Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism. Sci. Adv., 3: e1602785, 2017.

Ono E, Fukuchi-Mizutani M, Nakamura N, Fukui Y, Yonekura-Sakakibara K, Yamaguchi M, Nakayama T, Tanaka T, Kusumi T, Tanaka Y. Yellow flowers generated by expression of the aurone biosynthetic pathway. Proc. Natl. Acad. Sci. U.S.A., 103: 11075–11080, 2006.

Orhan I, Senol FS, Gülpinar AR, Kartal M, Sekeroglu N, Deveci M, Kan Y, Sener B. Acetylcholinesterase inhibitory and antioxidant properties of *Cyclotrichium niveum*. *Thymus praecox* subsp. *caucasicus* var. *caucasicus*, *Echinacea purpurea* and *E. pallida*. Food Chem. Toxicol., 47: 1304-10, 2009.

Phadungsawat B, Watanabe K, Mizuno, S, Kanekatsu M, Suzuki S. Expression of *CCD4* gene involved in carotenoid degradation in yellow flowered *Petunia* × *hybrida*. Scie. Hortic., 261: 108916, 2020.

Piero ARL, Puglisi I, Petrone, G. Gene characterization, analysis of expression and in vitro synthesis of dihydroflavonol 4-reductase from [*Citrus sinensis* (L.) Osbeck].

Phytochemistry, 67: 684–695,2006.

Pietta P, Simonetti P, Mauri P. Antioxidant activity of selected medicinal plants. J. Agric. Food Chem., 46: 4487-4490, 1998.

Qiu J, Gao F, Shen G, Li C, Han X, Zhao Q, Zhao D, Hua X, Pang Y. Metabolic engineering of the phenylpropanoid pathway enhances the antioxidant capacity of *Saussurea involucrata*. PLoS One, 8: e70665, 2013.

Saito K, Kobayashi M, Gong Z, Tanaka, Y, Yamazaki M. Direct evidence for anthocyanidin synthase as a 2-oxoglutaratedependent oxygenase: molecular cloning and functional expression of cDNA from a red forma of *Perilla frutescens*. Plant J., 17: 181–189, 1999.

Saito N, Cheng J, Ichimura M, Yokoi M, Abe Y, Honda T. Flavonoid in the acyanic flowers of *Pharbitis nil*. Phytochemistry, 35: 687–691, 1994.

Saito R, Fukuta N, Ohmiya A, Itoh Y, Ozeki Y, Kuchitsua K, Nakayama M.. Regulation of anthocyanin biosynthesis involved in the formation of marginal picotee petals in *Petunia*. Plant Sci., 170: 828-834, 2006.

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406–425, 1987.

Seitz C, Vitten M, Steinbach P, Hartl S, Hirsche J, Rathje W, Treutter D, Fork-mann G. Redirection of anthocyanin synthesis in *Osteospermum hybridaby* a two-enzyme manipulation strategy. Phytochemistry, 68: 824–833, 2007.

Sharma SM, Anderson M, Schoop SR, Hudson JB. Bactericidal and anti-inflammatory properties of a standardized *Echinacea* extract (Echinaforce ®): Dual actions against respiratory bacteria. Phytomedicine, 17: 563-568, 2010.

Shimada S, Inoue YT, Sakuta M. Anthocyanidin synthase in non-anthocyanin-producing Caryophyllales species. Plant J., 44: 950–959, 2005.

Shirley BW. Flavonoid biosynthesis: ‘new’ functions for an ‘old’ pathway. Trends Plant Sci., 1: 377–382, 1996.

Singh K, Kumar S, Yadav SK, Ahuja PS. Characterization of dihydroflavonol 4-reductase cDNA in tea [*Camellia sinensis* (L.) O. Kuntze]. Plant Biotechnol. Rep., 3: 95–101, 2009.

Smith SD, Wang SQ, Rausher MD. Functional evolution of an anthocyanin pathway enzyme during a flower color transition. Mol. Biol. Evol. 30: 602–612, 2012.

Stich K, Eidenberger T, Wurst F, Forkmann G. Enzymatic conversion of dihydroflavonols to flavan-3,4-diols using flower extracts of *Dianthus caryophyllus* L. (carnation). Planta, 187: 103–108, 1992.

Stimpel M, Proksch A, Wagner H, Lohmann-Matthes ML. Macrophage activation and induction of macrophage cytotoxicity by purified polysaccharide fractions from the plant *Echinacea purpurea*. Infect. Immun., 46: 845-849, 1984.

Stracke R, Jahns O, Keck M, Tohge T, Niehaus K, Fernie AR, Weisshaar B. Analysis of production of flavonol glycosides-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12-and MYB111-independent flavonol glycoside accumulation. New Phytol., 188: 985–1000, 2010.

Tanaka Y, Sasaki N, Ohmiya A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J., 54: 733–49, 2008

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

Thygesen L, Thulin J, Mortensen A, Skibsted LH, Molgaard P. Antioxidant activity of cichoric acid and alkamides from *Echinacea purpurea*, alone and in combination. Food Chem., 101: 74-81, 2007.

Tripathi AM, Niranjan A, Roy S. Global gene expression and pigment analysis of two contrasting flower color cultivars of *Canna*. Plant Physiol. Biochem., 127: 1–10, 2018.

Vazirian M, Dianat S, Manayi A, Ziari R, Mousazadeh A, Habibi E, Saeidnia S, Amanzadeh, Y. Anti-inflammatory effect, total polysaccharide, total phenolics content and antioxidant activity of the aqueous extract of three basidiomycetes. Res. J. Pharmacogn., 1: 15-21, 2014.

Veitch NC, Grayer RJ. Flavonoids and their glycosides, including anthocyanins. Nat. Prod. Rep., 28: 1626–1695, 2011.

Vogt T. Phenylpropanoid biosynthesis. Mol. Plant, 3: 2–20, 2010

Wacker A, Hilbig W. Virushemmung mit *Echinacea purpurea*. Planta Med., 33: 89-102,

1978.

Wang H, Fan W, Li H, Yang J, Huang J, Zhang P. Functional characterization of Dihydroflavonol-4-reductase in anthocyanin biosynthesis of purple sweet potato underlies the direct evidence of anthocyanins function against abiotic stresses. PLoS ONE, 8: e78484, 2013.

Weiss D. Regulation of flower pigmentation and growth: multiple signaling pathways control anthocyanin synthesis in expanding petals. Physiol. Plant., 110: 152–157, 2001.

Whittall JB, Voelckel C, Kliebenstein DJ, Hodges SA. Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in *Aquilegia*. Mol. Ecol. 15: 4645–4657, 2006.

Wichtl M. Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis, 3rd edn. Medpharm Scientific Publishers, Stuttgart, 2004 .

Wicker T, Sabot F, Hua-Van A, Bennetzen JL, Capy P, Chalhoub B, Flavell A, Leroy P, Morgante M, Panaud O, Paux E, SanMiguel P, Schulman AH. A unified classification system for eukaryotic transposable elements. Nat. Rev. Genet., 8: 973–982, 2007.

Winkel-Shirley B. Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol., 126: 485–493, 2001.

Xie DY, Jackson LA, Cooper JD, Ferreira D, Paiva NL. Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from *Medicago truncatula*. Plant Physiol. 134: 979–994,2004.

Xue L, Wang Z, Zhang W, Li Y, Wang J, Lei J. Flower pigment inheritance and anthocyanin characterization of hybrids from pink-flowered and white-flowered strawberry. Sci. Hortic., 200: 143–150, 2016.

Yang Q, Yuan HH, Sun XB. Preliminary studies on the changes of flower color during the flowering period in two tree peony cultivars. Yuan Yi Xue Bao, 42: 930–938, 2015. (in Chinese)

Yin T. Transcriptome analysis of differentially expressed genes relevant to variegation in peach flowers. PLoS One, 9: e90842, 2014.

Zhong PX, Wang LS, Li SS, Xu YJ, Zhu ML. The changes of floral color and pigments composition during the flowering period in *Paeonia lactiflora* Pall. A Yuan Yi Xue Bao, 39: 2271–2282, 2012. (in Chinese)

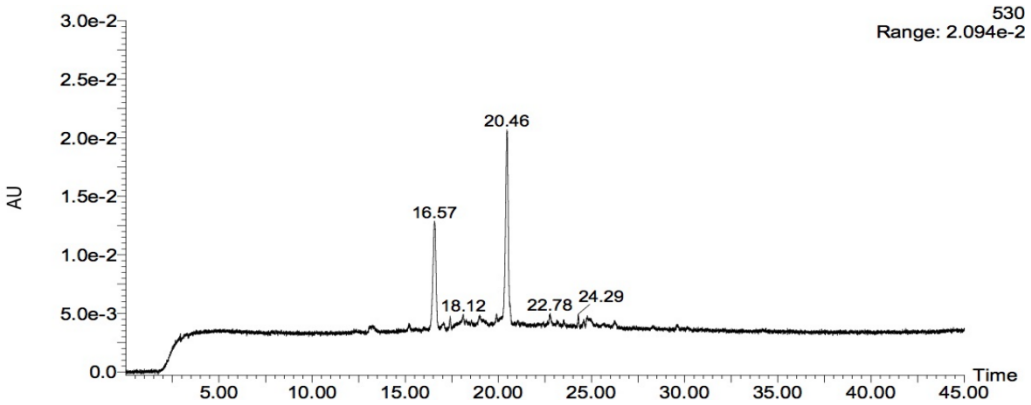
Zhou LL, Zeng HN, Shi MZ, Xie DY. Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. Planta. 229,37-51, 2008.

Zipor G, Duarte P, Carqueijeiro IM, Shahar L, Ovadia R, Teper-bamnolker P, Eshel D, Levin Y, Faigenboim A, Sottomayor M, Oren-Shamir M. In planta anthocyanin degradation by a vacuolar class III peroxidase in Brunfelsia calycina flowers. New Phytol., 205: 653–665, 2015.

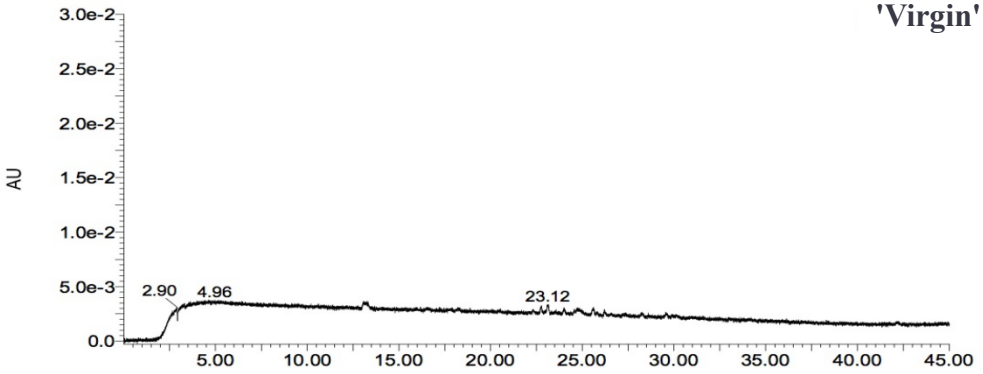
Table and Figure

Table 3.1. Primers used in this study

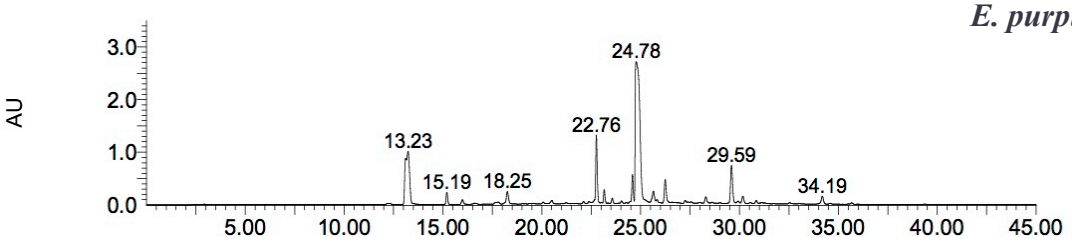
Primer	Sequence (5–3')
For 3'RACE	
EpDFR-FP1	CAAGAAACTGGTGTTCACCTCC
EpDFR-FP2	CCGGAGCTGTAAACGTACAG
EpANS-FP1	TGCACTCACGTTCAAACTCC
EpANS-FP2	AACGGACAATGGGTAACTGC
EpANS-FP3	AACGTGATTTGACCATTTGG
For 5' RACE	
EpDFR-RP1	TGCCAACTGATGAATGGTGG
EpDFR-RP2	AATGCACATACTGGCCTTGC
EpDFR-RP3	GATGAAGTCCAAATCGCTCC
EpDFR-RP4	CTTAATACTCCTTGTACAGTTGGC
EpANS-RP1	TTATTCACAAGCCCTCTGTGG
EpANS-RP2	AAGGATCTCGAGTGTGTCACC
EpANS-RP3	TTCGCAGTTACCCATTGTCC
EpANS-RP4	TAGAAACAGTCTCTTGGAGCG
EpANS-RP5	AGTGCTCTTAGCTGTCTAGC
EpANS-RP6	AGATCCCATGGTTGACAAGG
EpANS-RP7	CTGTGATGGTTGTGAGTTCC
For isolation of ORFs	
EpDFR-FP	ATGAAAGAGGATTCTCCGGC
EpDFR-RP	TCAGTTTGCAACTTTGCAGCC
EpANS-FP	ATGGTGATTTCAGCTAACACC
EpANS-RP	TCACCCAGTATCATTTCGG
For Real-time PCR	
EpDFR-FP2	CCGGAGCTGTAAACGTACAG
EpDFR-RP1	TGCCAACTGATGAATGGTGG
EpANS-FP1	TGCACTCACGTTCAAACTCC
EpANS-RP	TCACCCAGTATCATTTCGG
EpACT1-FP1	TATGTTGCTATTCAGGCCGTG
Ep ACT1-RP1	GTGATAACTTGTCCATCAGGC


530
Range: 2.094e-2
AU
Time
16.57
20.46
18.12
22.78
24.29

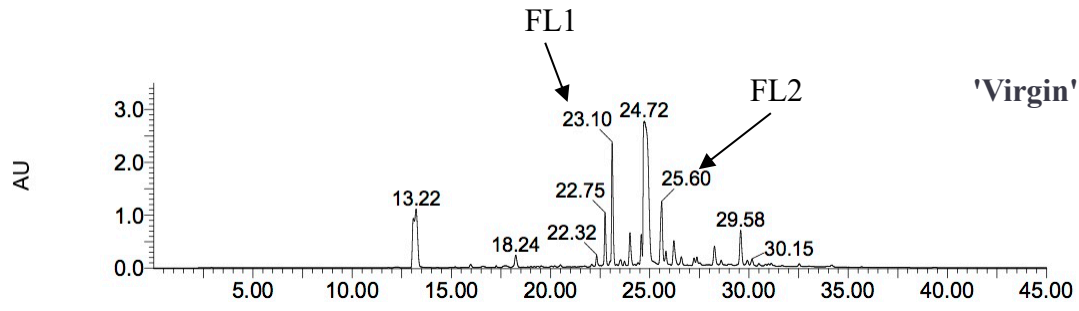
A


'Virgin'
AU
2.90
4.96
23.12

B


E. purpurea
AU
13.23
15.19
18.25
22.76
24.78
29.59
34.19

C


FL1
FL2
'Virgin'
AU
13.22
18.24
22.32
22.75
23.10
24.72
25.60
29.58
30.15

D

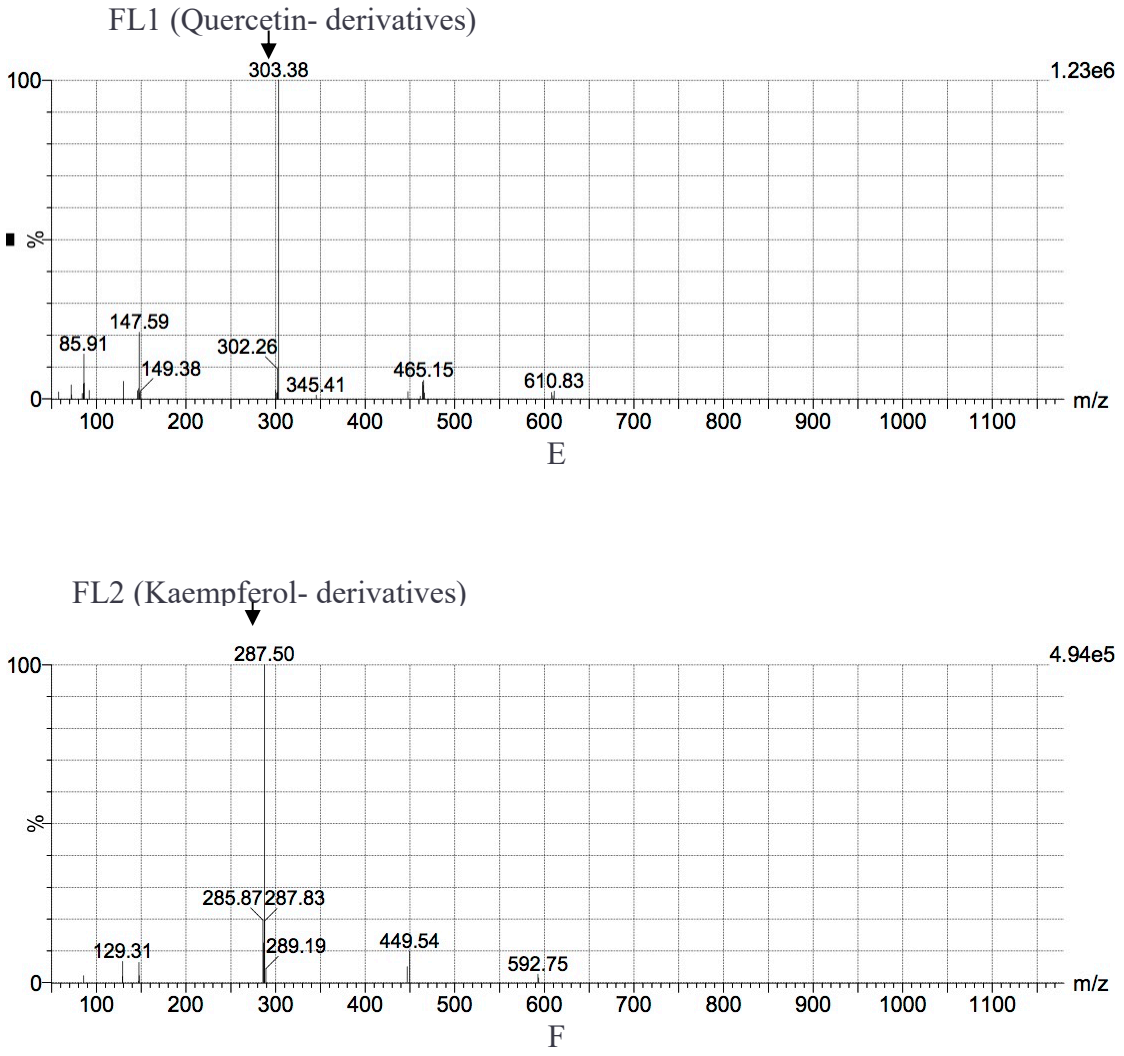
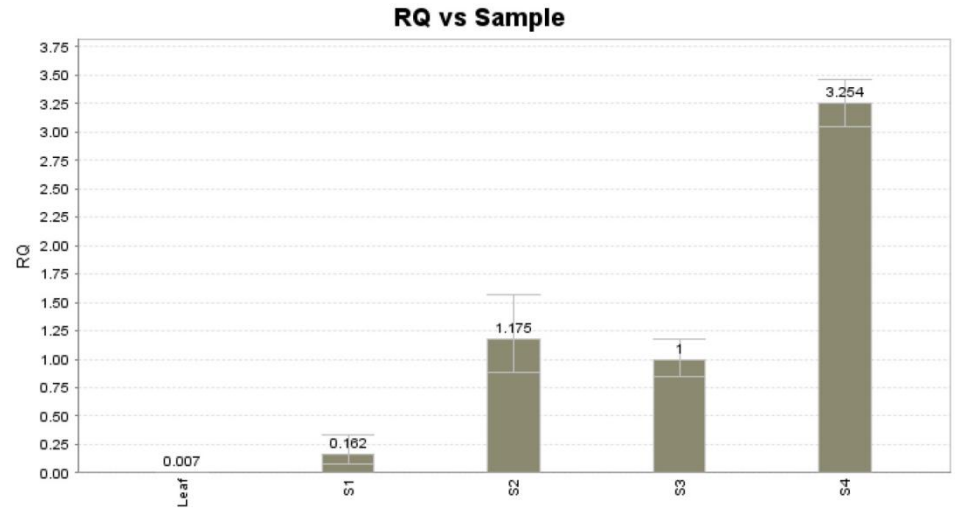


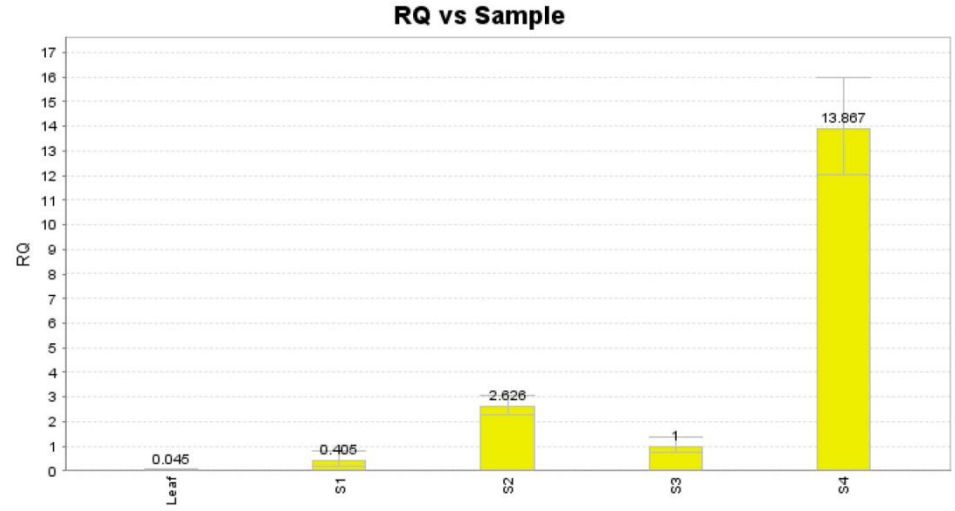
Fig. 3.1. The flavonoid composition analysis of the wild type and 'Virgin'.

Panels A and B show the the peak of anthocyanins (at 530 nm),panels C and D show the detection of flavonols (at 360 nm) in the petal of *E. purpurea* and 'Virgin'. The y-axes represent absorbance units, and the x-axes represent the retention times (min). Panels E and F show the specific accumulation of flavonols in petals of 'Virgin', the y-axes represent relative intensity of the spectrum , and the x-axes represent the mass-to-charge ratio. The amount of anthocyanin in each peak is shown as mg malvidin 3-glucoside equivalents mg^{-1} FW (Panel A,B). The amount of flavonols in each peak is shown as mg rutin 3-hydrate equivalents mg^{-1} FW (Panels C, D). Cy3G; Cyanidin 3-O-glucoside, Cy3MalG; Cyanidin 3-malonylglucoside.



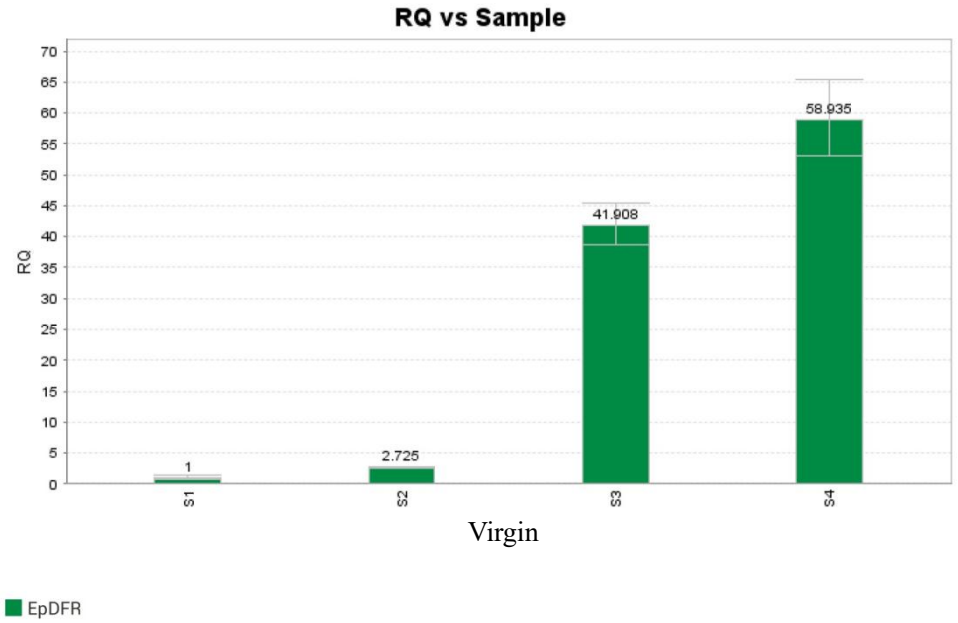
E. purpurea (the wild type)

EpDFR1



E. purpurea (the wild type)

EpANS1



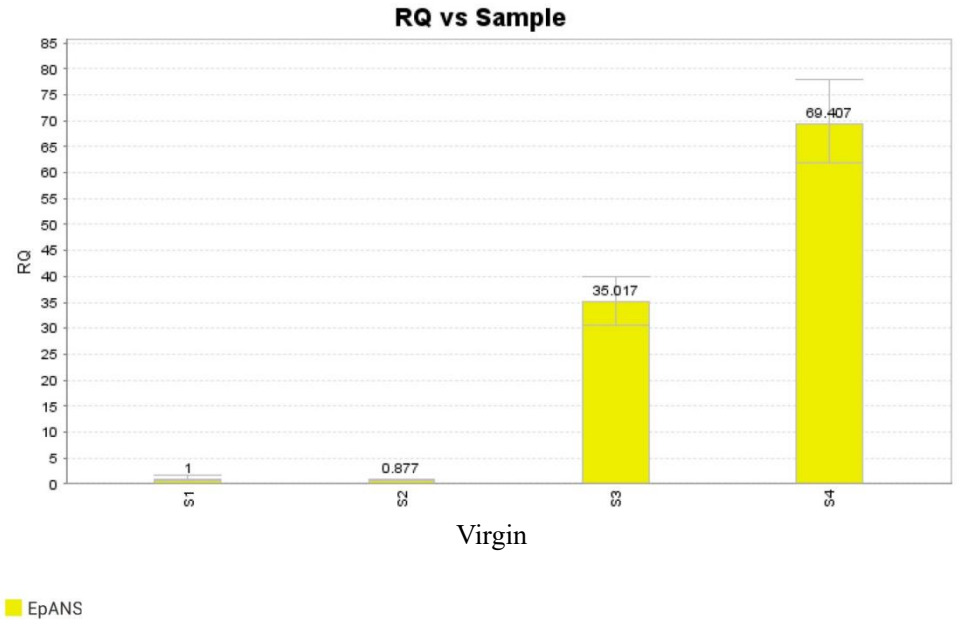


Fig. 3.2. Expression analysis of the *EpDFR* and *EpANS* genes in *E. purpurea*.

All images show RT-PCR products obtained after 40 cycles. S1, S2, S3 and S4 show different stages of flower. The *EpACT* gene was used as an internal control.

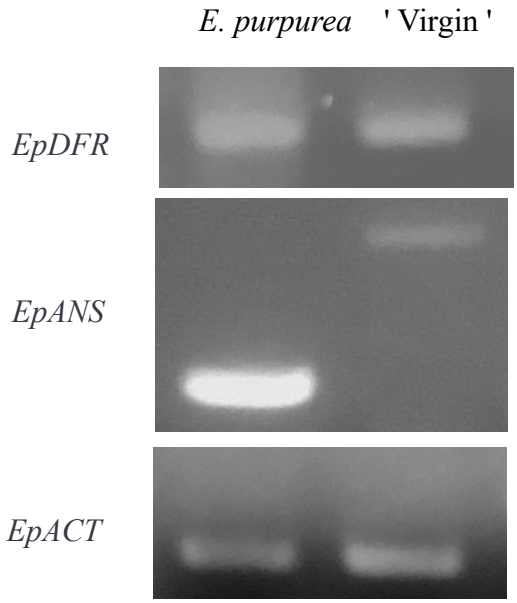
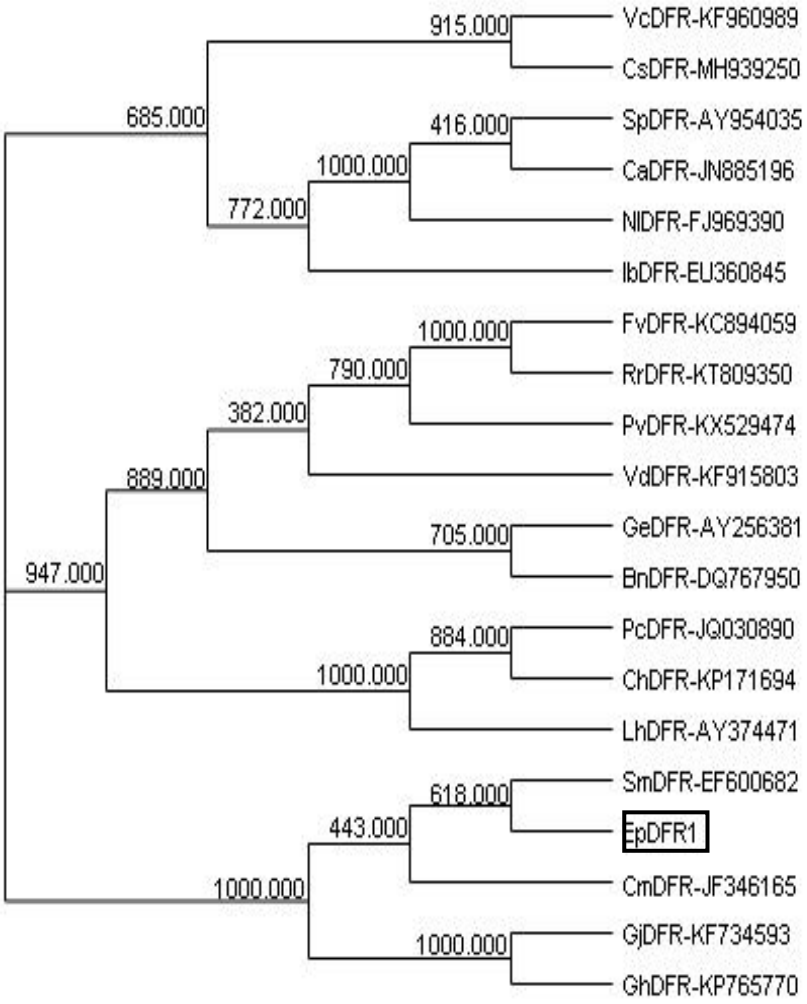


Fig. 3.3. RT-PCR analysis of *EpDFR*, *EpANS* expression in *E. purpurea* and 'Virgin'. *EpACT* was used as internal control.


915.000
VcDFR-KF960989
CsDFR-MH939250
685.000
416.000
SpDFR-AY954035
1000.000
CaDFR-JN885196
772.000
NlDFR-FJ969390
IbDFR-EU360845
1000.000
FvDFR-KC894059
790.000
RrDFR-KT809350
382.000
PvDFR-KX529474
889.000
VdDFR-KF915803
705.000
GeDFR-AY256381
947.000
BnDFR-DQ767950
884.000
PcDFR-JQ030890
1000.000
ChDFR-KP171694
LhDFR-AY374471
SmDFR-EF600682
618.000
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443.000
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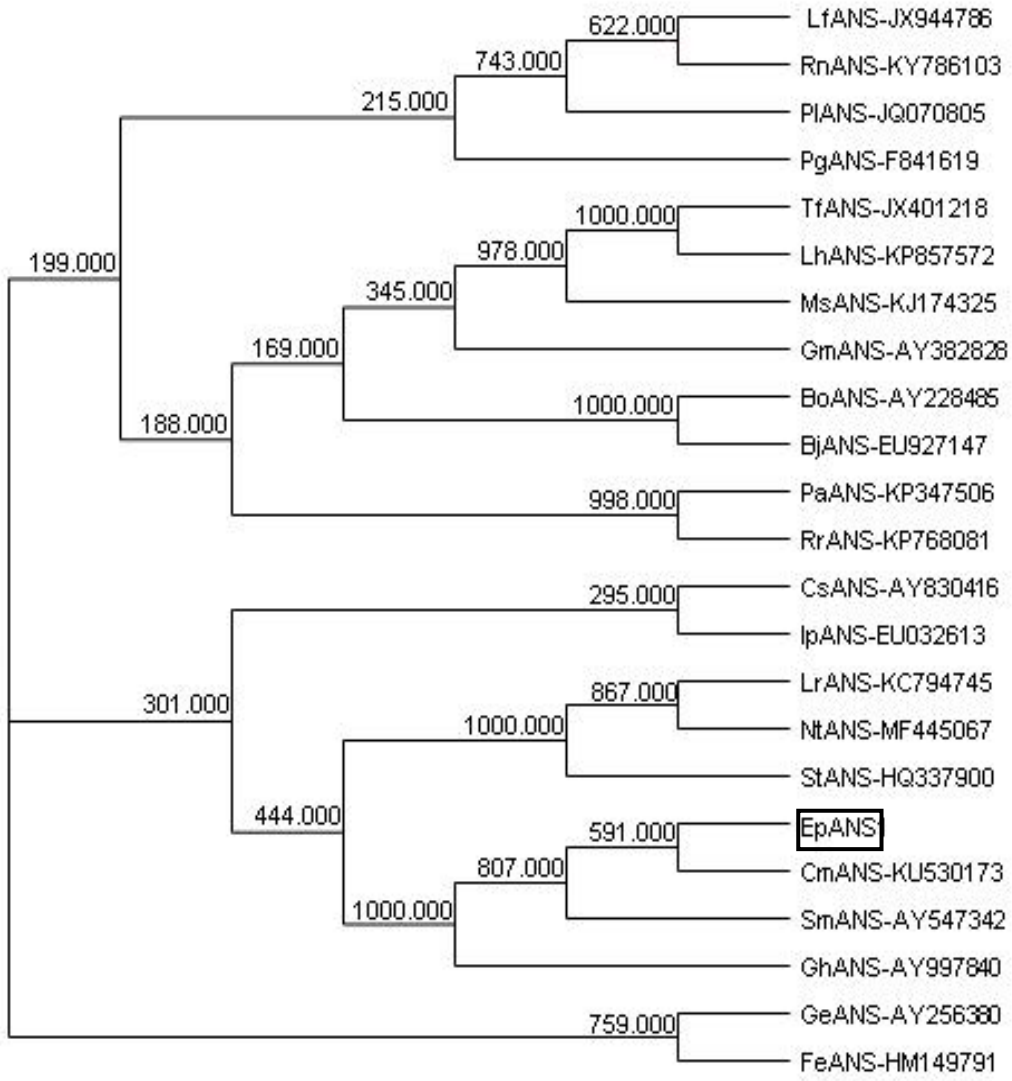
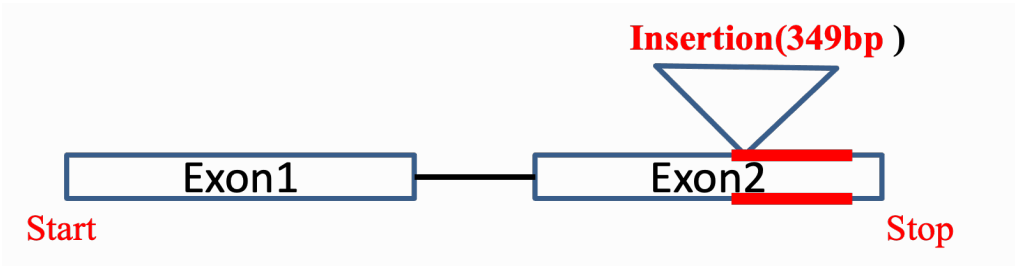


Fig. 3.4. Phylogenetic analysis of EpDFR and EpANS with other proteins respectively. The phylogenetic tree was generated using the neighbor-joining method in MEGA X software. Numbers at each interior branch indicate the bootstrap values of 1000 replicates. Plant species and GenBank accession numbers of their DFR and ANS proteins used for phylogenetic analysis were showed in figure. EpDFR and EpANS are boxed.

```
EpANS1          601 GAGAAGGAGGTTGGAGGTATGGAGGAGCTACTTCTTCAACTAAAAATCAACTATTACCCA 660
Virgin-ANS1     601 GAGAAGGAGGTTGGAGGTATGGAGGAGCTACTTCTTCAACTAAAAATCAACTATTACCCA 660

EpANS1          661 AAATGCCCTCAGCCTGAGCTAGCTCTCGGTGTTGAAGCTCACACTGATGTGAGTGCACTC 720
Virgin-ANS1     661 AAATGCCCTCAGCCTGAGCTAGCTCTCGGTGTTGAAGCTCACACTGATGTGAGTGCACTC 720

EpANS1          721 ACGTTCATACTCCACAACATGGTTCCTGGGCTCCAACTCTTTTACAACGGACAATGGGTA 780
Virgin-ANS1     721 ACGTTCATACTCCACAACATGGTTCCTGGGCTCCAACTCTTTTACAACGGACAATGGGTA 780

EpANS1          781 ACTGCGAAATGCGTCCCTGACTCCATAATTATGCATATTGGTGACACACTCGAGATCCTT 840
Virgin-ANS1     781 ACTGCGAAATGCGTCCCTGACTCCATAATTATGCATATTGGTGACACACTCGAGATCCTT 840

EpANS1          841 AGTAATGGCAAGTACAAAAGTATTCTCCACAGAGGGCTTGTGAATAAGGAGAAGGTTAGG 900
Virgin-ANS1     841 AGTAATGGCAAGTACAAAAGTATTCTCCACAGAGGGCTTGTGAATAAGGAGAAGGTTAGG 900

EpANS1          901 ATTTCTTGGGCGGTTTTCTGTGAACCA-------------------------------- 927
Virgin-ANS1     901 ATTTCTTGGGCGGTTTTCTGTGAACCATGTTGGGTAATCTTGATGGGTATGGGTCGAGCA 960

EpANS1          927 ------------------------------------------------------------ 927
Virgin-ANS1     961 GTTCCGGGTCAAGTACGGGTCGTCAAGCAACCCACGGATCTTTGGGTGGAAGTGGTCATG 1020

EpANS1          927 ------------------------------------------------------------ 927
Virgin-ANS1    1021 TGTTGACCAAGTTTTAAGGAGAAAGTGAACGTTATGGAACGGGTATAAAGGAGAAGTGGA 1080

EpANS1          927 ------------------------------------------------------------ 927
Virgin-ANS1    1081 GAATTGTTAGTTAGCATGAAATTAGGACGTGGAGTTCATATCAGCATGTGTTTAGTTTTA 1140

EpANS1          927 ------------------------------------------------------------ 927
Virgin-ANS1    1141 GTTTTTTTTGCTATAAATAGATAGGAGTACTTTATTTATTATTGTACCATTTTAATTTTT 1200

EpANS1          927 ------------------------------------------------------------ 927
Virgin-ANS1    1201 CAATTAAGTAAGAATTTCAGCAGTAACTAAATCGGTGATTTGTGAGTTTGATCTTTGGGC 1260

EpANS1          928 ----------------CCCAAGGATAAAATCATCCTGAAACCGCTCCAAGAGACTGTTTC 971
Virgin-ANS1    1261 CTGAACCAACAAACCACCCAAGGATAAAATCATCCTGAAACCGCTCCAAGAGACTGTTTC 1320

EpANS1          972 TAAGGAGGAGCCGCCACTCTTTCCGCCACGAACCTTCCATCAGCATATGGAACACAAGCT 1031
Virgin-ANS1    1321 TAAGGAGGAGCCGCCACTCTTTCCGCCACGAACCTTCCATCAGCATATGGAACACAAGCT 1380

EpANS1         1032 GTTTAGAAAGAACAATGACAAACTCAACCGAAATGATACTGGGTGA              1077
Virgin-ANS1    1381 GTTTAGAAAGAACAATGACAAACTCAACCGAAATGATACTGGGTGA              1426
```

A



B

Fig. 3.5. Comparison of sequences of *EpANS* in the white 'Virgin' and the wild type. Schematic representation of *EpANS* structure from the white 'Virgin'. The blue boxes indicate exons and the black lines indicate introns. The insertion in' Virgin' is shown as a blue triangle and located between positions 923–924 downstream of the ATG initiation codon.

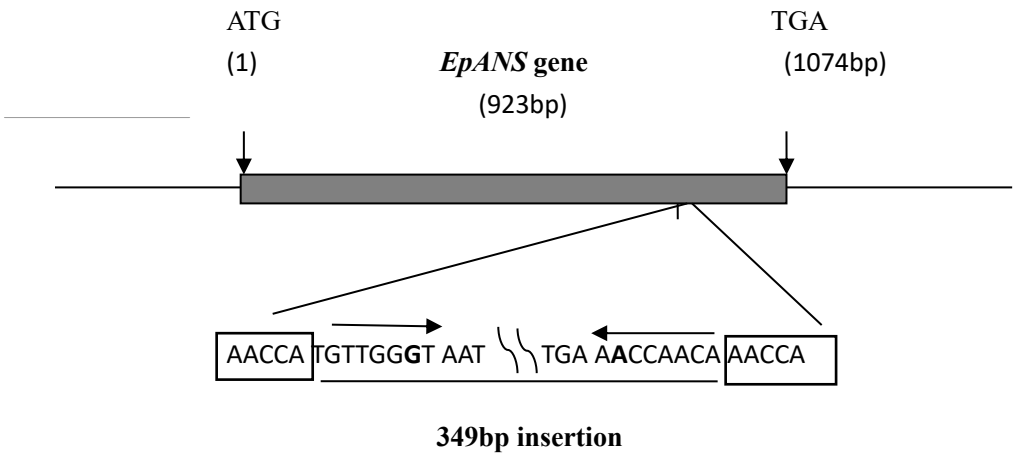


Fig. 3.6. Structure of *EpANS* in 'Virgin'. The shaded box represents the exon of the *EpANS* gene. The position of the *EpANS* insertion is indicated. The target site and its duplication are boxed. The terminal inverted repeats are shown by arrows. Nucleotides corresponding to the normal *EpANS* gene are numbered (beginning with the initiation codon in position 1) and their position numbers are shown in parentheses. The central part of the *EpANS* insertion element has been omitted from the figure. TSD = target site duplication, TIR = terminal inverted repeat.

Chapter 4

Summary

E. purpurea is a perennial herbaceous plant, purple coneflower, flower-tops. In recent years, *Echinacea* species have gained much attention due to their ornamental characteristics. The variety of colors in this plant warrants further genetic studies and improvement. Thus, efficient methods for its anthocyanin biosynthesis analysis are needed to improve ornamental qualities of this species.

In this study, I took *E. purpurea* molecular inheritance as a long-term goal. UPLC analysis showed that Cyanidin 3-glucoside (Cy3G) and cyanidin 3-malonyl glucoside (Cy3MalG) were the main anthocyanin in petals of *E. purpurea.* The structural gene F3'H,DFR, ANS of anthocyanin synthesis pathway was cloned by homologous cloning method and RACE technology in *E. purpurea* (the wild type) and the white 'Virgin'. The full length sequence of these genes was also verified by phylogenetic analysis with *Chrysanthemum* and other plants, the candidate genes were named as *EpF3'H EpDFR* and *EpANS* due to their similarity with their homologous genes, respectively. Their expression pattens were analyzed in different flower development stages and found genes expression were largely consistent with the relative content of anthocyanin. This suggested that they are functional components of the coloration in *E. purpurea. EpF3'H* gene contains 4 cytochrome P450-specific conserved motifs and 3 F3'H-specific motifs. And the expression pattern of it was basically consistent with the relative content of anthocyanin. This suggested that EpF3'H are functional components of the coloration and may be decisive for anthocyanins B-ring hydroxylation pattern in *E. purpurea*.

The gene structure sequence differences between the wild type and 'Virgin' were analyzed. To *EpDFR*, there was no changed amino acid sequence was found, which maybe not linked with the white color synthesis. There was a non-autonomous transposable elements insertion of *EpANS* in 'Virgin', which inhibited normal ANS

transcriptions and caused low levels of incomplete *EpANS* transcripts. So the insertion maybe responsible for the white anthocyanin-less phenotype.

Chapter 5

Future prospects and recommendations

My results contribute to investigate the possible function of the structural genes *EpF3'H, EpDFR* and *EpANS* from *E. purpurea* in the biosynthesis of anthocyanin. Thus here are some recommendations for future aspect.

1. Further experimental investigations has been recommended to find out the concrete confirmation about the function of *EpDFR* and *EpANS* in the model plants *Arabidopsis* should be experimentally verified.

2. The successful gene transformation method into *E. purpurea* should be find out and the investigated *EpF3'H, EpDFR* and *EpANS* genes should also transferred into mother plant to confirm the observed functions especially regarding anthocyanin synthesis.

3. Testcrosses between 'Virgin' and the wild type or other breeding lines should be carried out to identify the function of the nonautonomous transposable elements of *EpANS* and used to cultivate more new color varieties.

List of achievement

1. Insertion of a novel transposable element disrupts the function of an anthocyanin biosynthesis-related gene in *Echinacea purpurea* (**Scientia Horticulturae, 282: 110021, 2021**)

2. Isolation and functional analysis of flavonoid 3'-hydroxylase in echinacea (**生物化工, accepted**)

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