

Identification and adaptive evolution analysis of FAR1/FHY3 in *Eleutherococcus senticosus* and prediction of target genes regulating saponin synthesis

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Abstract

Light plays a significant role in affecting secondary metabolic processes in the medicinal plant *Eleutherococcus senticosus*. The FAR1/FHY3 gene family responds to variations in light quality and play diverse roles in plant growth and development. However, few studies have explored and functionally analyzed the *EsFAR1/FHY3* gene family. In this study, 21 *EsFAR1/FHY3* genes, exhibiting uneven distribution across 14 chromosomes, were identified in *E. senticosus* using bioinformatic tools. *EsFAR1/FHY3* genes were divided into five subfamilies. The gene structure and conserved domains within each subfamily were similar. There were many collinear relationships between FAR1/FHY3 in *E. senticosus* and FAR1/FHY3 in related species. Evolutionary analysis indicated that *EsFAR1/FHY3* underwent predominantly negative selection and was highly conserved. There were 5–15 light response elements in the *EsFAR1/FHY3* promoter. The results of RNA sequencing and real-time fluorescence quantitative PCR showed that 15 *EsFAR1/FHY3* genes were differentially expressed after irradiation with different light qualities. Molecular docking showed that four *EsFAR1/FHY3* genes stably bind to the promoter of the *farnesyl diphosphate synthase* (FPS) gene involved in saponin synthesis. These findings suggest that *EsFAR1/FHY3* responds to changes in light quality and interacts with the promoter of the saponin synthase gene in *E. senticosus*, offering insights into the molecular mechanism underlying the impact of light quality on the secondary metabolism of *E. senticosus*.

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Introduction

Light is an important abiotic factor affecting plant growth and development. In addition to providing energy for the plant's photosynthesis, they also provide the plant with valuable environmental information^[1]. Plants can perceive and utilize light of varying quality, duration, and wavelength using diverse photoreceptors, such as phytochromes and transcription factors^[2]. In *Arabidopsis thaliana*, genes encoding different phytochromes are named phyA to phyE. These phytochromes can detect red and far-red light, regulate various life activities and processes of plants^[3,4].

Far-red impaired response 1 (FAR1) and its homologous protein, far-red elongated hypocotyl 3 (FHY3), are homologous proteins derived from transposases^[4]. They are key members of the FAR1-related sequence (FRS) family. They function redundantly to regulate phyA signal transduction^[5]. FAR1/FHY3s are widely found in angiosperms and are recognized as founding members of the FRS and FRS-related factor gene families. Initially identified in *A. thaliana*, they are crucial components of the phyA-mediated far-red light-signaling pathway^[4]. The FAR1/FHY3 protein contains three domains: C2H2 zinc finger domain, central core transposase, and SWIM zinc finger domain. Among these, the C2H2 zinc finger domain binds to DNA, whereas the core transposase domain and SWIM zinc finger domain are required for homologous or heterologous dimerization and transcriptional activation activity^[6,7]. As a transcription factor originating from an ancient mutant-like transposase, FAR1/FHY3 binds to cis-acting elements within the functional gene promoter, either as a homodimer or heterodimer, to modulate the expression of downstream genes^[8]. At present,

studies have shown that the FAR1/FHY3 gene family plays an important role in response to light changes. The unique role of Arabidopsis FAR1/FHY3 gene family and its members in light control of Arabidopsis development^[8]. FAR1/FHY3 regulates flowering time by integrating ambient light signals with the miR156-SPL module-mediated senescence pathway^[9]. It can also directly up-regulate the expression of SMXL6 and SMXL7 in response to light changes to promote Arabidopsis branching^[10]. Light can significantly affect the secondary metabolism of plants. For example, the light signal transduction factor CsHY5 of tea plant can bind to the promoter of the anthocyanin synthesis-related gene *CsbHLH89* of *Camellia sinensis* and indirectly promote the accumulation of anthocyanin^[11]. Different light quality can regulate the accumulation of triterpenoid saponins in *E. senticosus* by regulating the expression of bZIP transcription factor^[12]. The study by Shohael et al. showed that different light conditions could affect the accumulation of biomass and secondary metabolites of *E. senticosus*^[13]. Therefore, we have reason to speculate that the FAR1/FHY3 gene family of *E. senticosus* can respond to light changes and affect the accumulation of secondary metabolites of *E. senticosus*. Previous studies have shown that FAR1/FHY3 transcription factors positively regulate salt stress and temperature stress responses in *Eucalyptus grandis*^[5]. Genome-wide identification and expression analysis of *Zea mays* FAR1/FHY3 gene family provide insights into maize inflorescence development^[14]. The whole genome identification, integration analysis, and gene expression analysis under methyl jasmonate treatment of *Panax ginseng* FAR1/FHY3 gene family provide an experimental reference for the subsequent verification of the function of the family

members under methyl jasmonate signal transduction^[15]. However, the genome-wide identification of the FAR1/FHY3 gene family in *E. senticosus* and the internal mechanism of how it responds to light quality changes and regulates the accumulation of secondary metabolites are rarely studied.

Eleutherococcus senticosus (Rupr. et Maxim) Maxim is an important traditional medicinal plant^[16,17]. It has various pharmacological effects, including anti-fatigue and anti-cancer properties, lowering blood sugar levels, and improving myocardial metabolism, making it a highly valuable medicinal plant^[18]. *E. senticosus* contains a variety of active ingredients, for instance, saponins, coumarins, flavonoids, and polysaccharides^[19]. Among these, triterpenoid saponins are the main active components^[16,17]. The East Asian, European and American markets have seen the commercialization of numerous *E. senticosus* products as dietary supplements or clinical drugs^[16,17,20]. Extensive research has led to a preliminary understanding of the mechanism of triterpenoid saponin synthesis at the genetic and epigenetic levels to enhance the quality of *E. senticosus*^[16,17,20]. Recent studies have shown that, in addition to genetic factors, light quality at different wavelengths significantly influences the growth^[21], and secondary metabolism^[13,22] of *E. senticosus*. There is, however, no clear understanding of how light quality impacts *E. senticosus*'s ability to synthesize and accumulate secondary metabolites. Therefore, the analysis of the transcriptional regulation of genes related to secondary metabolism by photoreceptors, such as the light-responsive transcription factors of *E. senticosus*, has become a crucial research focus.

In 2021, the sequencing of the *E. senticosus* genome was finalized^[23], creating advantageous circumstances for conducting a comprehensive analysis of the *EsFAR1/FHY3* transcription factor family on a genomic scale. In this study, the family members of *EsFAR1/FHY3* were systematically screened and identified from the genome sequencing data of *E. senticosus*. Based on this, a study of the physical and chemical properties of the protein was conducted, as well as a study of the chromosomal distribution and gene expansion of *EsFAR1/FHY3*. The gene structure and evolutionary characteristics of the *EsFAR1/FHY3* family were investigated. Simultaneously, *EsFAR1/FHY3* and its regulated target genes in response to light quality changes were screened using transcriptome sequencing data, which provided a reference for the functional identification and utilization of *EsFAR1/FHY3* in *E. senticosus*.

Materials and methods

Screening of *EsFAR1/FHY3*

The Pfam database was used to obtain the hidden Markov model corresponding to the FAR1/FHY3 protein (PF03101, PF10551, and PF04434). Genome sequencing data of *E. senticosus*^[23] were obtained according to a previously published method^[16], and the FAR1/FHY3 protein domain was identified. The above sequences were further confirmed by NCBI CD-Search tools, PFAM, and SMART tools^[24] to determine the number and composition of *EsFAR1/FHY3* family members.

Phylogenetic analysis, classification, and protein physicochemical property analysis of FAR1/FHY3

The AtFAR1/FHY3 protein sequence was downloaded from the Arabidopsis TAIR 9.0 genome database. ClustalW was utilized to conduct multiple sequence alignments of the selected *EsFAR1/FHY3* and AtFAR1/FHY3 protein sequences. Using MEGA X software and the method from previous literature^[14], the phylogenetic tree of *EsFAR1/FHY3* and AtFAR1/FHY3 gene families was constructed. Using the ProtParam software on ExPASy, the basic physicochemical properties of *EsFAR1/FHY3* were analyzed^[16,17].

Collinearity analysis and chromosomal localization of *EsFAR1/FHY3*

The intraspecific collinearity of the *EsFAR1/FHY3* protein sequence was analyzed using MCScanX^[16,25]. The *AeFAR1/FHY3* gene was screened from the genomic sequencing data of *Aralia elata*^[26], and collinearity between FAR1/FHY3 of *E. senticosus* and FAR1/FHY3 of *A. elata* was analyzed^[16,17]. *EsFAR1/FHY3* was mapped to the chromosome of *E. senticosus* using MapChart^[17].

Analysis of the basic structure of *EsFAR1/FHY3* gene and its promoter

The conserved domain of *EsFAR1/FHY3* was analyzed using the Conserved Domain Database tool, and the conserved motif of *EsFAR1/FHY3* was predicted using the MEME tool^[17]. The promoter sequence was obtained from the 2 Kb region that is upstream of the start codon of the *EsFAR1/FHY3* gene. Predictions of the cis-acting elements for *EsFAR1/FHY3* were made utilizing the PLANTCARE software^[16].

Evolutionary selection and positive selection site analysis of *EsFAR1/FHY3*

The phylogenetic tree was constructed using the method outlined in a previous study^[24]. The evolutionary trend and selection pressure of *EsFAR1/FHY3* gene were speculated by estimating the ratio (ω) of nonsynonymous substitution rate (dN) to synonymous substitution rate (dS) of nucleotide sequence^[24]. When $dN < dS$, that is, $\omega < 1$, the negative selection pressure is indicated. When $dN = dS$, that is, $\omega = 1$, the choice is neutral. When $dN > dS$, that is, $\omega > 1$, there is a positive selection^[27]. The site model of Comdelc program^[28] was used to analyze the selection pressure of *EsFAR1/FHY3* gene during evolution. The site model assumes that the ω values of different sites are different, but the ω values of each branch on the evolutionary tree are the same. In this study, three models were used for the LRT test: M1a (near neutral) to M2a (positive selection), M0 (single ratio) to M3 (discrete), and M7 (beta) to M8 (beta & ω) to verify whether there were differences^[27].

Plant materials and cultivation conditions

In this study, two-year-old *E. senticosus* from the same clone cutting propagation from Yilan County, Heilongjiang Province, China was used as the experimental material. The same growing *E. senticosus* was divided into four groups, nine plants in each group, and cultivated in a plant culture room with LED control light quality. The leaves of *E. senticosus* were 30 cm away from the light source, and the light intensity was $37.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The growth conditions of *E. senticosus* were as follows: constant temperature 22 °C, light 16 h, dark 8 h. A total of four light qualities, white light (W), blue light (B), red light (R), and green light (G), were used to irradiate *E. senticosus* plants, of which white light treatment was the control. The wavelength range was 300~800 nm. After 30 d of treatment, mature leaves with similar growth status, no disease, no mechanical damage, and complete morphology were collected as experimental materials for subsequent analysis. Transcriptome, metabolome analysis, and physiological index determination were performed in triplicate.

Expression analysis of *EsFAR1/FHY3*

According to the transcriptome sequencing data of *E. senticosus* under different light-quality conditions (PRJNA1031420), the *EsFAR1/FHY3* gene retrieved from the genome was mapped to the transcriptome sequencing data using the BLAST program. Based on the results of transcriptome sequencing, the expression of *EsFAR1/FHY3* under different light-quality conditions was analyzed, and a gene expression heat map was drawn using the FPKM value of *EsFAR1/FHY3* under different light-quality conditions^[16].

qRT-PCR of *EsFAR1/FHY3*

Leaves of *E. senticosus* cultured under white, red, blue, and green lights for 20 d were collected. RNA was extracted following the instructions of the RNA Prep Pure Plant Kit and reverse transcribed into complementary DNA. Complementary DNA was used as a qRT-PCR template, and the *GAPDH* gene of *E. senticosus* was used as an internal reference gene^[16,17,20]. Specific amplification primers required for qRT-PCR were designed for differentially expressed *EsFAR1/FHY3* genes (Table 1). The qRT-PCR reaction was repeated three times for each sample. Using a previously described method, the total reaction system and conditions were set^[16]. The total reaction system was 10 μ l: cDNA 0.5 μ l, upstream and downstream primers 0.3 μ l, 2 $\times$ SGExcel FastSYBR Mixture 5 μ l, RNase-Free dd H₂O 3.9 μ l. Reaction conditions: pre-denaturation at 95 °C 3 min, 5 °C 15 s, 60 °C 20 s, 72 °C 25 s, 40 cycles.

Molecular docking and molecular dynamics analysis

The online website of the Plant Transcriptional Regulatory Map was used to predict whether the *EsFAR1/FHY3* protein could bind to the promoters of farnesyl diphosphate synthase (FPS), squalene synthase (SS), and squalene epoxidase (SE)^[17,20], which are key enzyme genes in the triterpenoid saponin biosynthesis pathway of *E. senticosus*, along with their respective binding sites. The protein sequence was docked with the DNA promoter sequence using HDock SERVER^[17]. Using GROMACS 2022.2^[29], molecular dynamics (MD) simulations were performed. The ligand topology file and protein topology file were generated using SobTop 1.0 software and AMBER99SB-ILDN force field, respectively. In the MD simulation, the minimum distance between the simulation box and the protein in the XYZ direction was 1.0 nm. The steepest descent^[30] and conjugate gradient methods^[31] were used to minimize energy. Isothermal isovolumetric and isobaric equilibrium simulations were

performed for 2 ns. A total of 100 ns MD simulations were conducted. In the MD simulation, the minimum distance between the simulation box and the protein in the XYZ direction was 1.0 nm. Although the two descriptions are similar, one is the description of the analysis time, and the other is the description of the spatial distance. The root mean square deviation (RMSD) was calculated to analyze the trajectory of molecular dynamics^[32].

Results

Identification and physicochemical property analysis of *EsFAR1/FHY3*

A total of 21 *EsFAR1/FHY3* gene family members were identified from the genome of *E. senticosus* using the hidden Markov model and BLAST alignment of FAR1/FHY3 (Supplementary Table S1). The protein encoded by *EsFAR1/FHY3* ranged from 683 to 2,184. The molecular weight ranged from 78.54 and 246.27 kDa. The theoretical isoelectric points ranged from 6.16 to 8.67, including 12 basic and 10 acidic amino acids. The instability coefficients ranged from 37.9 and 58.23. Proteins with stability coefficients < 40 are known to be stable proteins^[33]. In *E. senticosus*, only *Ese01G001009.t1* was a stable protein, whereas the other proteins were unstable. The aliphatic coefficients were < 100, and the average coefficient of hydrophilicity was < 0; therefore, *EsFAR1/FHY3* proteins were hydrophilic proteins.

Phylogenetic analysis of the *EsFAR1/FHY3* family

To analyze the phylogenetic evolution of *EsFAR1/FHY3* family. Two to four FAR1/FHY3 transcription factors were selected from each classification of *AtFAR1/FHY3* in *A. thaliana* as a reference. MEGA X was used to analyze the phylogeny of the 21 *EsFAR1/FHY3* proteins. The results showed that the *EsFAR1/FHY3* family was divided into five subfamilies, namely, the I–V subfamilies (Fig. 1a). Subtribe I and subtribe II were first clustered together in one branch, whereas subtribe III and subtribe V were first clustered in one branch, indicating that their genetic relationships were closer. Notably, subfamily IV has the most members (8), whereas subfamily V has the fewest members with only two family members.

Mapping of *EsFAR1/FHY3* on chromosomes and collinearity analysis

The chromosome localization of *EsFAR1/FHY3* gene was analyzed. We found that 21 *EsFAR1/FHY3* genes were unevenly distributed across 14 chromosomes of *E. senticosus* (Fig. 1b). Among them, the highest number of *EsFAR1/FHY3* genes was found on chromosome 6 (4), whereas the remaining 13 chromosomes had 1–3 *EsFAR1/FHY3* genes each. There was no distribution of *EsFAR1/FHY3* on chromosomes 2, 11–14, 16–18, 20, and 24.

The collinearity within and between species was analyzed. The results of the intra-species collinearity analysis (Fig. 1c) revealed that out of the 21 *EsFAR1/FHY3* genes, only one collinear gene pair was identified between *Ese04G002049.t1* on chromosome 4 and *Ese10G002497.t1* on chromosome 10. To study the evolutionary relationship of the FAR1/FHY3 gene family between *E. senticosus* and its related species, we selected *A. elata* and *E. senticosus*, which have completed genome sequencing, for interspecies collinearity analysis. The results showed that a total of 18 pairs of collinear genes were identified between *E. senticosus* and *A. elata* (Fig. 1d). The number of collinear genes on chromosomes 1 and 6 was the highest, with three pairs, and there were two pairs of syntenic genes on chromosomes 19 and 21–23, respectively. There was only one pair of FAR1/FHY3 with collinearity on chromosomes 3, 4, 10, and 15. Moreover, the syntenic genes of *A. elata* and *E. senticosus* were

Table 1. qRT-PCR amplification primers of *EsFAR1/FHY3* genes.

Gene	Primer sequences (5'-3')	Segment size (bp)
Cluster-36682.1	CCAAACTCTACCGACAACAT AACCCACTCTTCTCAACCAG	183
Cluster-102049.6	AGCAGACTTGGCGACAGATA AACCGATAAAGGACTACGAAT	187
Cluster-89581.4	TTTTCTATCCGCCGTCATCG CTCCCAATTCAGTTGTCTTGCTTA	196
Cluster-69743.0	CAGTGGCTCCATTCACAGGG ATACAGTGGCGGGCTTCAGG	214
Cluster-102049.5	AGCAGACTTGGCGACAGATA GAACCGATAAAGGACTACGA	188
Cluster-84138.0	ACTATCTTGGTCCACGTTTC TTCCTGCTTACTGCACTCTG	191
Cluster-87448.8	ACTCTTGCGTTTAGTTCCAT CTCCGTCAGTTCACAGTCTTA	166
Cluster-71679.0	TGCTATTGCGGCAGTCTTTC GCAGTTTCCACCACCTCTTT	182
Cluster-89581.3	TTTTCTATCCGCCGTCATCG CTCCCAATTCAGTTGTCTTGCTTA	196
Cluster-89581.5	CCTGGTCATATCTTCGTCA CTAAGAATGCCTTCAGTGCC	207
Cluster-101653.3	GCACCCAAATCTCCCGTTCC TGCTTAGGGCTATGTCAGG	152
Cluster-101653.6	AATGTTACTTCGCCGCTCTC AATGGGTGGTTGATCGTCTT	192
Cluster-96057.0	GTCTAGTAGTCAGCGAGCAG TAGCCATTGTCTATCCATC	200
Cluster-86052.0	AAAAGTAAGTGCCCAACCA GAGTCGCAATGAGAAAGAAG	157
Cluster-87127.4	CCAGCCACTTCTCAATCTCA CTTCATCACCACACTACGCATG	220

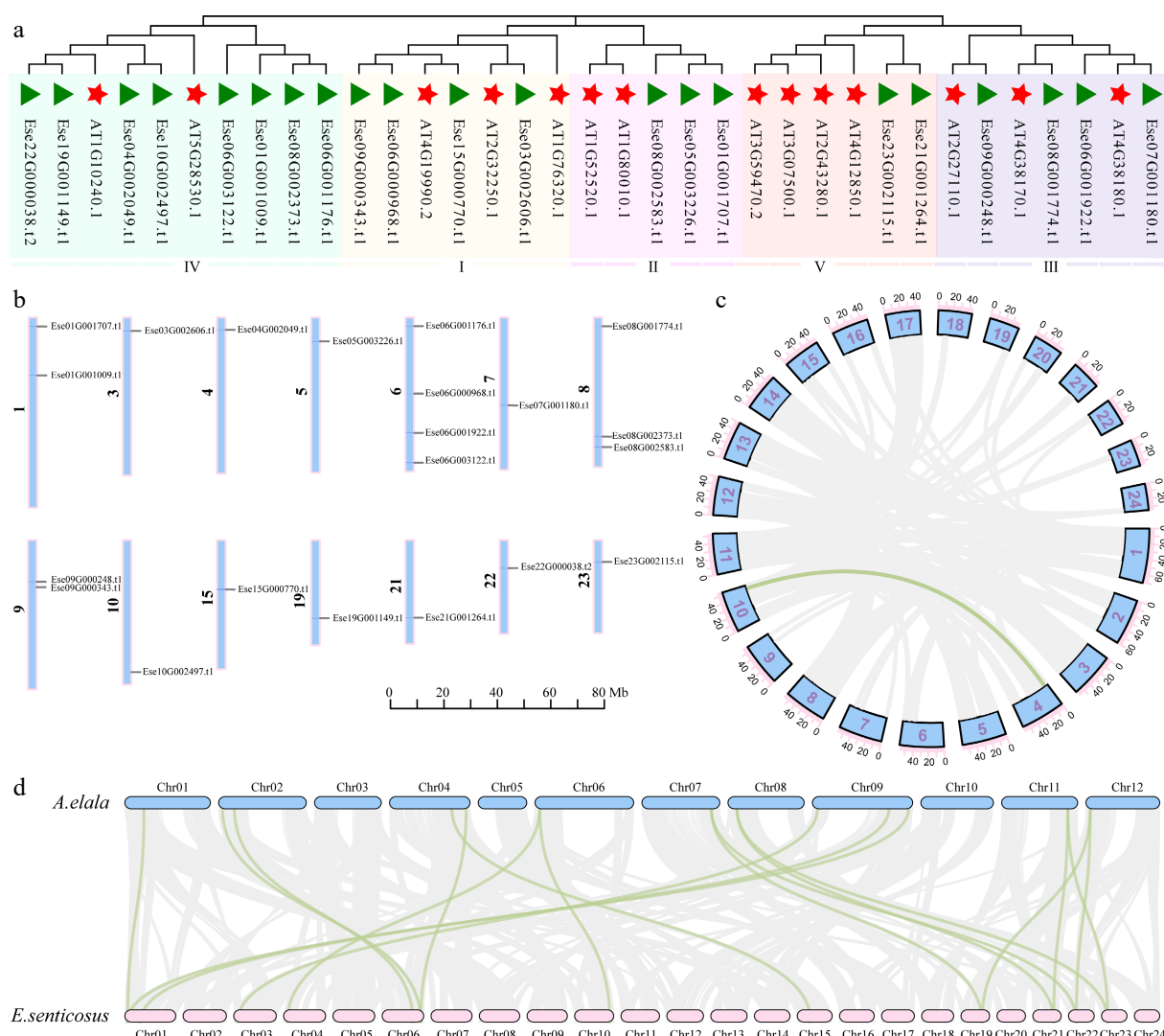


Fig. 1 Phylogenetic, chromosomal localization, and collinearity analysis of *EsFAR1/FHY3* gene family. The triangle represents *EsFAR1/FHY3* and the asterisk represents *AtFAR1/FHY3*. (b) Location of *EsFAR1/FHY3* on chromosomes. (c) Intrasppecific collinearity analysis of the *EsFAR1/FHY3*. (d) Collinearity analysis of species. Green represents *FAR1/FHY3* with collinearity, and gray represents other collinearity genes.

mostly distributed at both ends of the chromosome in the form of clusters, indicating that gene duplication and evolution in *A. elata* and *E. senticosus* were also similar.

Gene characterization of *EsFAR1/FHY3*

The conserved motifs, conserved domains, and gene structure of *EsFAR1/FHY3* were analyzed. Ten conserved motifs (named Motif 1 to Motif 10) were identified in the *EsFAR1/FHY3* gene family (Fig. 2a). Among them, except *Ese10G002497.t1*, *Ese04G002049.t1*, *Ese19G001149.t1*, and *Ese22G000038.t2* only contain Motif 1 to Motif 9, other *EsFAR1 / FHY3* contain all 10 conserved motifs. The position of each conserved motif in *EsFAR1/FHY3* was consistent and the gene structure was essentially the same, indicating that *EsFAR1/FHY3* was highly conserved throughout the evolutionary process. The reliability of the classification results was verified by phylogenetic analysis. The conserved domain types of the *EsFAR1/FHY3* gene family mainly belonged to the MULE, FAR1, SWIM, and FAR1 superfamily (Fig. 2b).

The results of the gene structure analysis of *EsFAR1/FHY3* showed that the length of *EsFAR1/FHY3* was mostly within 20 kb, with only *Ese15G000770.t1* being approximately 80 kb. Among the 21 *EsFAR1/FHY3* genes, four contained UTR structures: *Ese08G001774.t1*,

Ese09G000248.t1, *Ese23G002115.t1*, and *Ese22G000038.t2*. Among them, *Ese08G001774.t1* and *Ese23G002115.t1* contained the 5'-UTR, whereas *Ese09G000248.t1* and *Ese22G000038.t2* contained the 3'-UTR. All *EsFAR1/FHY3* genes contained 1–4 intron fragments (Fig. 2c). Motif sequences of *FAR1/FHY3* in *E. senticosus* were compared with those of *FAR1/FHY3* in *A. thaliana*. It was found that the Motif 2 sequences of *EsFAR1/FHY3* (Fig. 2d) and *AtFAR1/FHY3* (Fig. 2e) were similar, whereas those of other motifs were relatively different. Therefore, we speculated that Motif 2 was the most conserved domain among all *EsFAR1/FHY3* and *AtFAR1/FHY3* motifs. The gene structure of *EsFAR1/FHY3* in each subfamily was approximately the same and the conserved domains and positions were also similar, indicating that *EsFAR1/FHY3* is highly evolutionarily conserved.

Analysis of cis-acting elements in *EsFAR1/FHY3* promoter

The cis-acting elements of the promoter region of the *EsFAR1/FHY3* gene were analyzed. There were a large number of cis-acting elements in the promoter region of *EsFAR1/FHY3* (Supplementary Table S2), including light response elements, stress response elements, and plant hormone response elements. The number of

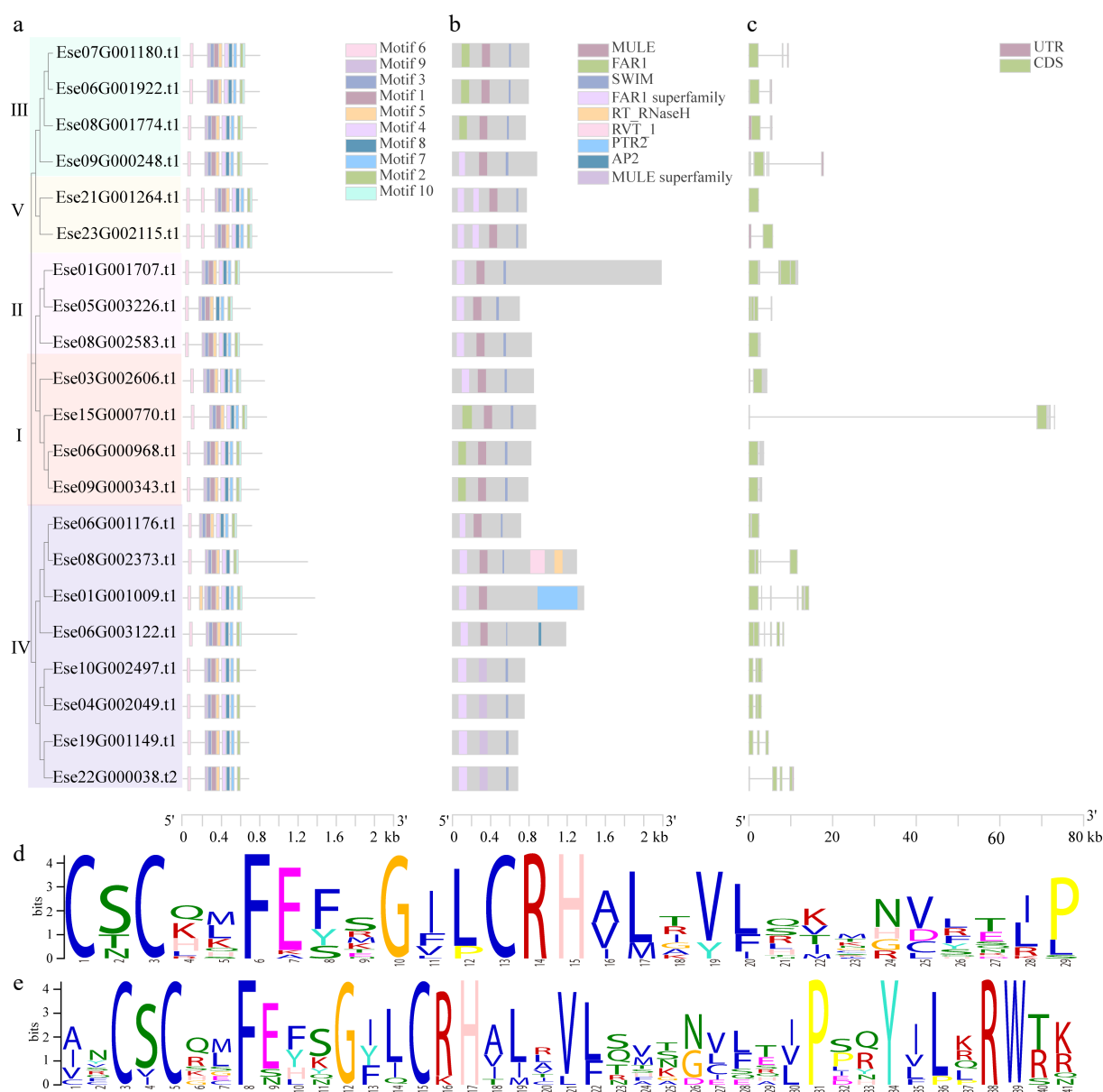


Fig. 2 Gene structure and conserved motif analysis of *EsFAR1/FHY3*. (a) Distribution of the *EsFAR1/FHY3* conserved motifs. (b) Conserved domain of *EsFAR1/FHY3*. (c) Intron structure of *EsFAR1/FHY3*. The green box represents an exon. The line represents an intron. (d) Seqlogo of core conserved Motif 2 in *EsFAR1/FHY3*. (e) Seqlogo of core conserved Motif 2 in *AtFAR1/FHY3*.

light-response elements was the highest (Supplementary Fig. S1, Supplementary Table S3). These light response elements mainly included Box 4, G-box, TCT-motif, GT1-motif, ATCT-motif, and GATA-motif. The number of light-responsive elements per *EsFAR1/FHY3* promoter ranged from 5 to 15. The *Ese23G002115.t1* promoter contained the most light-responsive elements (15), whereas the *Ese06G000968.t1* promoter contained the least light-responsive elements, with only five elements (Fig. 3).

Adaptive evolution analysis of *EsFAR1/FHY3*

The adaptive evolution of *EsFAR1/FHY3* gene was analyzed. Branch and site models were selected to analyze the selection pressure of *EsFAR1/FHY3*. The results showed that in the branch model, the LRT test result between the single-ratio model M0 and free-ratio model MF was $p < 0.001$. The LRT test results of the free-ratio and single-ratio models showed that the free-ratio model was superior to the single-ratio model, indicating that the selection pressures on different branches varied (Table 2).

To determine the possibility of multiple selections of specific sites in *EsFAR1/FHY3*, we selected the site model for the calculation (Supplementary Table S4). The results showed that the LRT test results of single-ratio model M0 and discrete model M3 were $p < 0.01$. This shows that the alternative hypothesis model M3 was established and there was no positive selection site. The result of the LRT between the M1a and M2a models was $p = 1.000$, indicating that M2a was invalid. In the comparison between models M7 and M8, the LRT test result was $p = 0.998$, indicating that model M8 was not established. The calculation results of the site model revealed that there were no positive selection sites in the evolution of *EsFAR1/FHY3* and that negative selection prevailed.

Effect of light quality on *EsFAR1/FHY3* expression

To study the effect of light quality on the expression of *EsFAR1/FHY3*, *EsFAR1/FHY3* identified from the genome was compared with the transcriptome sequencing data of *E. senticosus* under red-, green-, blue-, and white-light quality irradiation using BLAST.

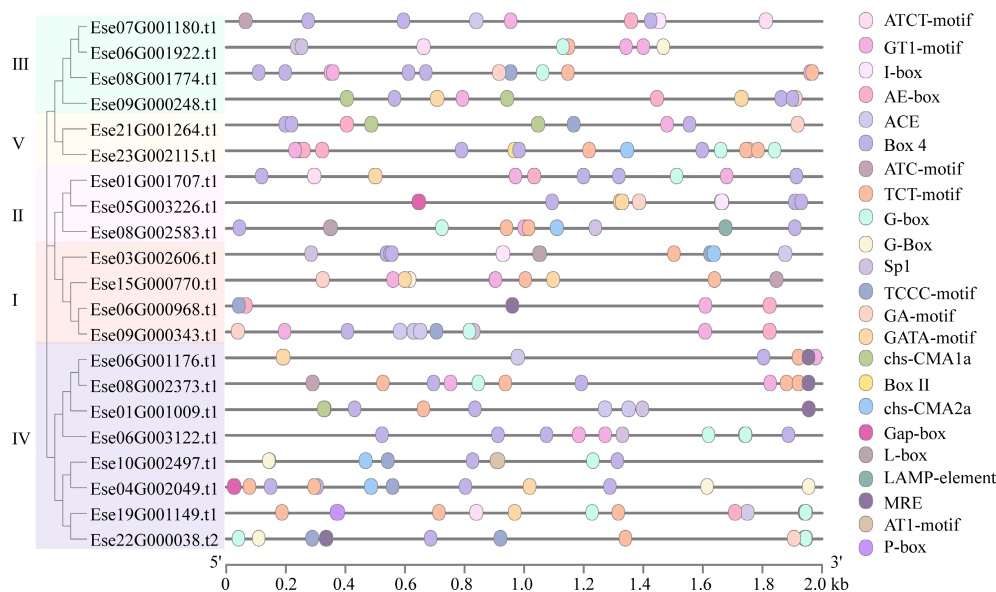


Fig. 3 Analysis of light response elements of *EsFAR1/FHY3* promoter.

Eighteen *EsFAR1/FHY3* transcripts were also identified. Among them, the expression levels of 15 *EsFAR1/FHY3* genes were differentially expressed under various light qualities ($|\text{Fold Change}| \geq 2$). Most of the differentially expressed *EsFAR1/FHY3* were upregulated under red or blue light. Only four *EsFAR1/FHY3* genes were significantly upregulated under green light, whereas the other *EsFAR1/FHY3* were downregulated under green light (Fig. 4a). This shows that *EsFAR1/FHY3* expression changes in response to different changes in light quality.

To ensure the reliability of the expression levels of *EsFAR1/FHY3* observed in the transcriptome sequencing data, we conducted qRT-PCR analysis on 15 genes that were identified as differentially expressed. The findings indicated that the transcriptome sequencing data for *EsFAR1/FHY3* expression closely aligned with what was obtained through qRT-PCR (Fig. 4b), suggesting that the differentially expressed *EsFAR1/FHY3* identified using transcriptome sequencing data was reliable.

Prediction of target genes of *EsFAR1/FHY3* regulating saponin synthesis in *E. senticosus*

To predict the regulatory effect of *EsFAR1/FHY3* on the target gene of *E. senticosus* saponin synthesis. The 15 differentially expressed *EsFAR1/FHY3* were predicted to bind to the promoters of

EsFPS, *EsSS*, and *EsSE*. Only four *EsFAR1/FHY3* members of subfamily I bound to the 'CTCTCGCGCTCT' site of the *EsFPS* promoter (Fig. 5a), whereas *EsFAR1/FHY3* in other subfamilies did not bind to the promoters of these saponin synthase genes. The results of molecular docking showed (Fig. 5b) that six amino acids, ASN (91), SER (92), SER (125), SER (127), ASN (236), and ASN (239), in the *Ese09G000343.t1* encoded protein formed hydrogen bonds with the *EsFPS* promoter. The eight amino acids ASP (41), LYS (74), ARG (77), GLU (84), LYS (89), LYS (117), SER (119), and GLU (138) in the protein encoded by *Ese06G000968.t1* formed hydrogen bonds with the *EsFPS* promoter. The seven amino acids LYS (45), LYS (53), ARG (69), ARG (165), ARG (168), ASN (169), and ASN (174) in the protein encoded by *Ese15G000770.t1* formed hydrogen bonds with the *EsFPS* promoter. The ten amino acids TYR (62), ARG (79), SER (81), ARG (82), ARG (83), LYS (86), SER (87), LYS (89), GLY (158), and CYS (168) in the protein encoded by *Ese03G002606.t1* formed hydrogen bonds with the *EsFPS* promoter.

MD simulations were conducted to demonstrate the stability of the interaction between *EsFAR1/FHY3* and specific binding sites of the *EsFPS* promoter (CTCTCGCGCTCT) (Fig. 6a). RMSD was used to evaluate the structural changes and stability of the complex (Fig. 6b). The RMSD fluctuation ranges of the complexes formed by *Ese09G000343.t1*, *Ese06G000968.t1*, *Ese15G000770.t1*, *Ese03G002606.t1*, and *EsFPS* were approximately 2 Å, 10 Å, 11 Å, and 12 Å, respectively. These results indicated that *EsFAR1/FHY3* exhibited strong stability after binding to the *EsFPS* promoter. Furthermore, root mean square fluctuation (RMSF) was used to analyze amino acid movement, revealing RMSF values ranging from 3 to 11 Å for the four complexes (Fig. 6c). Notably, the *Ese09G000343.t1*-*EsFPS* complex exhibited the lowest RMSD and RMSF values, indicating fewer fluctuations and higher relative stability. The surface visual conformation of the complex within 100 ns of MD simulation is shown in Fig. 6d. The simulation results showed that *Ese09G000343.t1*, *Ese06G000968.t1*, *Ese15G000770.t1*, and *Ese03G002606.t1* remained tightly bound to the *EsFPS* promoter-binding site throughout the simulation, indicating a stable interaction of these four *EsFAR1/FHY3* with the *EsFPS* promoter. This stable interaction is crucial for the binding of the *EsFPS* promoter and expression regulation of the *EsFPS* gene.

Table 2. Adaptive evolutionary analysis of *E. senticosus* FAR1 / FHY3 gene.

Model	Parameter	$\Delta\ln L$	Parameter estimation	
M0	41	-17546.660334	$\omega = 0.12627$	
MF	79	-17466.425540	$\omega A = 0.326188$	$\omega B = 0.168392$
			$\omega C = 0.6544$	
M1a	42	-17471.416569	$P0 = 0.91334$	$\omega0 = 0.12650$
			$P1 = 0.08666$	$\omega1 = 1.00000$
M2a	44	-17471.416569	$P0 = 0.91334$	$\omega0 = 0.12650$
			$P1 = 0.08665$	$\omega1 = 1.00000$
			$P2 = 0.00001$	$\omega2 = 1.00000$
M3	45	-17218.555062	$P0 = 0.12241$	$\omega0 = 0.00701$
			$P1 = 0.41439$	$\omega1 = 0.07580$
			$P2 = 0.46321$	$\omega2 = 0.23545$
M7	42	-17216.044985	$p = 1.10252$	$q = 6.38677$
M8	44	-17216.046811	$p0 = 0.99999$	$p = 1.10254$
			$q = 6.38697$	$p1 = 0.00001$
			$\omega = 1.00000$	

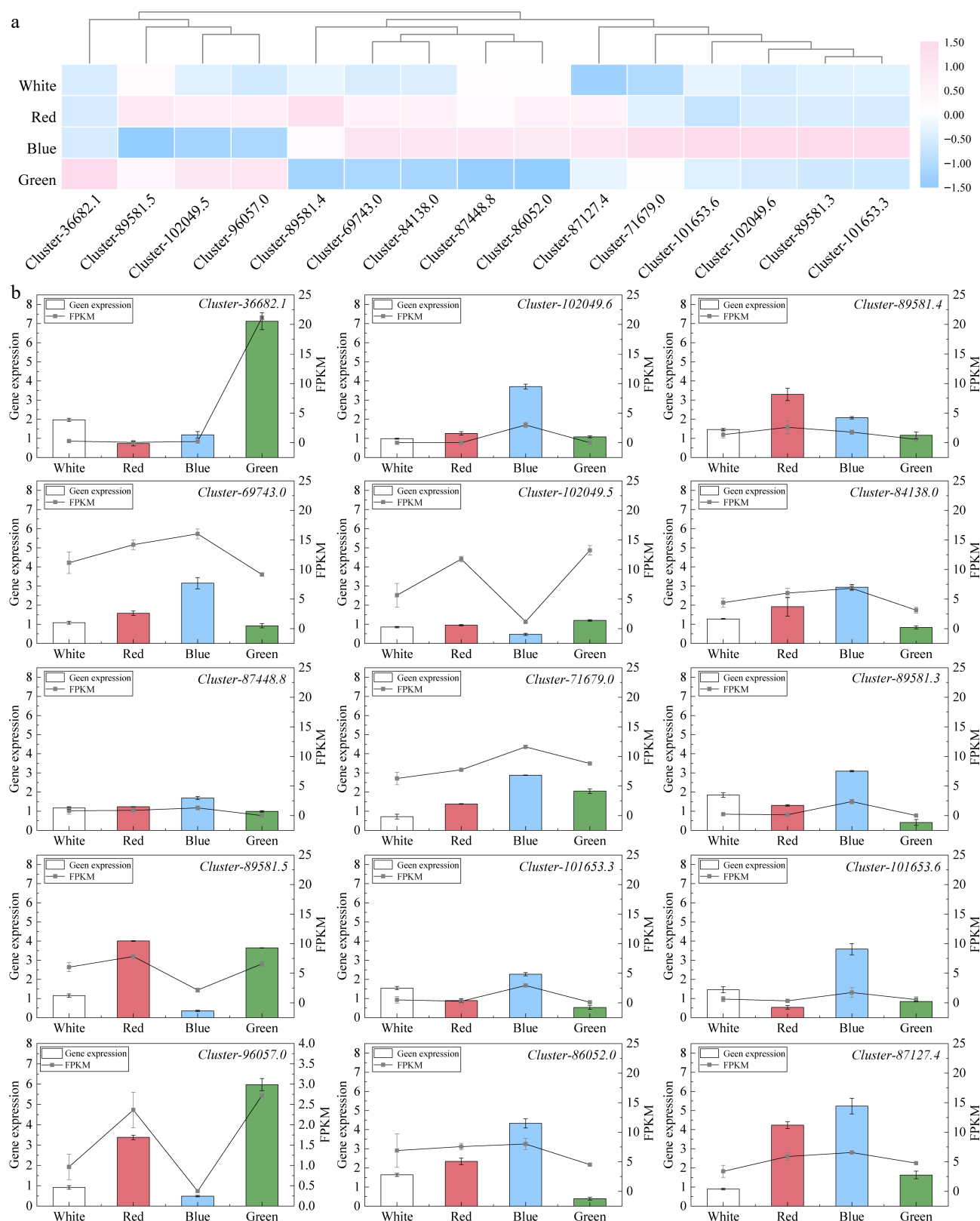


Fig. 4 Effect of light quality on *EsFAR1/FHY3* expression. (a) Expression of *EsFAR1/FHY3* in transcriptome sequencing results. (b) Expression of *EsFAR1/FHY3* was verified by qRT-PCR.

Discussion

The FAR1/FHY3 gene family plays multiple roles in plant physiological development, for instance responding to light signals^[4] and stress^[34], and regulating the biological clock and flowering time^[35].

A total of 59 FAR1/FHY3 genes, distributed across 21 chromosomes, have been identified in *Panax ginseng* by Jiang et al.^[15]. Li et al.^[33] identified 20 FAR1/FHY3 genes distributed across six chromosomes in *Cucumis sativus*. There are 21 *EsFAR1/FHY3* genes were identified in the genome of *E. senticosus*, which were distributed across 14

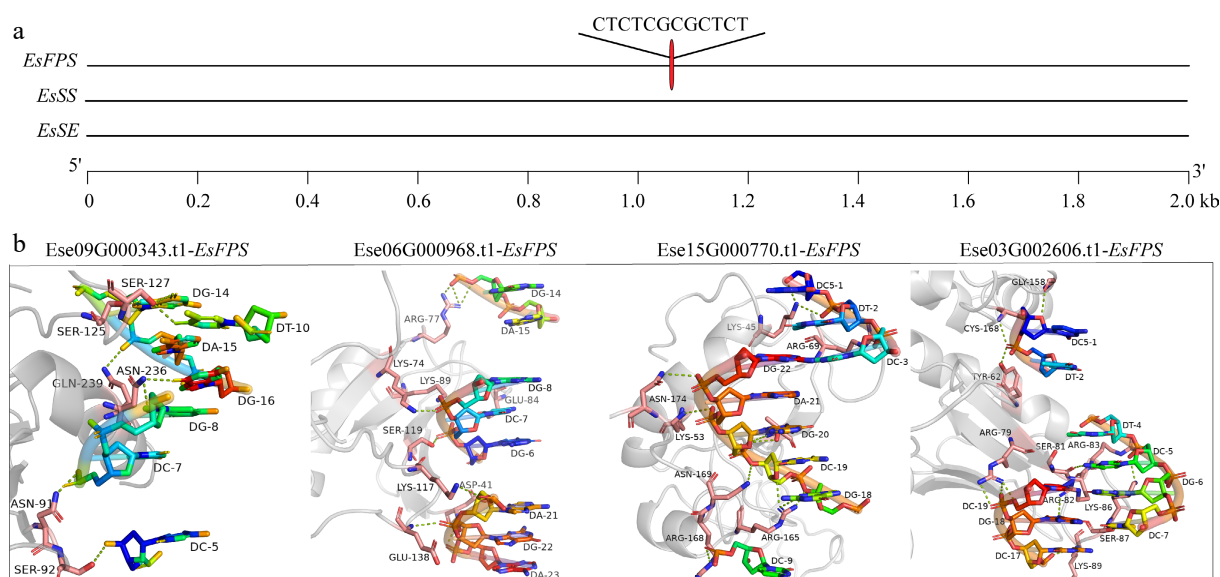


Fig. 5 Analysis of the binding of *EsFAR1/FHY3* to the promoter of *E. senticosus* saponin synthase gene. (a) Binding site assay. (b) Molecular docking of *EsFAR1/FHY3* and *EsFPS*.

chromosomes. Studies have shown that genes of enzymes involved in the biosynthetic pathway of secondary metabolites are organized into clusters in the genome^[36–38]. Anti-nutritive triterpenoids (cucurbitacins) related to bitterness in cucumber are typical examples of natural compound gene clusters^[39]. Gene clusters of plant defense compounds were also found in monocots and dicots. For example, the momilactones biosynthetic gene cluster in rice^[39]. *EsFAR1/FHY3* distribution on only a few chromosomes may be similar to this feature. Analysis of physicochemical properties revealed that most FAR1/FHY3 proteins in *E. senticosus* were unstable, with the exception of Ese01G001009.t1, which was consistent with the findings in *A. thaliana*^[34]. Based on these above results, we found that as the number of FAR1/FHY3 genes in a specific species increased, the number of chromosomes also increased. Although the number of FAR1/FHY3 genes identified in different species varied, the number of FAR1/FHY3 genes in *E. senticosus* did not change significantly compared with that in other species^[14]. This indicates that the number of FAR1/FHY3 family members is relatively conserved among different species^[8,15,33].

The subfamily classification of *FAR1/FHY3* varies among different species. For example, *FAR1/FHY3* in *C. sativus* has been divided into three subfamilies^[33], whereas *FAR1/FHY3* in *Z. mays* has been divided into five subfamilies^[14]. In our study, *EsFAR1/FHY3* genes were divided into five subfamilies. We speculate that this variation is due to the different numbers of FAR1/FHY3 genes in different species and the variability in the evolutionary process^[40].

In *E. senticosus*, *EsFAR1/FHY3* is mostly located within 20 kb and each subfamily contains a varying number of intron sequences. This variation in gene structure indicates that introns may have been constantly acquired or lost during the evolution of *EsFAR1/FHY3* family members^[41]. The 5'-UTR plays an important role in gene regulation, whereas the 3'-UTR serves as the binding site for the messenger RNA degradation complex, which is associated with messenger RNA stability^[42]. In *E. senticosus*, except for subfamilies I and II, *EsFAR1/FHY3* of subfamilies III, IV, and V contained a UTR structure similar to that in *Camellia sinensis*^[42]. In this study, we identified the first ten conserved motifs of *EsFAR1/FHY3*. Except for Ese10G002497.t1, Ese04G002049.t1, Ese19G001149.t1, and Ese22G000038.t2 only contained Motif 1 to Motif 9, whereas the remaining *EsFAR1/FHY3* genes contained all 10 conserved motifs. This reflects the highly conserved structural characteristics of

EsFAR1/FHY3. This was also observed in *Juglans sigillata*^[43] and *Z. mays*^[14]. The combination of these motifs gives rise to the MULE, SWIM, and FAR1 protein domains found within *EsFAR1/FHY3*. These domains serve as the fundamental architectural framework underlying the various biological functions associated with *EsFAR1/FHY3* transcription factors^[4]. The 'Cu fist' domain and the Cys-rich motifs at the N and C termini of Mac1 are common features of Cu starvation responsive transcription factors. Studies have shown that the 'Cu fist' domain is essential for Mac1-mediated Cu uptake in *Aspergillus nidulans*^[44]. Mutations in highly conserved motifs in the conserved iRhom homeodomain eliminate ADAM17 maturation and activity^[45].

The analysis of interspecies collinearity between *E. senticosus* and *A. elata* revealed a significant level of collinearity between FAR1/FHY3. This phenomenon also occurs in species, such as *Zea mays*^[14] and *Arachis hypogaea*^[46]. Studies have shown that in the past 200 million years, the entire genome has been repeated, accompanied by large-scale gene loss, resulting in the breakage of ancestral gene linkages on multiple chromosomes^[47]. However, the analysis of gene collinearity in *E. senticosus* species found that only one pair of *EsFAR1/FHY3* genes had a collinearity relationship. This seems to indicate that the FAR1/FHY3 gene family is relatively conserved during evolution. Further analysis of the adaptive evolution of *EsFAR1/FHY3* showed the absence of any positive selection sites within this gene. Instead, negative selection predominantly influenced the evolution of the *EsFAR1/FHY3* gene family. This indicates that *EsFAR1/FHY3* underwent strong negative selection during evolution. In the evolutionary process, the structure and function of *EsFAR1/FHY3* are likely to have been fixed in the early stages, and its adaptive evolution may have stabilized early, making it difficult to detect sites of positive selection^[48]. This further illustrates the highly conserved evolutionary characteristics of the *EsFAR1/FHY3* gene family, and the conserved structure of the *EsFAR1/FHY3* gene is an important part of the light response. Therefore, we preliminarily speculate that the *EsFAR1/FHY3* gene can affect the secondary metabolism of *E. senticosus* through its conserved light response components.

A significant number of light-responsive cis-acting elements, such as Box 4, G-box, TCT-motif, GT1-motif, ATCT-motif, and GATA-motif, were identified in the promoter region of *EsFAR1/FHY3*, which indicates that *EsFAR1/FHY3* may play a role in the response of *E.*

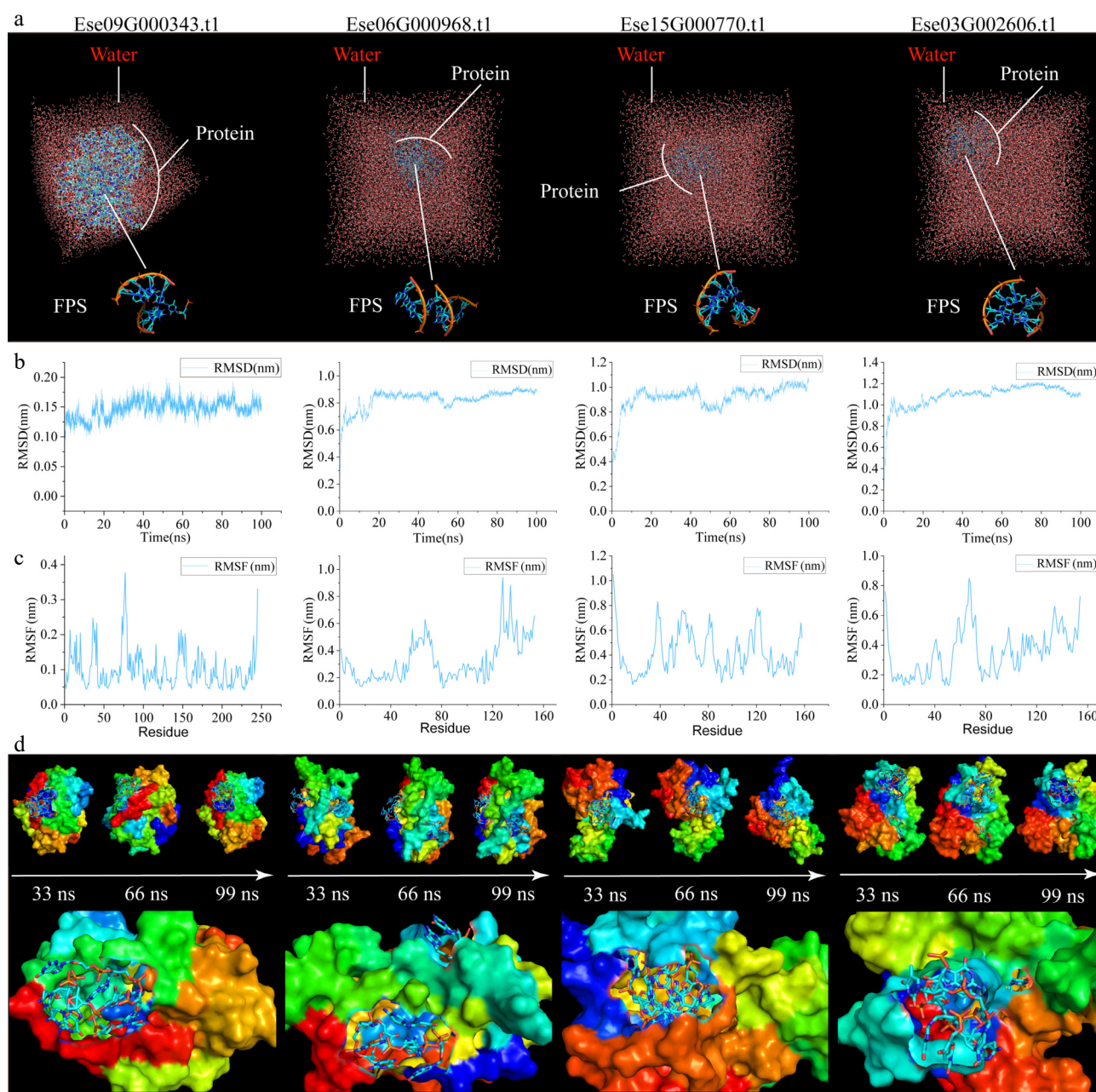


Fig. 6 MD simulation results of *EsFAR1/FHY3* binding to *EsFPS* promoter. (a) Visualization of MD simulation. (b) RMSD of all atoms in the complex. (c) RMSF of all atoms in the complex. (d) Surface conformation of the complex between 0 and 100 ns.

senticosus to light changes^[49,50]. Studies have shown that light quality can regulate secondary metabolic processes in most plants, including *E. senticosus*^[13,22]. Plants have evolved various photoreceptors to adapt to varying light conditions^[51]. After sensing light signals, these photoreceptors trigger complex signaling cascades, prompting plants to adapt to specific light conditions and generate adaptive responses at the gene expression level through specific transcription factors and their complexes^[52]. For instance, FAR1/FHY3 in *A. thaliana* can regulate plant growth, development, and metabolic processes by regulating phyA signaling^[53].

Liu et al.^[54] showed that FAR1/FHY3 protein can bind to DNA and affect plant growth and development. However, it is unclear whether *EsFAR1/FHY3* directly regulates the expression of secondary-metabolism-related genes in *E. senticosus*. In this study, we found that the promoter of *EsFPS*, a key enzyme gene for triterpenoid saponin biosynthesis in *E. senticosus*, binds to *EsFAR1/FHY3*

proteins of subfamily I. This may be related to ACGT-containing elements (ACEs) in *EsFAR1/FHY3*^[3]. Some other subfamilies may have lost this structure during evolution and therefore could not bind to the promoter of *EsFPS*. Further MD analysis revealed that *EsFAR1/FHY3* proteins from the four I subfamilies of *E. senticosus* formed stable complexes with the *EsFPS* promoter region. Therefore, we speculated that the *EsFAR1/FHY3* protein directly binds to the *EsFPS* promoter and regulates the expression of this gene, thereby influencing the accumulation of triterpenoid saponins in *E. senticosus*. This is consistent with the results obtained for *A. thaliana*^[10,54]. In addition, it should be noted that the saponin synthesis pathway in *E. senticosus* is catalyzed by at least ten enzymes^[23], and this study only analyzed the promoters of three key enzyme genes. Therefore, although the remaining 11 *EsFAR1/FHY3* did not exhibit the ability to bind to the promoters of the three enzyme genes specifically discussed, it is plausible that these genes

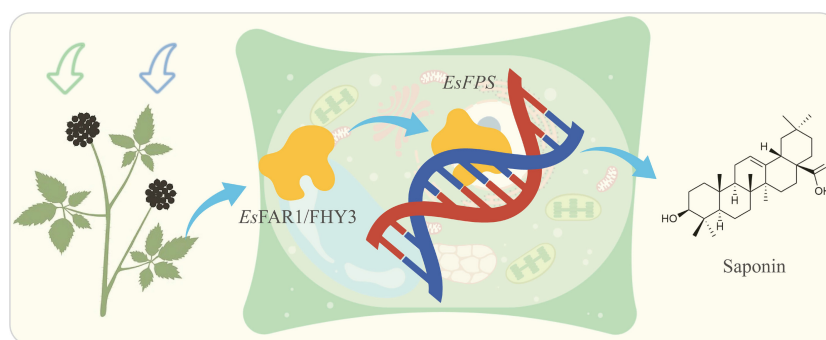


Fig. 7 Schematic diagram of *EsFAR1/FHY3* regulating the synthesis of *E. senticosus* saponins in response to light changes.

could interact with the promoters of other enzyme genes that play a role in the synthesis of saponins. This interaction could potentially regulate the overall production of saponins in *E. senticosus*.

Conclusions

Using the hidden Markov model, 21 *EsFAR1/FHY3* proteins, distributed across 14 chromosomes, were identified in the *E. senticosus* genome. *EsFAR1/FHY3* was divided into five subgroups, and the conserved motifs, domains, and gene structures within each subgroup were similar. *EsFAR1/FHY3* has mainly been affected by negative selection during evolution, and its promoters contain a lot of light-responsive cis-acting elements. Under irradiation with red, green, blue, and white light, 15 *EsFAR1/FHY3* showed differential expression, of which only four *EsFAR1/FHY3* classified as subgroup I was bound to the promoter of the *E. senticosus* saponin synthesis key enzyme gene *EsFPS* through varying numbers of hydrogen bonds. Therefore, we speculated that *EsFAR1/FHY3*, which can bind to the *EsFPS* promoter, was specifically expressed under irradiation with specific wavelengths of light. After binding to the *EsFPS* promoter, they regulate the expression of *EsFPS*, thereby affecting the synthesis of triterpene saponins in *E. senticosus* (Fig. 7).

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Xing Z, Long Y; figure and table production: Jiao M, Wu P; sample collection: Zhao X; draft manuscript preparation: Xing Z; manuscript revision: Ma J, Cui Y, and Kou X; research: Li C. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The sequence data from RNA-Seq experiments reported in this study have been deposited in the NCBI with Accession No. PRJNA1031420.

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Conflict of interest

The authors declare that they have no conflict of interest.

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