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RESEARCH ARTICLE

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

Hongsong Wu

Abstract *Echinacea purpurea* L. is one of the most important medicinal plants which is widely grown as flowering potted or garden plants in many countries. However, variation in flower color is somewhat limited in the cultivars. Anthocyanin is responsible for producing many floral colors in the visible spectrum. Therefore, understanding the genetic networks of flower coloration to broaden our understanding of anthocyanin biosynthesis pathways in *E. purpurea* is of great significance. In our study, we studied the expression patterns of *EpF3'H*, the phylogenetic tree, the multi-alignment of deduced amino acid sequences and *35S*::*EpF3'H* in *Arabidopsis tt7* mutants. The *35S*::*EpF3'H* exhibited apparent phenotypic alterations to restored the ability of the *Arabidopsis tt7* mutants, suggesting that the *EpF3'H* participated in anthocyanin synthesis. This study lay a foundation for understanding the anthocyanin biosynthesis pathways in *E. purpurea*.

Keywords: *E. purpurea*, Flavonoid 3'-hydroxylase (F3'H), Molecular cloning, Genetic transformation, Anthocyanin biosynthesis.

Introduction

Echinacea purpurea L. is one of the world's most important and well-known medicinal plants, belonging to the *Asteraceae* (Compositae) family. At present, the demand for *E. purpurea* is greater than the wild supply, so it is widely cultivated all over the world, including Japan. *E. purpurea* species have gained much attention in recent years due to their ornamental characteristics. The cyanidin was identified using high speed counter-current chromatog (Li et al. 2012). Molecular studies of *E. purpurea* have focused on genetic diversity (Jiang et al. 2021). The genes related to anthocyanin biosynthesis are rarely reported in *E. purpurea*.

Flower color is one of the most outstanding, important traits of a flower. The most abundant and predominant flavonoid pigments are anthocyanins, which produce a wide range of colors, such as orange, pink, red, magenta, purple, violet and blue (Jing et al. 2021). The biochemical functions of F3'H are usually identified by measuring the colour changes in plants. A single-base deletion in the *F3'H* gene resulted in deletion of the haem-binding domain of the F3'H and color change of pubescence colour from brown to grey in soybean (Toda et al. 2002). In addition, a *F3'H* mutant produced pelargonidin-based anthocyanin (Hoshino et al. 2003).

In this study, we isolated the *F3'H* cDNA from the wild-type flowers of *E. purpurea* and confirmed its function as a flavonoid 3'-hydroxylase that functions in cyanidin synthesis by transgenic complementation of the *Arabidopsis F3'H* (*tt7-1*) mutant plants. This is the report of molecular basis underlying the color in *E. purpurea*, and our 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. purpurea* in the future.

Materials and methods

Plant materials

Cultured plants of *E. purpurea* were purchased from from Sakata Seed Co. (Japan) and cultivated outdoor. The petals were collected from flowers at 4 different stages. We used at least three pots for biological repeats. *Arabidopsis tt7*

Department of Life Science and Green Chemistry, Graduate School of Engineering, Saitama Institute of Technology, 1690 Fusaiji, Fukaya, Saitama, Japan.

***Corresponding Author:** Hongsong Wu, Department of Life Science and Green Chemistry, Graduate School of Engineering, Saitama Institute of Technology, 1690 Fusaiji, Fukaya, Saitama, Japan, E-Mail: hellowhs2021@126.com

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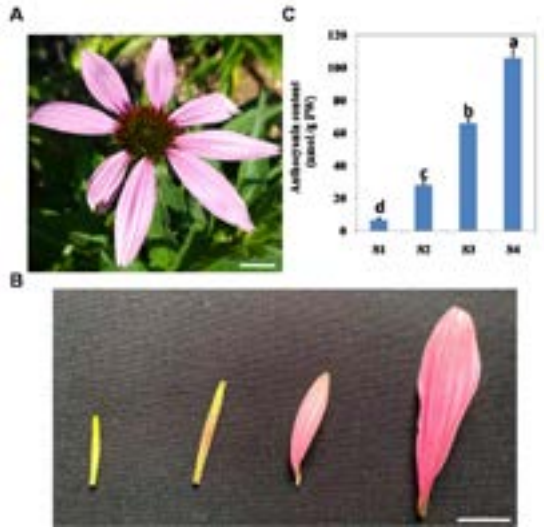

Fig. 1. The photograph and harvested at developmental stages of *E. purpurea*. **A:** Photograph of the *E. purpurea.* B: *E.purpurea* were harvested at developmental stages defined as follows: Stage 1: closed bud (less than 15 mm in length); Stage 2: enlarged closed bud with slight pigmentation (about 20 mm in length); Stage 3: bud just opening (clearly pigmented) and Stage 4: fully opened flower stage (more than 30 mm in length). C= The content of anthocyanin in developmental stages of *E.purpurea*.

Scale bars = 1cm

mutant, and T_2 transgenic seeds were germinated on MS medium (22°C, 16-h/light, 8-h/dark).

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*. A pair of primers was designed in Supplementary Table S1 and PCR amplification was performed using the cDNA of *E. purpurea* as the template. DNA sequencing was performed using the automated sequencer (ABI 3730xl Analyzer; Applied Biosystems) according to the manufacturer's protocol.

Phylogenetic analysis

Phylogenetic trees were constructed using Genetyx-ver12 software with the neighbour-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. The reliability of the trees was evaluated using the bootstrap resampling method (Felsenstein 1985) with 1000 replicates.

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. purpurea*. The gene expression was quantitated with the comparative Ct method (Bogs et al. 2006). *EpACT1* was used as an internal reference for comparison of expression level. The primers are displayed in Supplementary Table S1.

Vector construction and genetic transformation

The CDS of *EpF3'H* was inserted into the pCAMBIA1300 vector downstream of 35S promoter. The *35S::EpF3'H* vector was genetically transformed into *Arabidopsis tt7* mutant. The homozygous T_2 transgenic seeds were individually harvested and used for further investigation.

Results

The photograph and the content of anthocyanin in developmental stages of E. purpurea

The *E. purpurea* has been used in the present study. Photographs of the *E. purpurea* flowers were pink (Fig. 1A). *E. purpurea* were harvested at developmental 4 stages. Stage 2-4 of flowers gradually turned pink (Fig. 1B). The total anthocyanin content of the developmental 4 stages increased from S1 to S4 (Fig. 1C). These results suggested that stage 2-4 of flowers gradually turn red due to anthocyanin synthesis.

Isolation of the full-length cDNA of EpF3'H gene

To investigate the function of EpF3'H in regulating the anthocyanin synthesis of *E. purpurea*, we obtained an integrity cDNA sequence of *EpF3'H* (Fig 2A; Fig S1). It contains an ORF of 1533bp, 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. Real-time quantitative PCR analysis showed that *EpF3'H* were detected in the leaves and flowers with different expression levels. *EpF3'H* was most abundantly expressed in both S4, followed by, S2, S1, S3 and the leaf of *E. purpurea* (Fig 2B). The expression pattern of *EpF3'H* was approximately corresponds to the anthocyanin accumulation pattern in *E. purpurea*.

Homology and structural characters of EpF3'H

A phylogenetic tree of F3'Hs revealed that EpF3'H and the F3'Hs of *Gerbera* hybrid cultivar, *Chrysanthemum morifolium*, clustered together and formed a subgroup (Fig. 3). Protein multiple sequence alignment analysis showed a high

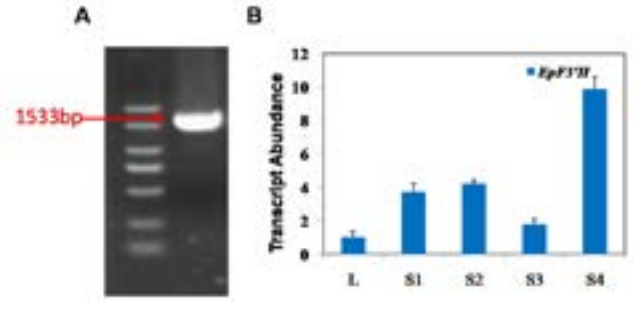

Fig. 2. The molecular cloning and expression analysis of the *EpF3'H* **gene. A:** The complete open reading frames of flavonoid 3'-hydroxylase (*F3'H*) gene. B: All images show RT-PCR products obtained after 40-cycles. S1, S2, S3 and S4 show different stages of flower. L defined as the leaf. Error bars indicate ± SD (n=3, from three technical replicates)

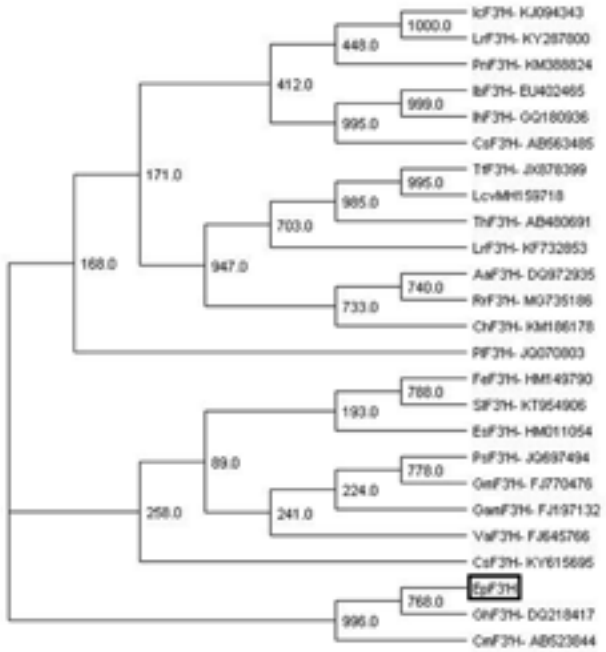

Fig. 3. Phylogenetic tree of F3′H sequences, constructed using the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The black box indicate F3′H isolated from *E. purpurea* in this study. EMBL/DDBJ/GenBank DNA database accession numbers and species names (except EpF3′H) are shown

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, indicating that *EpF3′H* is a member of the F3′H family and might have the same catalytic function as the other F3′Hs (Fig. 3).

EpF3′H has four cytochrome P450-specific conserved motifs and three F3′H-specific conserved motifs according to NCBI conserved domain search (Fig. 4). The presence of all these F3′H-specific conserved motifs indicated that *EpF3′H* is a typical F3′H protein.

Ectopic expression of EpF3′H contributes to anthocyanin accumulation in the Arabidopsis tt7 Mutant

The *tt7* mutants of *Arabidopsis* have pale brown seeds and reduced proanthocyanidin content (Zhang et al. 2020). *EpF3′H* was overexpressed in *Arabidopsis tt7* mutants. qRT-PCR analysis showed that high level expression of *EpF3′H* gene could be examined in *Arabidopsis* T_2 transformants (OE-2, OE-4 and OE-6) (Fig. 5A). The *35S::EpF3′H* exhibited apparent phenotypic alterations to restored the ability of the Arabidopsis *tt7* mutants, and the phenotypic of transgenic line (OE-2) were shown. (Fig. 5B). In addition, non-transgenic *Arabidopsis tt7* seed coats exhibited pale brown, whereas the *35S::EpF3′H* plants seed coats was

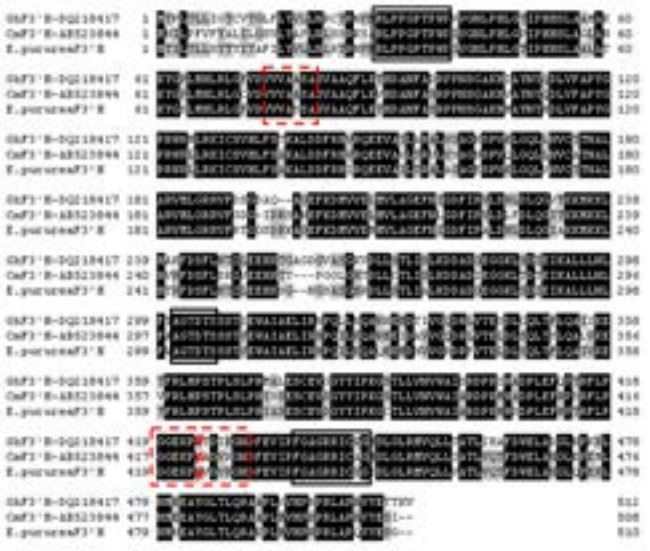

Fig. 4. Multi-alignment of deduced amino acid sequences of *EpF3′H* in *E. purpurea*. Identical amino acids are indicated in white foreground and black background; Blocks of similar amino acids are indicated in white foreground and gray background. Black line boxes are cytochrom P450-specific conserved 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

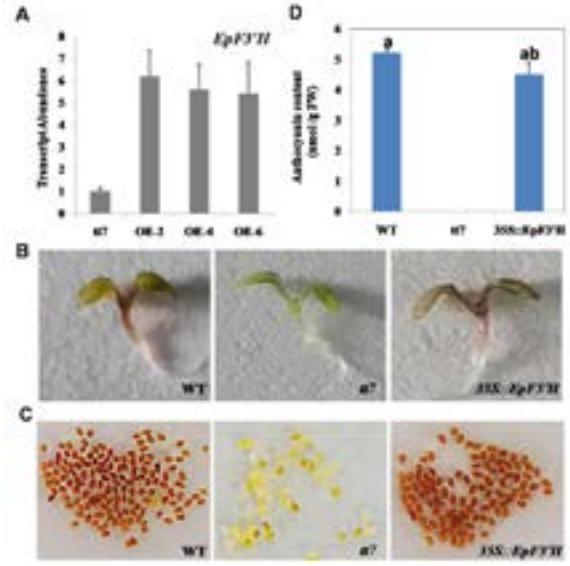

Fig. 5. Ectopic expression of *EpF3′H* contributes to anthocyanin accumulation in the *Arabidopsis tt7* Mutant. A: *EpF3′H* expression in *tt7* and *35S::EpF3′H Arabidopsis* seedlings. B: Phenotypes of wild-type, *tt7* and *35S::EpF3′H Arabidopsis* seedlings. C: Phenotypes of wild-type, *tt7* and *35S::EpF3′H Arabidopsis* seeds. D: The content of anthocyanin in wild-type, *tt7* and *35S::EpF3′H Arabidopsis* seedlings. Error bars indicate ± SD (n=3, from three technical replicates).

brown (Fig. 5C). The *35S::EpF3′H* transgenic *Arabidopsis* lines accumulate significantly higher levels of anthocyanin (Fig. 5D). These results strongly suggest that *EpF3′H* encode a functional protein, which had a special catalytic activity of flavonoid 3′-hydroxylase.

Discussion

In the wild type of *E. purpurea*, two anthocyanin derivatives cyanidin 3-glucoside and cyanidin 3-malonyl glucoside were reported to be the main pigmentation component. In this study, we sought to identify a F3'H homolog that functions in anthocyanin hydroxylation and flower coloration. The *F3'H* gene was 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 1533 bp long ORF encoding a 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.

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. For instance, F3'H is supposed to be involved in anthocyanin biosynthesis and flavone biosynthesis (Park et al. 2021).

From the analysis of results obtained in the present study, it is reasonable to speculate that F3'H function in anthocyanin hydroxylation which is involved in determining flower coloration of *E. purpurea*, similar to the function of F3'Hs in morning glory species (Hoshino et al. 2003), *G. triflora* (Nakatsuka et al. 2005), *Antirrhinum* (Ishiguro et al. 2012), etc. In conclusion, the results of the present study suggested that *EpF3'H* functional components of the coloration and this study lay a foundation for understanding the anthocyanin biosynthesis pathways in *E. purpurea*.

Authors' contributions

Conceptualization of research (HW); Designing of the experiments (HW); Contribution of experimental materials (YA); Execution of field/lab experiments and data collection (YA); Analysis of data and interpretation (HW); Preparation of the manuscript (HW).

References

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

Carla S., Stefan M., Christian E., Gert F. and Bernd W. 2000. Identification of the Arabidopsis thaliana Flavonoid 3'-Hydroxylase Gene and Functional Expression of the Encoded P450 Enzyme. Biol. Chem., **381**: 749–753.

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

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

Ishiguro K., Taniguchi M. and Tanaka Y. 2012. 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.

Jiang L., Fan Z., Tong R., Yin H., Li J. and Zhou X. 2021. Flavonoid 3'-hydroxylase of Camellia nitidissima Chi. promotes the synthesis of polyphenols better than flavonoids. Mol Biol Rep., **48**: 3903–3912.

Jing Z.H., Yin M.J., Wang Q., Bao K., Zhou P.N., Liu C.C. and Wu Q.N. 2021. Expression profiling and functional verification of flavonoid 3'-hydroxylase gene from leaves of Euryale ferox. Zhongguo Zhong Yao Za Zhi., **46**: 4712–4720.

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

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

Li J.Y., Luo J., Li M.L., Lu Y. and Liu Z.H. 2012. Separation of anthocyanins in echinacea purpurea flower by high speed counter-current chromatography and the research on the antioxidant activity of the anthocyanins. Fenxi Ceshi Xuebao., **31**: 45–50.

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

Park S., Lee H., Min M.K., Ha J., Song J., Lim C.J., Oh J., Lee S.B., Lee J.Y. and Kim B.G. 2021. Functional Characterization of *BrF3'H*, Which Determines the Typical Flavonoid Profile of Purple Chinese Cabbage. Front Plant Sci., **12**: 793589.

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

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

Toda K., Yang D., Yamanaka N., Watanabe S., Harada K. and Takahashi R. 2002. A single-base deletion in soybean flavonoid 3 0-hydroxylase gene is associated with gray pubescence color. Plant Mol. Biol., **50**: 187–196.

Zhang Y.Z., Cheng Y.W., Xu S.Z., Ma H.P., Han J.M. and Zhang Y. 2020. Tree peony variegated flowers show a small insertion in the F3'H gene of the acyanic flower parts. BMC Plant Biol., **20**: 221.

Supplementary Table S1. **The list of Primers used in the present study**

Primer	Sequence (5–3′)
EpF3′H-FP	ATGACTATTCTAACCCTACTATCATACACC
EpF3′H-RP	TTAACCACTTTCATATACTTGAGG
For RT-PCR	
EpF3′H-FP1	ACAGTGGAATGGGCAATAGC
EpF3′H-RP	GTCTCCTTTACGATGGCTTG
EpACT-FP1	TATGTTGCTATTCAGGCCGTG
EpACT-RP1	GTGATAACTTGTCCATCAGGC
For Heterologous expression SalI--EpF3′H1-FP KpnI-EpF3′H1-RP	GTCGACATGACTATTCTAACCCTACTATC GGTACCTTAACCACTTTCATATACTTGAGG

Supplementary Figure S1. **Isolation of the full-Length cDNA and amino acid of *EpF3′H* gene**