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Cloning and sequence analysis of a flavanone 3-hydroxylase gene from *Prunus persica* (L.) Batsch

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Abstract

Anthocyanins as a type of plant flavonoids, is a kind of important plant secondary metabolites, which is catalyzed by a series of enzymes such as flavanone 3-hydroxylase (F3H). Here a *F3H* gene, designated as *PpF3H*, was cloned from *Prunus persica* (L.) Batsch. The full-length cDNA of *PpF3H* gene was 1453 bp in length, containing a 1086 bp open reading frame (ORF) which encodes 361-amino-acid proteins with a calculated molecular weight of 40.50 kDa and isoelectric point of 5.45. Protein analysis and phylogenetic tree analysis indicated that *PpF3H* shared the same ancestor in evolution with other F3Hs and had a further relationship with other angiosperms species. Use SWISS-MODEL to perform three-dimensional structure, the results showed that *PpF3H* had a jerry roll in the enzyme core consisted of *β*-fold, and all of the 2-oxoglutarate-dependent dioxygenase share a common structure. The model analysis showed that the PpF3H protein contains 17 α-helixes and 17 *β*-sheets. Molecular cloning and sequence analysis of *PpF3H* were carried out to further study the molecular mechanisms of biosynthese of anthocyanins.

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Introduction

Prunus persica (L.) Batsch is a kind of perennial xylophyta, which belongs to *Rosaceae*. The species of peach can be divided into two categories, edible and ornamental peach. Edible pulp is delicate and juicy, aromatic flavor, rich nutrition, which is not only used as fresh food, also be processed into preserved peach, peach juice, dried and canned peach, so peach is loved by the masses of the people. Ornamental peach possesses exuberant flowers, gorgeous color, which plays a key role in beautifying the city landscape, improving the ecological environment, and increasing people's quality of life (Debeaujon *et al.*, 2000). China harbor is the largest producer of peach in the world, the plant area and production are the highest in the world, based on the excellent and rich resources, it is convenient for us to carry out our research (Chai and Hu, 2003).

Flavonoids is a class of important plant secondary metabolites, it exist in fruits, vegetables, beans and tea and many other plants in the form of conjugate, whose compounds including flavonols, flavones, chalcones, condensed tannins or proanthocyanidins and anthocyanins (Shirley, 2001). Since the flavonoid biosynthetic genes often coordinated regulatory approach flower or fruit development stage, which result in the accumulation of specific flavonoids. It plays an important role in various biological functions in plants, such as in medical treatment to prevent gastric cancer, protect the function of the heart (Peer *et al.*, 2004), and symbiotic microbes signaling (Wasson *et al.*, 2006), also can prevent the ultraviolet ray (Li *et al.*, 1993), influence the fertility of plant (Stam *et al.*, 1992) and act as antitoxin of plants. Flavonoid is one of the major flower pigments in plants (Matthew *et al.*, 1996). As a signal molecule in plant - microbe interactions, flavonoids provide pigmentation attracts pollinators (Hahlbrock *et al.*, 1989).

Flavanone3-hydroxylase (F3H) is one of the key enzymes in the accumulation process of dihydroflavonol, which catalize the synthesis of flavonoid and anthocyanin (Fig.1). The expression of F3H will determine and influence flavonoids and anthocyanins substances in plants and the content of flavonoids and anthocyanins may change the plant organs color depth, variety (Elomaa *et al.*, 1993). Studies have shown that flavanone3-hydroxylase (F3H) gene was expressed in plant roots, stems, leaves and flowers (Jeong *et al.*, 2006), so far, flavanone 3-hydroxylase (F3H) genes have been cloned from many plants (Charrier *et al.*, 1995; Deboo *et al.*, 1995; Pelt *et al.*, 2003) such as tea (Hu *et al.*, 2014) and *Juglans regia* (Xu, 2010). Previous study on fruit crops suggested that a lot of structure genes encoding enzymes in the anthocyanin biosynthesis pathway have been cloned (Boss *et al.*,1996; Cultrone *et al.*,2010 and Honda *et al.*, 2002) and some functions of plant genes F3H also been described in detail, such as F3H gene determines the rendering of flowers and fruit, like carnations (Zuker *et al.*, 2002), *Reaumuria trigyna* (Zhang *et al.*, 2014) and apple (kim *et al.*, 2003), will also affect the growth and development of plants, such as arabidopsis (Pelletier *et al.*, 1996), will also affect the concentration of other hormones in plants, such as F3H gene content and concentration of catechin in tea positively correlated (Singh *et al.*, 2008) lotus effect *in vivo* formation of some substances have health effects (Grotewold *et al.*, 2005), also affects the metabolic pathway of gymnosperms (Shen *et al.*, 2006). However, up to now, no *F3H* gene was cloned and characterized from *P. persica* (L.) Batsch. Therefore, in this report, a *F3H* gene was cloned from *P. persica* (L.) Batsch. At the first time, we analyzed the structure of the sequence, aiming to provide the gene resource for increase the content of anthocyanins in *P. persica* (L.) Batsch using genetic engineering.

Materials and methods

Plant material

The fruits of *P. persica* (L.) Batsch were taken in the Peach Blossom Village in Jingzhou city and immediately placed in a -80 °C freezer to preserve. cDNA Prime Script® 1st Strand cDNA Synthesis Kit, DNA agarose gel purification equipment Ver.4.0, pMD18-T vector kit, dNTPs RNasinlamv reverse transcriptase ,where in the primer synthesis and DNA

sequencing were carried out by Shanghai Sangon biotechnology company in China, and Tap DNA polymerase purchased from the Takara Company (Dalian, China).

RNA isolation and cDNA synthesis

Using CTAB method to extract the total DNA of *P. persica* (L.) Batsch (Testolin *et al.*, 2000), and using the RNA QIAGEN - RNeasy MiNiKit to extract the RNA of *P. persica* (L.) Batsch leaves. After using 1% agarose gel electrophoresis detection, the RNA was used for reverse transcription cDNA synthesis follow the cDNA Prime Script® 1st Strand cDNA Synthesis Kit instructions.

Full-length cDNA clone of F3H gene

A pair of specific primers PpF3HUP (5'-ATGGCTCCTGCTACTACTCTCAC-3') and PpF3HDP (5'-TAACACGAGCAAGCCTTGAGA-3') were designed with the EST sequence of chamomile F3H gene. Using gradient PCR to set up a total of 10 gradient and confirm the best annealing temperature is 59°C, and then the RT-PCR was performed using the one-step RT-PCR kit (Dalian TaKaRa, China) under the following reaction system: ddH_2O 16.75μl, buffer 2.5μl, $MgCl_2$ 2μl, Primer(F) 1μl, Primer(R) 1μl, dNTP 0.5μl, Taq polymerase 0.25μl, cDNA 1μ, and the reaction conditions : 94°C 4min initial denaturation; 94°C 30S, 59°C 30s, 72°C 90s, 32 cycles, 72°C 10min. The PCR products were separated on a 1% agarose gel electrophoresis. (Flugge and Gao, 2005) to get purified PCR product, and the PCR product was cloned into the pMD18-T vector (Dalian TaKaRa, China), and then sequenced.

Bioinformatics analysis

The obtained sequences were analyzed using online bioinformatics tools (http:// www.ncbi. nlm.nih.gov and http://www.expasy.org). The software DNAman 6.0 was used to analyze *PpF3H* gene sequence and amino acid composition. The software Clustalx 2.0 was used for multi-alignment, and the software MEGA 6.0 was used to construct the evolution tree. The three-dimensional structure of PpF3H protein was analyzed using online bioinformatics tools (http://swissmodel.expasy.org/).

Results

PpF3H gene sequence analysis

Use the RT-PCR and the cDNA fragments of peach as templates encoding F3H gene (designated as *PpF3H*) were BLastn analysis on NCBI. The results showed that the sequence and other angiosperms F3H gene nucleotide sequence similarity is higher, Respectively 96%, 97%, 90%, 89%, 82%, 81%, 82%, 81%, 80%, 76%, 88%(Table .1).The *PpF3H* had a closer relationship with F3H from prunus plants than from others. These results implied *PpF3H* is a member of F3H gene family in *P. persica* (L.) Batsch.

Table 1. Sequence similarity between Peach *PpF3H* gene and other plants F3H nucleic acid.

Species	GenBank Accession No.	Identity	E-value
Prunus avium	JF740092.1	96%	0.0
Prunus ceras	JQ622245.1	97%	0.0
Pyrus communis	KC460396.1	90%	0.0
Malus domestica	FJ817486.1	89%	0.0
Theobroma cacao	XM_007046636.1	82%	0.0
Dimocarpus longan	EF468104.1	81%	0.0
Gossypium hirsutum	GU434116.1	82%	0.0
Mangifera indica	KF929410.1	81%	0.0
Vitis vinifera	FQ392497.1	80%	0.0
Daucus carota	AF184270.1	76%	7e-136
Rubus coreanus	EU255776.1	88%	1e-138

PpF3H protein sequence analysis

Using DNAman and online website (http://web.expasy.org/protparam/) for protein sequence analysis and structure prediction, the results showed that the full-length cDNA of *PpF3H* gene is 1453 bp and encoding a 361-amino-acid (Fig.2), the protein molecular formula is derived for $C_{1797}H_{2842}N_{488}O_{550}S_{13}$, the theory of isoelectric point is 5.45, the molecular weight of 40.50 KDa, the positively charged amino acid residue base is 46, the negatively charged amino acid base of residual charge also is 55, instability index is 35.27, classified as stable protein, the hydrophilicity of -0.468 on average, the protein presumably hydrophobic protein.

Table 2. The GenBank accession no. of F3H genes in this paper.

Abbreviation	Species	GenBank Accession No.
MdbF3H	*Malus domestica*	AAX89397.1
Md232	*Malus domestica*	NP_001280854.1
MhcF3H	*Malus* hybrid cultivar	ACP30361.1
Msp23	*Malus sp*	CAA49353.1
PcsF3H	*Pyrus communis*	AAX89399.1
PcsF3H2	*Pyrus communis*	AGL81347.1
PcsF3H3	*Pyrus communis*	AAM18084.1
Md231	*Malus domestica*	NP_001280883.1
PxbF3H	*Pyrus x bretschneideri*	AGZ15309.1
PpyF3H	*Pyrus pyrifolia*	ADP09378.1
PcsF3H1	*Pyrus communis*	AGL50918.1
Pxb23	*Pyrus x bretschneideri*	XP_009363496.1
PTUPE	*Prunus persica*	XP_007202107.1
PppF3H	*Prunus persica*	BAC98346.1
PcPF3H	*Prunus cerasus*	AGG18109.1
PaF3H1	*Prunus avium*	AJO67967.1
PaN3D	*Prunus mume*	ADZ54782.1
PapF3H	*Prunus armeniaca*	AGG18093.1
PaF3H2	*Prunus avium*	AEO79981.1
Pmn23	*Prunus mume*	XP_008241821.1
RcoF3H	*Rubus coreanus*	ABW74548.1
PaF3H1	*Prunus avium*	AJO67967.1
PmF3H	*Prunus mume*	XP_008241821.1
PpF3H	*Prunus persica*	BAC98346.1
PcF3-H	*Prunus cerasus*	AGG18109.1
PaF3H2	*Prunus armeniaca*	AGG18093.1
PcF3H	*Pyrus communis*	AGL50918.1
PxbF3H	*Pyrus x bretschneideri*	AGZ15309.1
Md203d	*Malus domestica*	NP_001280883.1
PpyF3H	*Pyrus pyrifolia*	ADP09378.1
RcF3H	*Rubus coreanus*	ABW74548.1
AlF3H	*Arabidopsis lyrata*	XP_002876075.1
LcF3H	*Lycium chinense*	AID50182.1
PaF3bH	*Pimpinella anisum*	AAX21535.1
IpF3H	*Ipomoea purpurea*	ABW69678.1
AgF3H	*Ampelopsis grossedentata*	AFN70721.1
VrF3H	*Vitis rotundifolia*	AGS57503.1
MiF3H	*Mangifera indica*	AIY24993.1
DlF3H	*Dimocarpus longan*	ABO48521.1
GhF3H	*Gossypium hirsutum*	ADC96713.1
GbF3H	*Gossypium barbadense*	ABL86673.1
FxaF3H	*Fragaria x ananassa*	BAE17126.1
DcF3H	*Daucus carota*	AAD56577.1
SpF3bH	*Solanum pinnatisectum*	AAX63401.1.

Characterization of the deduced PpF3H protein

A database search with Blast P (NCBI) and multi-alignment by DNAman 6.0. showed that the deduced *PpF3H* polypeptide had considerable high homology with other plant F3Hs (Fig. 3). The multiple comparison showed that the *PpF3H* protein gene protein and other reference F3H protein consistency of 93.08%, with conservative domain structure, therefore maintain a strong protection in molecular evolution. All of the data indicated that *PpF3H* was mentioned above a member of the family of F3Hs.

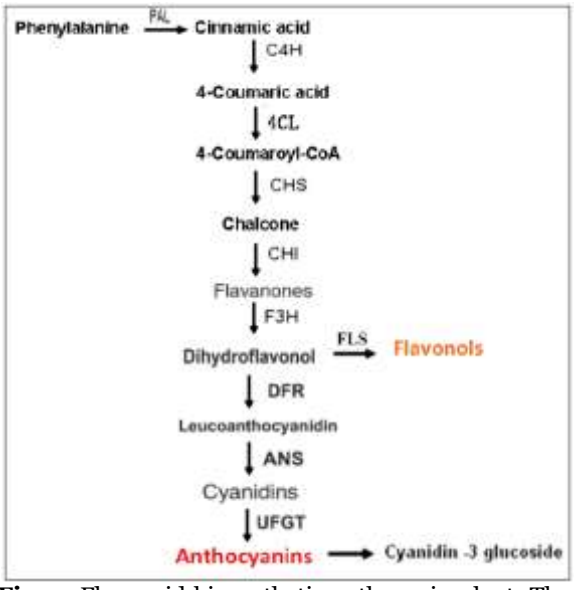

Fig. 1. Flavonoid biosynthetic pathway in plant. The abbreviations are as follows: *PAL*, *phenylalanine ammonia-lyase*; *C4H*, *cinnamate 4-hydroxylase*; *4CL*, *4-coumarate-CoA ligase*; *CHS*, *chalcone synthase*; *CHI*, *chalcone isomerase*; *F3H*, *flavanone 3-hydroxylase*; *FLS*, *flavonol synthase*; *DFR*, *dihydroflavonol 4-reductase*; *ANS*, *anthocyanidin synthase*; *UFGT*, *UDP-glucose-flavonoid3-O-glycosyltransferase*.

PpF3H gene molecular evolution analysis

A phylogenetic tree was constructed based on the deduced amino acid sequences of *PpF3H* and other F3H family proteins from other plant species. Using online bioinformatics tools (http://www.ncbi. nlm.nih.gov and (http://www.expa sy .org) to elect different plants F3H gene family as multiple functional gene sequences and a phylogenetic tree was constructed with the software ClustalX 2, MEGA 5.1 and use the Neighbor - joining(NJ) method (Kumar *et al.*, 2004) to statistics. Phylogenetic tree consists of *PpF3H* protein together with Rosaceae other plants, bindweed plants and Solanaceae（Fig.4). The results showed that similar species is classified as a class, and all the plants F3H genes, whether they belong to woody plants or herb share the same ancestry, at the same time, *PpF3H* have further relationship with other species of angiosperm and the F3H gene of *PpF3H* and *Prunus* correlation is higher than other plants.

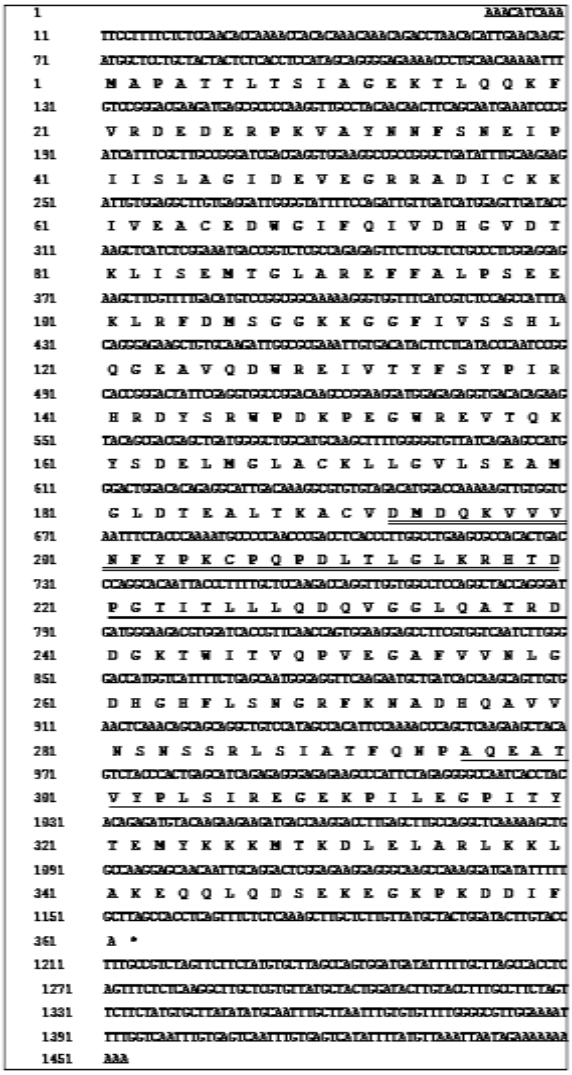

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *PpF3H*. The completely identical amino acids and conservative amino acids and underlined and defined by double, single line.

Three-dimensional model analysis

The three-dimensional structure of *PpF3H* protein was analyzed using online bioinformatics tools (http:/t/swissmodel.expasy.org/). In order to better

understand *PpF3H* protein the three-dimensional structure is modeled *PpF3H* gene, Switzerland execution mode (SWISS-MODEL), the three-dimensional structure model with weblab viewerlite. Analysis, as shown in Fig.5, *PpF3H* had a jerry roll in the enzyme core consisted of b-sheet, all of the 2-oxoglutarate-dependent dioxygenase share a structure, This figure cited in Structural and Mechanistic Studies on Anthocyanidin Synthase Catalysed Oxidation of Flavanone Substrates: The Effect of C-2 Stereochemistry on Product Selectivity and Mechanism. The model showed that the protein contains 17 α-helixes and 17 *β*-sheets.

Fig. 3. Sequence multi-alignment of the deduced *PpF3H* protein with other plants F3H proteins. The completely identical amino acids were indicated by white letters on a mazarine background, whereas those conservative amino acids were indicated by black letters on a red background and those non-similar amino acids were indicated by black letters on a white background.

Discussion

Many studies have shown that anthocyanin content determines the coloring of fruit, and it ultimately depends on the accumulation of regulating gene expression during the synthesis of key genes involved in a variety of control factors (Koes *et al.*, 2005). F3H play an important role in the early anthocyanin biosynthetic pathway. This study cloned *PpF3H* gene by using one step RT-RCR. *PpF3H* cDNA fragments length is 1453 bp encoding 459 amino acid sequence,

isoelectric point of 9.1. Protein sequence analysis, 3D structure model and phylogenetic tree analysis suggested that PpF3H protein has similar functions with other angiosperm F3H proteins. However, the biological significance of F3H gene is still unclear regulation in anthocyanin biosynthetic pathway. Although some plants for F3H gene anthocyanin biosynthesis has been little research, the related genes used in plant genetic engineering (El Kereamy *et al.*, 2002; Holton *et al.*, 1995). The study on F3H genes especially regulatory function in the anthocyanin biosynthesis on *P. persica* (L.) Batsch is remain limited. This research provide some foundation for verify the function of F3H in *P. persica* (L.) Batsch anthocyanin biosynthesis, and done a step for changing fruit coloring by biotechnology methods.

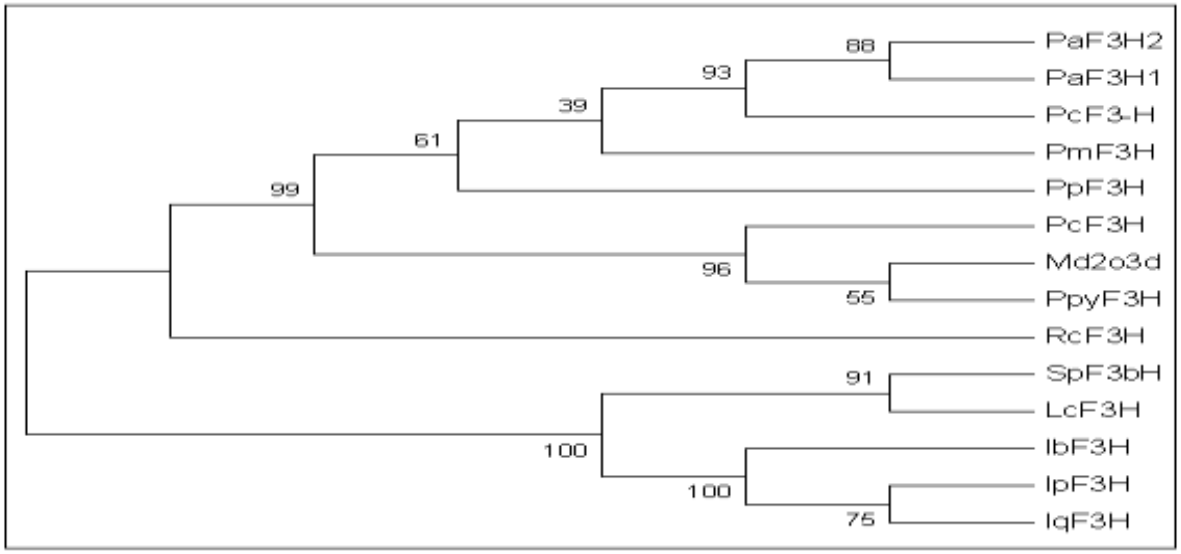

Fig. 4. Phylogenetic tree of the deduced PpF3H protein and other species F3H protein. The length of the line representing the evolutionary distance, each node marked the bootstrap value, repeat 1000 times.

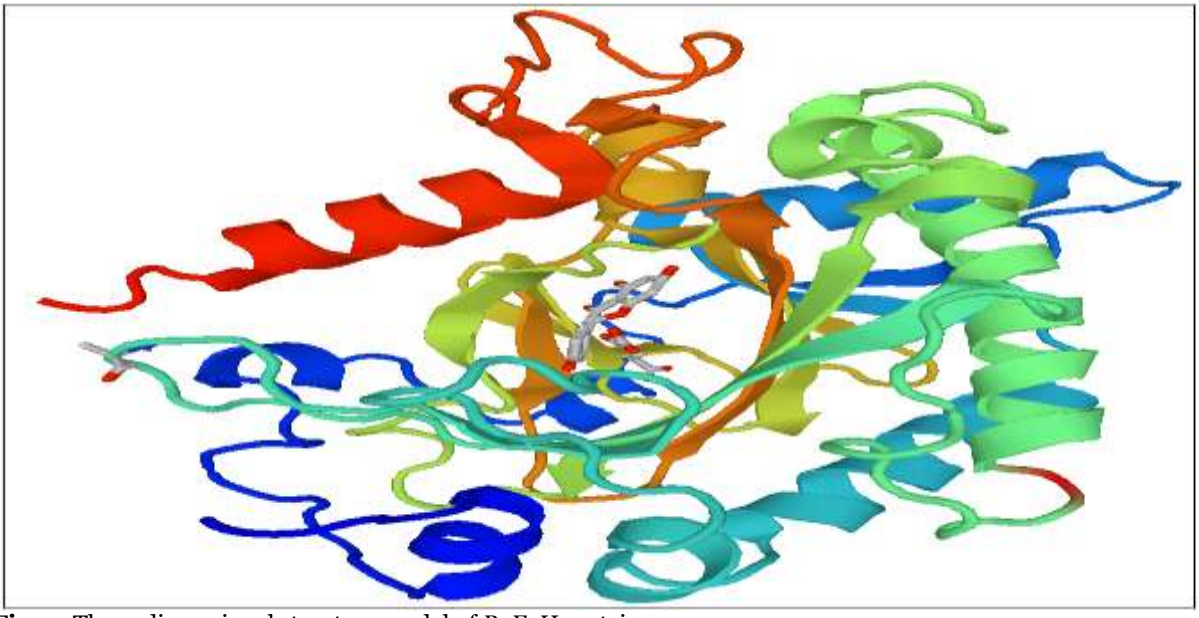

Fig. 5. Three-dimensional structure model of *PpF3H* protein.

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