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

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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 lays 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 chromatography (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 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

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mutant, and T₂ 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-вер12 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 T₂ 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 pl 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
Flavonoid 3'-hydroxylase gene from purple coneflower (Echinacea purpurea)

The 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 T2 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 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 monfolium* 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*. Which Determines the Typical Flavonoid Profile of Purple Chinese Cabbage. Front Plant Sci., 12: 793–797. 

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 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


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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tr>
<td>EpF3'H-FP</td>
<td>ATGACTATTCTAACCCTACTATCATACACC</td>
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<tr>
<td>EpF3'H-RP</td>
<td>TTAACCTTGTTATCTACCC</td>
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<tr>
<td>For RT-PCR EpF3'H-FP1</td>
<td>ATGAC TAT TC TAACCC TAC TATCATACCC</td>
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<tr>
<td></td>
<td>TTAACCTTGTTATCTACCC</td>
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<tr>
<td></td>
<td>EpACT-FP1: ACAGTGGAATGGGCAATAGC</td>
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<tr>
<td></td>
<td>GTCTCCTTTACGATTGCTTG</td>
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<tr>
<td>EpACT-RP1</td>
<td>EpACT-FP1: TATTTGCTATTCAAGGCGTG</td>
</tr>
<tr>
<td></td>
<td>GTGATAACTTGTCCATACCC</td>
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<tr>
<td>For Heterologous expression</td>
<td>SalI--EpF3'H1-FP: GTCGACATGACTATATCTAACCCTACTATC</td>
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<tr>
<td></td>
<td>KpnI-EpF3'H1-RP: GGTACCTTAAACCTTTTATATACTTGAGG</td>
</tr>
</tbody>
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**Supplementary Figure S1. Isolation of the full-Length cDNA and amino acid of EpF3’H gene**