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Over-expression of *JcSEUSS1* from *Jatropha curcas* Induces the Accumulation of Anthocyanin in Leaves

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Abstract Anthocyanins are a class of important secondary metabolites in plants and play important roles in photoprotection and antioxidation. In this study, we cloned the *JcSEUSS1* gene from *Jatropha curcas*. Sequence alignment indicates JcSEUSS1 contains a highly conserved LIM domain, and the sequence of JcSEUSS1 is similar to other SEUSS proteins. The expression pattern of *JcSEUSS1* in different organs of *Jatropha* plants was analyzed by real-time fluorescence quantitative PCR, and it was found that the gene was expressed in all tissues. Furthermore, *JcSEUSS1* over-expression significantly promoted the accumulation of anthocyanin in leaves of *Jatropha*. The expression of *chalketone synthase* (*JcCHS*) and *chalketone isomerase* (*JcCHI*), which are involved in anthocyanin biosynthesis, were significantly up-regulated in *JcSEUSS1*-transgenic lines. Our results indicate that *JcSEUSS1* may positively regulate the anthocyanin synthesis in leaves of *Jatropha*.

Keywords *Jatropha curcas*; *JcSEUSS1*; Over-expression; Anthocyanin

Anthocyanins are widely found in various tissues of plants and are water-soluble flavonoids pigments (Cuyckens and Claeys, 2004). It helps plants resist oxidation and attract insect pollination, and can also be used to make anticancer drugs (Holton and Cornish 1995; Wu et al., 2004; Li et al., 2007). At present, anthocyanin pathway is well studied in model plants such as *Arabidopsis thaliana* and maize. Its metabolic pathway can be roughly divided into three stages: In the first stage, phenylalanine, the precursor of anthocyanin synthesis, was synthesized in phenylalanine ammonia (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate--CoA ligase (4CL) catalyzed the formation of 4-coumaryl-CoA. The second stage is 4-coumaryl-CoA and malonyl-CoA in chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone-3-hydroxylase (F3H) to produce flavanone and dihydroflavonol, which is also the key stage of anthocyanin metabolism. In the third stage, dihydroflavonol was catalyzed by dihydroflavonol 4-reductase (DFR) to produce colorless anthocyanins, followed by anthocyanin synthetase (ANS) and flavonoid-3-O-glucosyltransferase (3GT) to produce colored anthocyanins (Holton and Cornish, 1995; Ferrer et al., 2008). In addition, environmental factors such as light, temperature and drought, as well as levels of sucrose, jasmonic acid and ethylene, all affect anthocyanin synthesis in plants (Christie and Jenkins, 1996; Zhang et al., 2002; El-Kereamy et al., 2003; Hara et al., 2003; Mori et al., 2007; Sperdouli and Moustakas, 2012).

Jatropha curcas L. is a perennial woody oil crop in the family of Euphorbiaceae. Its seed oil has high commercial value and can be used to produce biodiesel (Pandey et al., 2012) and bio-aviation fuel (Du et al., 2019). Meanwhile, the efficient genetic transformation system of *Jatropha* seed lays a solid foundation for improving its biological traits (Pan et al., 2010; Fu et al., 2015). However, current studies on functional genes of *Jatropha* mainly focus on screening genes that affect its branching, flowering induction, flower organ development and seed oil content (Li et al., 2014; Kim et al., 2014; Ni et al., 2015; Li et al., 2017; Ni et al., 2017; Khan et al., 2018; Govender et al., 2019), there are few studies on genes that regulate anthocyanin biosynthesis. So far, only over-expression of *Jatropha curcas* gene JcTPS1 could cause the accumulation of anthocyanin in *Arabidopsis*

thaliana leaves (Zhao et al., 2018, Molecular Plant Breeding, 16(1): 255-261). Anthocyanins can protect plants from various biotic and abiotic stresses (Zhang et al., 2013; Ahmed et al., 2014). The content of anthocyanins in the young leaves of wild *Jatropha* was significantly higher than that in the mature leaves, and studies showed that the higher content of anthocyanins had a protective effect on photosystem I and photosystem II of the young leaves of *Jatropha* (Ranjan et al., 2014). Jasmonic acid can induce anthocyanin synthesis in *Jatropha* leaves, thus increasing the antioxidant capacity of plants (Lucho-Constantino et al., 2017).

SEUSS (SEU) is a homologue of the animal LDB (Lim-domain-binding) protein, consisting of two glutamine rich domains and a highly conserved domain. This highly conserved domain has a high sequence similarity with the animal LIM binding domain (Franks et al., 2002). *SEU* is expressed in seedlings, leaves, flowers and other tissues of *Arabidopsis thaliana* and is involved in a series of growth and development processes (Franks et al., 2002; Bao et al., 2010). It is mainly involved in the regulation of root, flower organ, ovule and embryo development of *Arabidopsis thaliana* (Sridhar et al., 2006; Azhakanandam et al., 2008; Wynn et al., 2014; Gong et al., 2016). In this study, we cloned cDNA sequence of *SEUSS* from *Jatropha curcas* (*JcSEUSS1*), analyzed its expression pattern, and preliminarily studied the effect of *JcSEUSS1* on anthocyanin synthesis in *Jatropha* leaves by overexpressing the gene in *Jatropha curcas*.

1 Results and Analysis

1.1 Cloning and sequence analysis of *JcSEUSS*

Taking *Arabidopsis thaliana* SEU (NP_175051) protein sequence as a reference, two sequences with high similarity to Arabidopsis SEU were found through homologous sequence alignment in *Jatropha curcas* genome database (http://www.kazusa.or.jp/jatropha/). The encoded proteins were named JcSEUSS1 (XP_012066097) and JcSEUSS2 (XP_012066572), respectively, and their sequence identity percentage was 58.44%. *JcSEUSS1* cDNA (XM_012210707) was 3 842 bp in length and CDS was 2 748 bp in length, encoding 915 amino acids. DNAMAN software was used to compare *JcSEUSS1* amino acid sequences with SEU protein sequences of several other species, and it was found that *JcSEUSS1* had highly conserved LIM binding domain with other species (Figure 1), so it could be inferred that JcSEUSS1 belonged to LIM domain protein. Phylogenetic tree analysis of JcSEUSS1 homologous proteins from other species was performed using MEGA 10.0 software. The results showed that JcSEUSS1 was closely related to RcSEUSS, MeSEUSS and PtSEUSS (Figure 2).

1.2 Tissue-specific expression pattern of *JcSEUSS1* in *Jatropha curcas*

Tissue-specific expression analysis of *JcSEUSS1* was performed by real-time quantitative PCR in 8 tissue parts including root, stem, young leaf, mature leaf, inflorescence, female flower, male flower and fruit. The results showed that *JcSEUSS1* was expressed in all tissues of *Jatropha curcas* (Figure 3). The relative expression level was higher in roots, young leaves and inflorescence, the highest in roots, and higher in leaves than in male and female flowers.

1.3 Over-expression of *JcSEUSS1* promoted anthocyanin accumulation in *Jatropha curcas* leaves

In order to explore the effect of *JcSEUSS1* gene on the growth and development of *Jatropha curcas*, this study constructed over-expressed vector *35S:JcSEUSS1* (Figure 4A) and transformed *Jatropha curcas* by agrobacterium-mediated method. Fourteen independent transgenic plants were obtained (Figure 4B), and 3 T0 generation transgenic lines, L3, L4 and L7, were randomly selected for phenotypic analysis. qRT-PCR showed that *JcSEUSS1* gene expression in petioles of these 3 transgenic plants was significantly up-regulated (Figure 4C). These results indicated that L3, L4 and L7 were indeed positive plants. The expression level of L4 was the highest and 40 times higher than that of wild type, while that of L3 was the lowest and about 3 times higher than that of wild type.

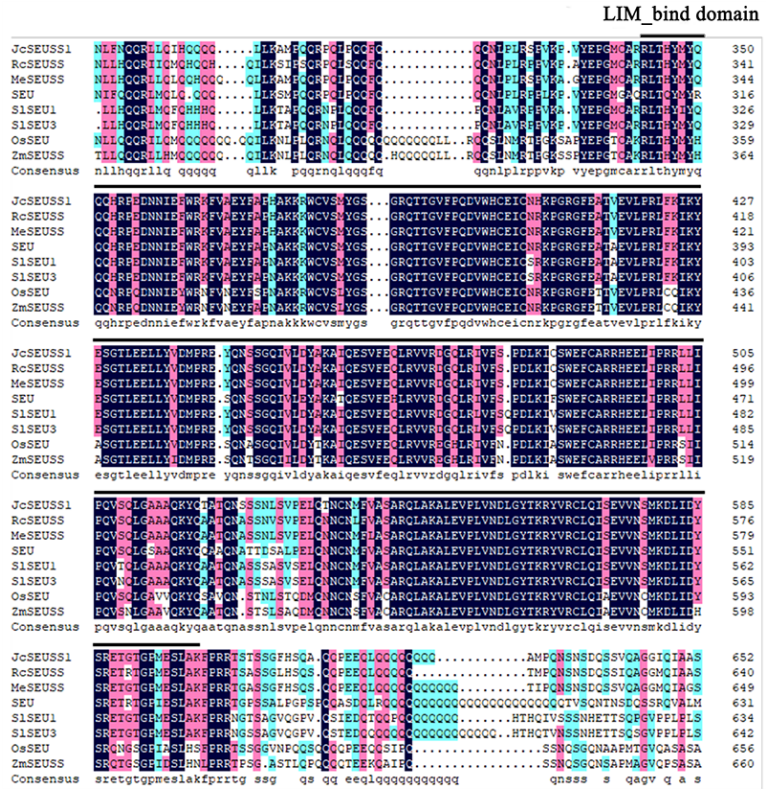

Figure 1 Conserved domain analysis of JcSEUSS1

Note: These homologs including *Arabidopsis thaliana* SEU (Accession No. NP_175051.1); *Solanum lycopersicum* SlSEU1 (Accession No. NP_001352551) and SlSEU3 (Accession No. XP_010322951); *Ricinus communis* RcSEUSS (Accession No. EEF42030) and *Manihot esculenta* MeSEUSS (Accession No. XP_021601723); *Oryza sativa Japonica Group* OsSEU (Accession No. XP_015616772); *Zea mays* ZmSEUSS (Accession No. NP_008670718); The dark blue shows the identically conserved protein sequences, and the partially conserved amino acid sequences are shown in light blue and red; LIM_bind domain in these amino acid sequences is overlined

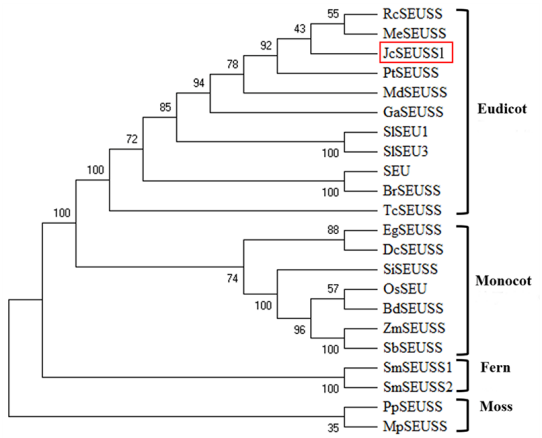

Figure 2 The phylogenetic analysis of JcSEUSS1

Note: JcSEUSS1: *Jatropha curcas*; RcSEUSS: *Ricinus communis*; MeSEUSS: *Manihot esculenta*; SlSEU1,SlSEU3: *Solanum lycopersicum*; PtSEUSS*: Populus trichocarpa* (accession No. NP_024456170); MdSEUSS: (accession No. XP_008381721); TcSEUSS*: Theobroma cacao* (accession No. NP_007019358); GaSEUSS*: Gossypium arboretum* (accession No. NP_017633449); SEU: *Arabidopsis thaliana*; ZmSEUSS: *Oryza sativa;* ZmSEUSS: *Zea mays*; BrSEUSS*: Brassica rapa* (accession No. XP_009145061); SbSEUSS*: Sorghum bicolor* (accession No. XP_021316829); SiSEUSS*: Setaria italic* (accession No. XP_004977184); BdSEUSS*: Brachypodium distachyon* (accession No. XP_003568560); EgSEUSS*: Elaeis guineensis* (accession No. XP_010921708); DcSEUSS*: Dendrobium catenatum* (accession No. XP_020680333); SmSEUSS1, SmSEUSS2: *Selaginella moellendorffii* (accession No. XP_024535463, No. XP_024520157); PpSEUSS: *Physcomitrium patens* (accession No. XP_024399004); MpSEUSS: Marchantia polymorpha subsp. Ruderalis (accession No. OAE35334); Red frame: JcSEUSS1

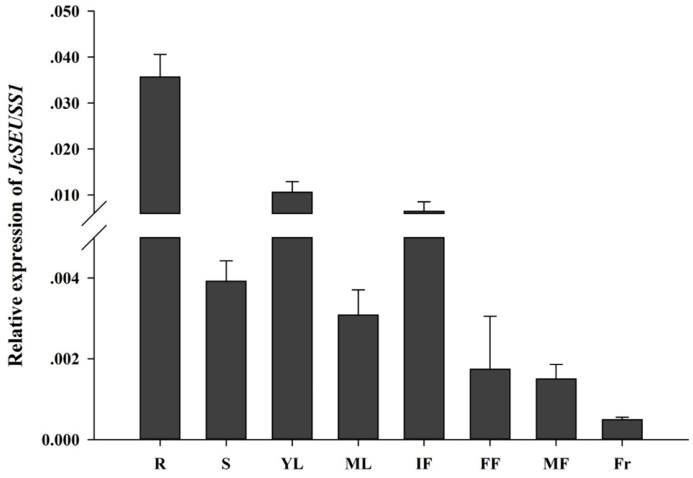

Figure 3 Expression level of *JcSEUSS1* in various organs of adult *Jatropha curcas*

Note: R: Roots; S: Stems; YL: Young leaves; ML: Mature leaves; IF: Inflorescences; FF: Female flowers; MF: Male flowers; Fr: Fruits at 40 d after pollination

Subsequently, phenotypes of transgenic lines of T0 generation were observed and compared, and the results showed that both leaves and petioles of transgenic plants turned red significantly (Figure 5A; Figure 5B; Figure 5C; Figure 5D). After observation and analysis of young and mature leaves, it was found that the color of young leaves became dark and obviously red than that of the wild type. In addition, the veins and petioles of young leaves of the transgenic plants were dark red, different from the tender green veins and petioles of the wild type (Figure 5E). Extraction and measurement results of anthocyanin showed (Figure 5F) that the anthocyanin content of the young leaves and petioles of the transgenic plants was significantly higher than that of the wild type. The anthocyanin content in the young leaves of the three transgenic plants was about 2 times higher than that in the wild type, but the anthocyanin content in the petiole of the three transgenic plants was more obvious, and the anthocyanin content in the transgenic plants was about 9 times higher in L3, 15 times higher in L4 and 13 times higher in L7. These results suggested that the over-expression of *JcSEUSS1* can promote the synthesis of anthocyanins in young leaves of *Jatropha curcas*.

By comparing the mature leaf morphology of transgenic plants, it was found that the mature leaf segments of wild-type and different transgenic plants were all green, but the veins and petioles of transgenic plants were obviously red (Figure 5G). After the determination of anthocyanin content (Figure 5H), the anthocyanin content of mature leaves and petioles of transgenic lines was significantly higher than that of wild type, and the increase of anthocyanin content of petioles was more obvious. The anthocyanin content of L3 transgenic plants was not significantly increased, while that of L4 and L7 was significantly increased compared with that of wild type, while the anthocyanin content of petiole of 3 transgenic plants was increased by about 10 times. The results showed that anthocyanin content was highest in L4 lines with the highest *JcSEUSS1* expression, and lowest in L3 lines with the lowest *JcSEUSS1* expression in both mature and young leaves and petioles. These results indicated that the over-expression of *JcSEUSS1* promoted anthocyanin accumulation in *Jatropha curcas* leaves.

1.4 The expression of anthocyanin synthesis-related genes in petioles of transgenic *Jatropha* seedlings was up-regulated

The relative expression levels of *JcCHS* and *JcCHI*, which encode key enzymes of anthocyanin synthesis, were detected in petioles of young *JcSEUSS1* transgenic *Jatropha*. The results showed that the content of anthocyanin in petiole of young wild-type *Jatropha* was low, and the expression levels of these two genes were also low. However, the expression levels of these two genes were significantly up-regulated in transgenic plants (Figure 6), and the relative expression trends of *JcCHS* and *JcCHI* were consistent with *JcSEUSS1*. These results suggested that over-expression of *JcSEUSS1* gene may promote anthocyanin accumulation in *Jatropha* leaves by promoting the expression of anthocyanin synthesis-related genes.

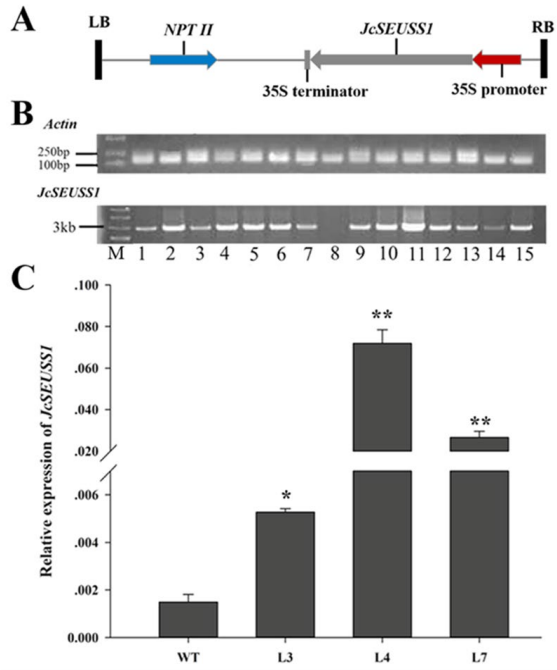

Figure 4 Relative expression of *JcSEUSS1* in transgenic *Jatropha*

Note: A: Over-expressed vector of *JcSEUSS1*; *NPT II*: Kanamycin gene; B: PCR identification of *35S:JcSEUSS1* transgenic plants; M: Trans2K Plus II DNA Marker, 1~15: 15 independent transgenic plants (L1~L15); C: The expression level of *JcSEUSS1* in wildtype and transgenic plants; *Actin*: positive control; WT: Wildtype *Jatropha*; *35S: JcSEUSS1*: Transgenic *Jatropha* with over-expressing *JcSEUSS1*; L3,L4,L7: The strain 3, 4 and 7 of *JcSEUSS1* transgenic *Jatropha*; Reference gene: *JcACT1*; Error bars represent SE (n=3); *: $p \leq 0.05$; **: $p \leq 0.01$

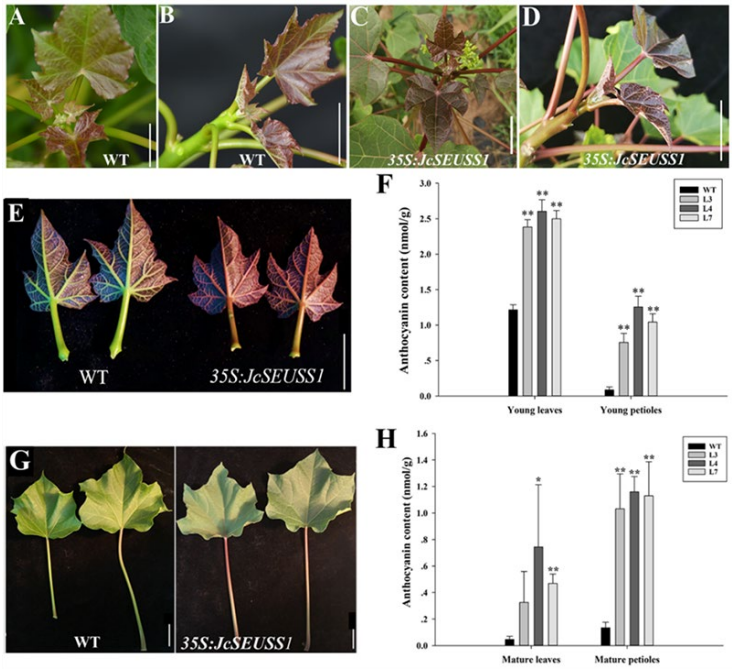

Figure 5: Over-expression of *JcSEUSS1* induced the increase of anthocyanin content in leaves and petioles of transgenic *Jatropha*

Note: A,B: Young leaves (A) and petioles (B) in wildtype *Jatropha*; C,D: Young leaves (C) and petioles (D) in *35S:JcSEUSS1* transgenic *Jatropha*; E: Phenotype of abaxial side of young leaves of wildtype and transgenic *Jatropha*; F: the anthocyanin content of young leaves and petioles in wildtype and transgenic *Jatropha*; G: Phenotype of adaxial side of mature leaves of wildtype and transgenic *Jatropha*; H: The anthocyanin content of mature leaves and petioles in wildtype and transgenic *Jatropha*; WT: Wildtype *Jatropha*; *35S:JcSEUSS1*: Over-expressed *JcSEUSS1* in transgenic *Jatropha*; L3,L4,L7: The strain 3, 4 and 7 of *JcSEUSS1* transgenic *Jatropha*; *: $p \leq 0.05$; **: $p \leq 0.01$; Bar=3 cm

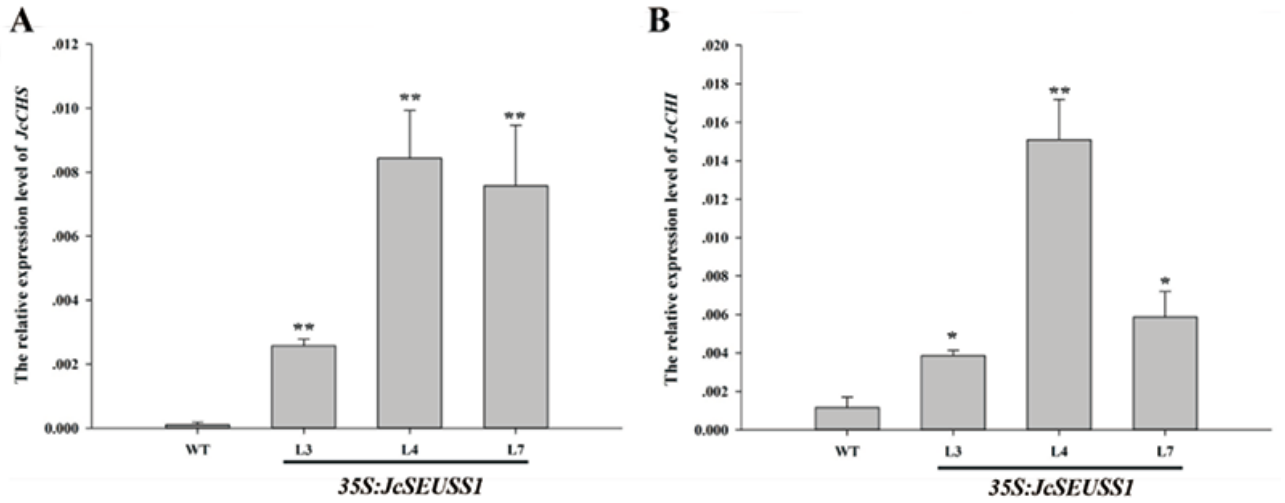

Figure 6 Relative expression of *JcCHS* and *JcCHI* in young petioles of *35S:JcSEUSS1* transgenic *Jatropha*

Note: WT: Wildtype *Jatropha*; *35S:JcSEUSS1*: Over-expressed *JcSEUSS1* in transgenic *Jatropha*; L3, L4, L7: The strain 3, 4 and 7 of *JcSEUSS1* transgenic *Jatropha*; Reference gene: *JcACT1*; *: $p \leq 0.05$; **: $p \leq 0.01$

2 Discussion

SEU gene has been widely studied in *Arabidopsis thaliana*. *SEU* gene is expressed in seedlings, leaves, inflorescence buds, flowers and other tissue parts of *Arabidopsis thaliana*, so *SEU* gene regulates multiple growth and development stages of Arabidopsis vegetative growth and reproductive growth (Franks et al., 2002; Bao et al., 2010). In this study, we found that *JcSEUSS1* is also a similar constitutively expressed gene in *Jatropha*, and regulates the growth and development of different tissue parts of *Jatropha*, including leaves, male flowers, fruits and seeds. Compared with other species, *JcSEUSS1* is highly conserved not only in protein sequence but also in gene function. In Arabidopsis, the expression level of *SEU* gene in flowers is significantly higher than that in leaves, so *SEU* mainly regulates the development of *Arabidopsis* flowers (Franks et al., 2002). However, *JcSEUSS1* expression level in *Jatropha* leaves is significantly higher than that in male and female flowers, so there may be some differences in the function of *SEU* gene between *Jatropha* and *Arabidopsis*. *JcSEUSS1* may have stronger effect on leaf development of *Jatropha* than on flower development.

In this study, we found that over-expression of *JcSEUSS1* gene can promote anthocyanin accumulation in *Jatropha* leaves, and *SEU* gene has not been reported to regulate anthocyanin synthesis in other species. Although the function of *SEU* in regulating anthocyanin synthesis has not been reported in *Arabidopsis thaliana*, the regulatory relationship between *SEU* and key enzymes of anthocyanin synthesis has been found compared with the wild type, the expression of *CHS*, a key enzyme gene encoding flavonoid synthesis in the mutant *seu-6*, was significantly upregulated under dark or continuous light conditions (Huai et al., 2018). SEU can interact with PIF4, both of which negatively regulate photomorphogenesis and positively regulate temperature-mediated hypocotyl elongation (Huai et al., 2018). PIF4, a member of the helix-loop-helix (bHLH) transcription factor family, is a negative regulator of anthocyanin accumulation induced by red light (Liu et al., 2015). In this study, we found that over-expression of *JcSEUSS1* resulted in significantly upregulation of *JcCHS* and *JcCHI* genes (Figure 6). qPCR results showed that the expression of *JcSEUSS1* was significantly increased in transgenic plants without gene silencing (Figure 4). This contrasts with the effect of *SEU* on *CHS* expression in Arabidopsis (Huai et al., 2018), suggesting that the function of *JcSEUSS1* in *Jatropha* may differ from that of its homologue in model plant Arabidopsis. This difference may be related to the fact that transgenic *Jatropha* seeds in this study were planted in tropical areas with strong light and high temperature, because previous studies have shown that light intensity and light quality have important regulatory effects on anthocyanin accumulation in plants (Dong et al., 1998). *CHS* gene encodes chalcone synthase, the first key enzyme in flavonoid synthesis (Tropf et al., 1995). *CHS* gene expression requires UV-B and UV-A/blue light induction (Christie and Jenkins, 1996). In Arabidopsis,

over-expression of *CHS* gene can increase the tolerance of leaves to strong light (Zhang et al., 2018). CHI encodes chalcone isomerase, whose main function is to convert yellow chalcone produced by *CHS* into colorless flavanones. CHI is also a key enzyme in the anthocyanin synthesis pathway (Burbulis and Winkel-Shirley, 1999). Its expression also requires light induction (Song et al., 1998). In conclusion, both *CHS* and *CHI* are necessary enzymes for photoinduced anthocyanin synthesis pathway in plants, and the expression levels of *JcCHS* and *JcCHI* in *Jatropha JcSEUSS1* overexpressed plants were significantly up-regulated, suggesting that *JcSEUSS1* may be involved in photoinduced anthocyanin synthesis pathway in *Jatropha* plants. Regulation of *JcCHS* and *JcCHI* gene expression promotes anthocyanin accumulation in *Jatropha* leaves.

Jatropha is mainly cultivated in tropical and subtropical areas where both light and UV are strong, and anthocyanins have a photoprotective effect (Lucho-Constantino et al., 2017; Pang et al., 2018). In this study, the content of anthocyanin in the leaves and petioles of *JcSEUSS1* transgenic plants with overexpression increased by 2~10 times (Figure 5F; Figure 5H), so that these transgenic materials could be used to develop excellent stress-resistant *Jatropha* seeds.

3 Materials and Methods

3.1 Plant materials and cultivation

The seedlings of *Jatropha* seeds were cultured in tissue culture room (25°05′N, 102°69′E), Xishuangbanna Tropical Botanical Garden, Kunming, Yunnan Province, at (26±2)°C and 14 h/d of light. Adult plants were planted in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun Town, Mengla County, Xishuangbanna Autonomous Prefecture, Yunnan Province (21°41′N, 101°25′E), with an average annual temperature of 21.4°C and annual rainfall of 1 556 mm.

3.2 Cloning of full-length cDNA of *JcSEUSS1* from *Jatropha*

Silica gel adsorption method was used to extract total RNA from roots, stems, young leaves (the second young leaf from stem tip), mature leaves (the eighth leaf from stem tip), inflorescences, female flowers, male flowers and fruits grown for 40 days of *Jatropha* (Ding et al., 2008). The quality and concentration of *Jatropha* RNA were determined by agarose gel electrophoresis and NanoDrop 2000 spectrophotometer. Then refer to the instructions of TAKARA (Dalian, China) PrimeScript TH Reagent Kit with gDNA Eraser for reverse transcription of RNA into cDNA. Primers XC772 and XC773 were designed using Primer5 based on the nucleotide sequence of *JcSEUSS1* obtained from *Jatropha curcas* genome database (http://www.kazusa.or.jp/jatropha/) (Table 1), and the full-length cDNA of the *JcSEUSS1* was amplified.

Table 1 PCR primer sequences used in this study

Target templates	Primers	Sequences (5′-3′)	Usages
JcSEUSS1	XC772	CGGGATCCATGGTACCCTCGGGGCCG	Primers for full-length cDNA
	XC773	GCGTCGACAGGGGATGGTTTCCAATCAA	
JcACT1	XK191	CTCCTCTCAACCCCAAAGCCAA	Reference gene
	XK192	CACCAGAATCCAGCACGATACCA	
JcSEUSS1	XD437	CACAGCAGCTGCAGTCA	Primers for qRT-PCR
	XD438	AGAAACAATGACTGATCTGA	
JcCHS	XE489	GCCCGAGTTCTTGTTGTTTGC	Primers for qRT-PCR
	XE490	GACGCTCAACGGAAGTATCTG	
JcCHI	XE491	CCATTAACGGGCCAACAATAC	Primers for qRT-PCR
	XE492	AGAGGAGCCAGGAGGAAAGAC	

3.3 Real-time fluorescence quantitative PCR

Firstly, the primers of qRT-PCR were designed on the NCBI website (https://www.ncbi.nlm.nih.gov/). Then taking cDNA as template, qRT-PCR was performed with the help of Roche's LightCycler 480 SYBR Green I Master kit. The exported data were analyzed using LightCycler 480 software and mapped using Sigmablot software. *JcACT1* gene of *Jatropha* was used as an internal reference, and three biological replicates were carried out in the experiment.

3.4 *35S*:*JcSEUSS1* plasmid construction and transformation of *Jatropha*

The full-length *JcSEUSS1* cDNA was cleaved by *BamH*I and *Sal*I restriction endonuclease and then ligated to pOCA30 vector containing CaMV 35S promoter and 35S enhancer. The over-expression vector *35S*:*JcSEUSS1* was constructed and transformed into *Jatropha* by agrobacterium-mediated transformation. In this study, the cotyledon of *Jatropha* seeds was used as transformed explants for agrobacterium infection, and kanamycin resistance and PCR amplification were used to screen the positive plants (Pan et al., 2010; Fu et al., 2015).

3.5 Extraction of anthocyanins from *Jatropha* seeds

In this experiment, according to the modified extraction method of anthocyanin proposed by Gou et al. (2011), 1 g of annual *Jatropha* tissue sample was taken. After freezing and milling with liquid nitrogen, 4 mL of anthocyanin extract (5% hydrochloric acid and 80% methanol) was added, mixed and placed overnight at 4°C, centrifuged at 14 000 r/min for 20 min, and supernatant was obtained. NanoDrop2000 spectrophotometer was used to measure the OD values at 530 nm, 620 nm and 650 nm respectively. Anthocyanin content (nmol/g)=$OD_\lambda/\varepsilon \times v/m \times 1\ 000\ 000$

Optical density of anthocyanin $OD_\lambda=(OD_{530}-OD_{620})-0.1\ (OD_{650}-OD_{620})$

OD_λ: optical density value of anthocyanin, ε: molar extinction coefficient of anthocyanin 4.62×10^6, v: total volume of extraction liquid (mL), m: sample mass (g).

Authors' Contributions

WJX was the executor of this experimental study, completed the analysis of experimental data and the writing of the first draft. MX, TYB, TMY and XZF provided help in the experiment and participated in the revision of the paper. TMY and XZF were the architects and principals of the project, directing the experimental design and data analysis. All authors read and approved the final manuscript.

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