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Molecular cloning and functional analysis of one *ZEITLUPE* homolog *GmZTL3* in soybean

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Abstract *ZEITLUPE* (*ZTL*) plays an important role in the control of flowering time and photomorpogenesis in *Arabidopsis* and is highly conserved throughout the plant kingdom. Here, we report the characterization of a soybean *ZTL* homolog *GmZTL3* (*Glycine max ZTL 3*). The absorption spectrum of the recombinant GmZTL3 proteins indicates that it may be a UV/blue photoreceptor. The *GmZTL3* expression is independent of diurnal cycles and varies in different tissues along with developmental stages. Before the unifoliolates open fully, *GmZTL3* transcripts concentrate in the roots and hypocotyls, while at flowering *GmZTL3* accumulates at higher abundance in stems and petioles. Furthermore, the *GmZTL3* mRNA accumulates in all kinds of leaves before flowering and concentrates in maturation seeds. In *Arabidopsis,* the ectopic expression of *GmZTL3* delays flowering, implicating *GmZTL3* is an inhibitor of flowering induction. Our data indicate that *GmZTL3* probably functions as a photoreceptor and plays a role in multiple developmental processes, including the control of flowering time.

Keywords *ZEITLUPE* (*ZTL*) · Photoreceptor · Circadian clock · Flowering · Soybean

Introduction

The circadian clock regulates 24-h biological rhythms for adapting to the diurnal cycles and temperature fluctuations, which is highly conserved among organisms [1]. The mechanism of operation consists of a series of processes. First, an input pathway perceives and transfers environmental cues relevant to temperature and light to the circadian clock. Second, the endogenous oscillator integrates the environmental cues and transforms them into internal signals. Finally, the output pathway transmits the internal signal into target cells to regulate the physiological and developmental processes [2]. Many physiological and developmental processes, such as cell elongation, stomata opening, leaf movement and flowering are controlled by the circadian clock [1]. The circadian clock consists of one central feedback loop and two lateral feedback loops [3, 4].

In *Arabidopsis*, *ZEITLUPE* (*ZTL*) encodes a 627 amino acid peptide containing three domains: LOV domain, F-box domain and six kelch repeats domain [6]. The ZTL protein was identified as a member of a new photoreceptor family in *Arabidopsis*, which plays a role in light input to the circadian clock [7, 12]. The ZTL protein is regulated by circadian rhythms and can be recruited to the SCF (Skp1-Cullin-F-box) proteasome complex, which targets degradation of Timing of CAB Expression 1 (TOC1), a component of

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central oscillator of clock [5, 8]. ZTL can interact with the photoreceptors Cryptochrome 1 (CRY1) and Phytochrome B (PHYB) in *Arabidopsis* [9], as well as with GIGANTEA (GI) protein and blue light enhances this interaction through the LOV domain [7]. Additionally, the circadian phase-specific degradation of ZTL is mediated by the proteasome and it may be ubiquitinated itself [10]. Moreover, ZTL can negatively regulate the Pseudo-Response Regulator 5 (PRR5) abundance to regulate the circadian oscillator [11].

Currently, there are no reports on *ZTL* homologs in soybean. In this study, we isolated one *ZTL* homolog in soybean (*Glycine max*) and named it *GmZTL3* (*Glycine max ZTL3*). We purified the recombinate GmZTL3 proteins from yeast, measured the absorption spectra, and analyzed the expression profiles of *GmZTL3* transcripts in different photoperiods as well as different organs during the developmental progress. Finally we investigated the subcellular localization of GmZTL3 proteins and *GmZTL* flowering activity through expressing *GmZTL3* constitutively in *Arabidopsis*. The results suggest that *GmZTL3* is one *ZTL* homolog in soybean and involved in control of the flowering process.

Materials and methods

Plant materials and growth conditions

The soybean cultivar Kennong 18 [*Glycine max* (Merr.) cv. Kennong 18] was grown in artificial climate chambers under both short day (8 h light/16 h dark) and long day conditions (16 h light/8 h dark) at 28°C under a light frequency of 100 μmol m^{-2} s^{-1}. Seedlings were harvested before the expansion of the unifoliolate leaves. Tissues were sampled independently at the following developmental stages: unifoliolate and 1st, 2nd, 3rd, and 4th trifoliolate fully expanded, and at flowering. Seeds and pods (excluding seeds) were sampled at 7, 14, 21 days after flowering and also at maturity. For circadian analysis, the fully expanded unifoliolates were sampled under short day and long day conditions for two successive 24 h light/dark cycles at 2 h interval. All samples were immediately frozen in liquid nitrogen and stored at −80°C for later use.

Seeds of *Arabidopsis* wild type C24 and *ztl* mutants were grown on MS plates for 3 days, grown under long days at 20–22°C for 10 days, and then transplanted to the soil.

RNA isolation, cDNA synthesis and gene cloning

Total RNA was extracted [13] by TRIzol regent (Invitrogen, USA) and cDNA synthesis was performed according to RevertAid first-strand cDNA synthesis kit manual (Fermentas, Germany). The Glymal15g17480 sequence, including UTR and CDS regions, was cloned using RT-PCR with primers of *GmZTL3*-U-F and *GmZTL3*-U-R (Table 1). Three independent clones were sequenced, and the resulting consensus sequence was used as the template to amplify the CDS region with primers of *GmZTL3*-C-F and *GmZTL3*-C-R (Table 1). The amplicons were inserted into the pGWC vectors [14] and confirmed by sequencing. The yeast expression vector *GmZTL3*-pYES-DEST52, the ectopic expression vector *GmZTL3*-pLeela and the subcellular localization vector pEXSG-*GmZTL3*-YFP were established separately by LR recombination reaction (Invitrogen, USA).

Bioinformatics analysis

The amino acid sequence alignment was carried out using ClustalW software with default parameters, and a Neighbor-joining tree was built using the MEGA software (version 4.0). The bootstrap method was used for assessing the phylogenetic tree, and the number of bootstrap replicates was set to 1000. The search for GmZTL3 LOV domains and pairwise alignment were performed at NCBI using the BLAST network service. The three-dimensional structure of GmZTL3 LOV domain was built using the Swiss Model (http://www.swissmodel.expasy.org) with the *Arabidopsis*

Table 1 Primers used in qPCR and PCR

Names of primers	Sequence (5′–3′)
GmZTL3-U-F	GCCACTGCGACGCCGTTTCCGTTAA
GmZTL3-U-R	CCTTTGCAACAGCATGCCCGTTTATTAA
GmZTL3-C–F	ATGGAGTGGGACAGCAATTCCGATCTCAGC
GmZTL3-C-R	CTAGATGACAGAACTTGCCAAGGAAAGTTC
qGmZTL3-F	GGAGGAATGGATGCTAAG
qGmZTL3-R	ACCAATCAGAGAATCACC
GmACTIN11-F	ATCTTGACTGAGCGTGGTTATTCC
GmACTIN11-R	GCTGGTCCTGGCTGTCTCC
pLeela-F	GTTATGGGTCAACGGTTTC

Phototropin2 LOV domain [15, 16] as template (PDB ID: 2Z6D), and the result was analyzed using PyMOL software.

Expression, purification, and absorption spectroscopy measurement of the GmZTL3 protein

The yeast expression vector *GmZTL3*-pYES-DEST52 was transformed into *Saccharomyces cerevisiae* strain INV*Sc*1 according to the *S.c.* EasyComp™ Kit protocol (Invitrogen, USA). Expression of the recombinant fusion protein GmZTL3, which contained polyhistidine (6xHis) tag at the C-terminus, was induced with galactose under dark conditions for 24 h at 28°C. The cells from culture about 1 l was lysed with the breaking buffer (50 mM sodium phosphate, pH 7.4, 5% Glycerol, 1 mM PMSF), using acid-washed glass beads and a bead beater (BiospecProducts, USA). The purification procedure was accomplished following ProBond™ Purification System manual (Invitrogen, USA)under dim red light. The GmZTL3 protein was monitored by anti-His antibody (Abmart, China) and anti-GmZTL raised by our lab in Western blotting. The purified protein was dialyzed against the elution buffer to remove imidazole, which could interfere the absorption spectra of proteins. The purified protein was quantified using a Bradford quantification kit (Bio-Rad, USA). About 100 μg purified GmZTL3 protein was used to measure the absorption spectrum with Back-Man DU-800 spectrophotometer (BeckMan Coulter, USA) at room temperature (25°C) and analyzed with the supporting software and Origin software (Version 7.0). The parameter was set as follows: scan wavelength, 200–800 nm; scan speed, 1200 nm/min; scan interval, 1 nm.

Analysis of gene expression

For *GmZTL3* expression analysis, specific primers (Table 1) were used for the quantification of *GmZTL3* and *GmACT11* (Table 1) was used as the internal control gene [17]. Quantitative real time RT-PCR (qPCR) reactions were performed with the ABI StepOne Detection System (Applied Biosystems, USA). The qPCR reaction consisted of 4 μl template, 7.5 μl 2× SYBR Premix, 300 nM of each primer, and 0.3 μl ROX (TaKaRa, Japan).

Subcellular localization of GmZTL3 proteins

The vector pEXSG-*GmZTL3*-YFP driven by the *Cauliflower mosaic virus* 35S promoter and the vector pENSG-CFP-AHL22 served as a maker of nuclear proteins [18] were biolistically co-transformed into *Arabidopsis* mesophyll protoplasts and the result was recorded by confocal laser scanning microscope (Leica, USA).

Plant transformation

The *GmZTL3*-pLeela vector was transformed into *Arabidopsis* C24 and *ztl* mutant backgrounds via *Agrobacterium*-mediated transformation. The transgenic lines were screened using 50 mg l^{-1} glufosinate ammonium and confirmed by PCR with the primers specific to the pLeela and *GmZTL3* (Table 1). Three transgenic lines T1 plants (*ztl* background), together with ten *ztl* plants, were grown under LD conditions in growth chamber room. T2 plants from three transgenic lines T1 plants (C24 background), together with ten wild-type C24 plants, were grown under the same conditions.

Results

GmZTL3 is a member of the *ZTL* family

We screened soybean peptide sequences in the Phytozome database (http://www.phytozome.org) using the *Arabidopsis* ZTL protein sequence as a query. Six peptide sequences were identified which shared three same domains with all AtZTL family: LOV domain, F-box domain and six kelch repeats domain. A further phylogenetic analysis showed four soybean sequences close to AtZTL. Therefore, they were assigned the names *GmZTL1, GmZTL2, GmZTL3*, and *GmZTL4*, respectively (Fig. 1). The sequence of *GmZTL3,* including the UTRs and CDS, was cloned in this study. Alignment of the peptides of GmZTL3 and AtZTL showed a very high similarity and the majority of key residues (including GXNCRFLQ motif) and the spacer of three domains were also conserved (Fig. S1). The CDS of *GmZTL3* covered 1,833 bp and encoded a 611-residue polypeptide. The critical amino acids in three domains indicated conserved functions between GmZTL3 and AtZTL.

Absorption peaks of the GmZTL3 protein appear in UV/blue light region

The three dimensional structure model of the GmZTL3 protein LOV domain shows the analogous hydrophobic structure as the AtPhot2 (Arabidopsis phototropin2) LOV domain, which could bind FMN (flavin mononucleotide) chromophore (Fig. 2b). This result indicated that the GmZTL3 protein may perceive the light. Based on cloning of *GmZTL3* into a yeast expression vector with a C-terminal His-tag and galactose induction, the recombinant GmZTL3 protein was purified from yeast (Fig. 2a) and used to analyze the biological activity. As expected, the GmZTL3 protein exhibited a particular spectroscopical characteristic (Fig. 2c). The absorption peaks appeared at

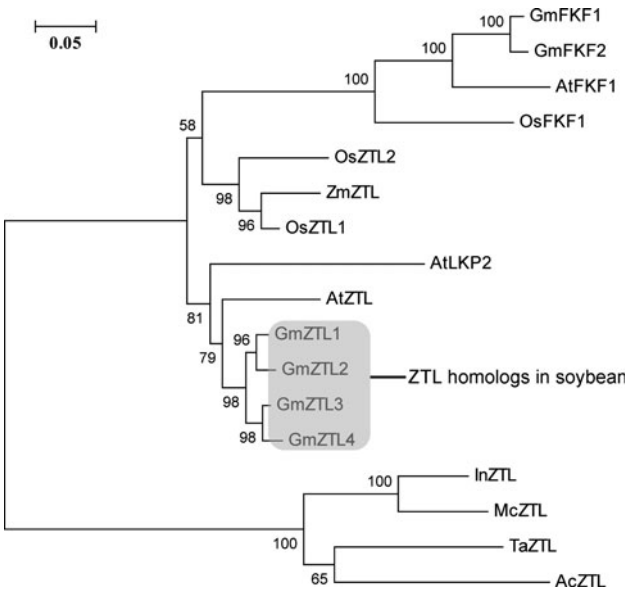

Fig. 1 Phylogenetic tree demonstrating the evolutionary relationships among members of ZTL protein family. The *gray shadow* indicates the soybean ZTL homologs. Accession numbers: GmFKF1 (*Glycine max*), Glyma05g34530; GmFKF2 (*G. max*), Glyma08g05130; GmZTL1 (*G. max*), Glyma13g00860; GmZTL2 (*G. max*), Glyma17g06950; GmZTL3 (*G. max*), Glyma15g17480; GmZTL4 (*G. max*), Glyma09g06220; OsFKF1 (*Oryza sativa*), Os011g34460; OsZTL1 (*O. sativa*), Os06g47890; OsZTL2 (*O. sativa*), Os02g05700; ZmZTL (*Zea mays*), GRMZM2G113244; InZTL (*Ipomoea nil*), ABC25060; McZTL (*Mesembryanthemum crystallinum*), AAQ73527; AcZTL (*Allium cepa*), ACT22763; TaZTL (*Triticum aestivum*), ABR14627; AtZTL, AT5G57360; AtFKF1,AT1G68050; AtLKP2, AT2G18915

450 nm (blue light region) with a small peak at 340 nm (ultraviolet-A region). These results demonstrated that the GmZTL3 protein possessed properties of a blue-light photoreceptor and may be able to perceive blue light in soybean.

GmZTL3 expression is independent of the diurnal cycle

To study the relationship between the expression of *GmZTL3* and light/dark cycles, we tested the mRNA abundance in unifoliolates under long day and short day conditions by quantitative real time RT-PCR (qPCR). The expression level of *GmZTL3* kept almost constant excluding a little fluctuation in both LD and SD conditions. Thus the *GmZTL3* mRNA shows no obvious circadian rhythm in different photoperiodic conditions in soybean as *AtZTL* does in *Arabidopsis* (Fig. 3).

Spatial and temporal expression profiles of *GmZTL3*

To study the specific expression profiles of *GmZTL3* in tissues/organs, we examined the transcripts in most of soybean organs by qPCR. The *GmZTL3* transcripts were detectable in different organs and seedlings (Fig. 4). At the unifoliolate stage (vegetative phase), roots accumulated the highest amount of *GmZTL3* transcripts. When plants entered the reproductive phase, the stem, the third trifoliolates, and petioles expressed high levels of *GmZTL3*. As seed development progressed, the *GmZTL3* transcript abundance in the pod wall also increased. The data reflected a wide role of *GmZTL3* in the broad developmental processes in soybean.

To elucidate the expression profiles of *GmZTL3* during leaf development, we monitored the expression of *GmZTL3* in different leaves across different developmental stages. In general, *GmZTL3* mRNA accumulated in all kinds of leaves including unifoliolates and different trifoliolates during vegetative growth, but decreased at flowering (Fig. 5a). In seeds, *GmZTL3* expression maintained relatively low levels, but increased significantly at maturation (Fig. 5b).

GmZTL3 proteins localize in both the cytoplasm and nucleus

To visualize the subcellular localization of GmZTL3 proteins, a fusion gene GmZTL3-YFP driven by a 35S promoter was constructed, and then co-transformed with the nuclear marker gene CFP-AHL22 into *Arabidopsis* leaf protoplasts. The results showed that YFP-associated fluorescence was found in both the cytoplasm and nucleus, while the CFP signal was seen only in nucleus (Fig. 6).

Constitutive expression of *GmZTL3* delayed flowering in *Arabidopsis*

To study the function of *GmZTL3* in flowering regulation, we carried out an ectopic expression experiment in both *Arabidopsis* C24 and *ztl* mutant backgrounds. The transgenic lines, in both backgrounds, produced more rosette leaves and flowered later in LD conditions (Fig. 7). The transgenic lines T1 plants (*ztl* background) produced more rosette leaves (Fig. 7a) and flowered later (Fig. 7c) than the *ztl* plants. The number of rosette leaves produced by the transgenic lines T2 plants in C24 background was also more than their parents (Fig. 7b), and the plants flowered later (Fig. 7d).

Discussion

In *Arabidopsis*, three different families of blue-light receptors have been characterized: cryptochromes, phototropins, and the ZTL family. LOV domains are highly conserved in all blue light photoreceptors [19]. In our results, the GmZTL3, holding conserved LOV domain, had

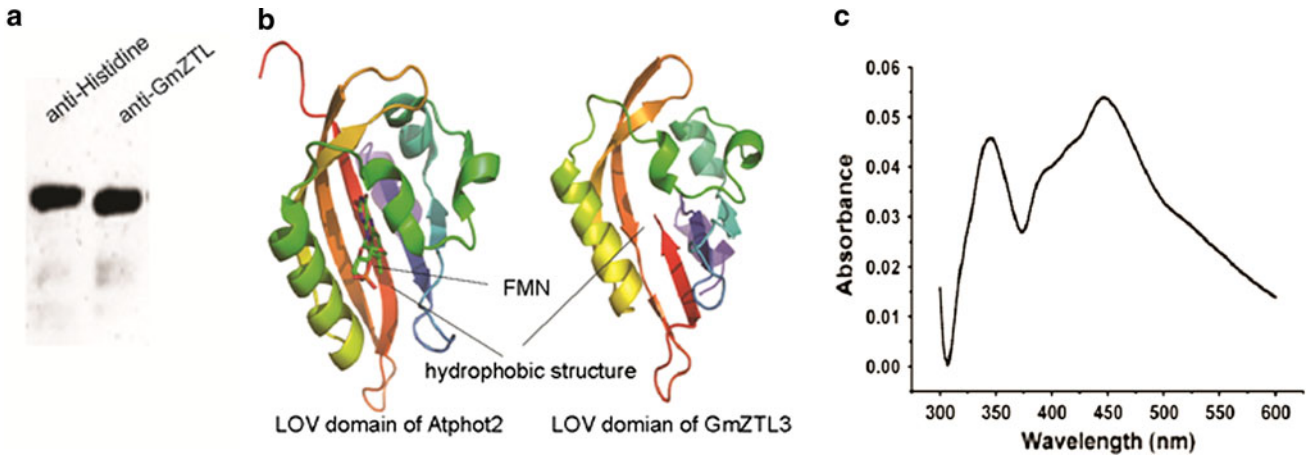

Fig. 2 The absorption spectrum of the GmZTL3 protein. **a** Identification of the purified recombinant GmZTL3 protein monitored by anti-Histidine and anti-GmZTL in Western blotting. **b** Three dimensional structure model of GmZTL3 LOV domain with the AtPhototropin2 LOV domain as a template (PDB ID: 2Z6D), showing that the three dimensional structure of GmZTL3 LOV domain possesses analogue hydrophobic structure as the Phototropin2 LOV domain. **c** The absorption spectrum of the GmZTL3 protein

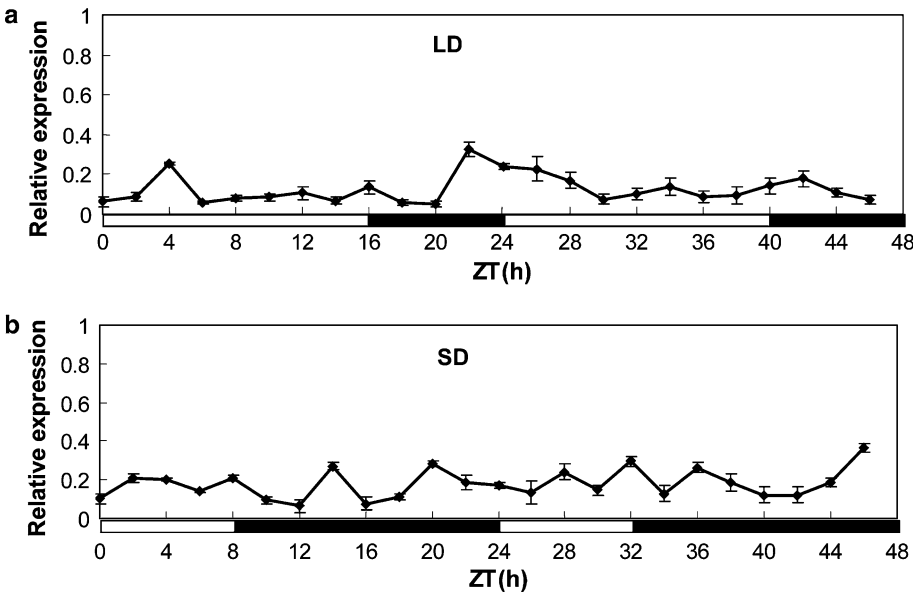

Fig. 3 The expression of *GmZTL3* transcripts in response to both short days (SD) and long days (LD) over 48 h in soybean leaves. *Open* and *closed boxes* represent light and dark periods respectively. *Error bars* represent standard deviation of three replicates. *GmACT11* was employed as a control

a similar sequence and structure, and possessed the analogical absorption spectra. Therefore, we speculate that GmZTL3 may act as a photoreceptor to perceive the UV/blue light in soybean.

Similar to ZTL homologs in *Arabidopsis*, rice and onion [22–24], the *GmZTL3* mRNA was constantly expressed independent of photoperiod in soybean. However, the ZTL protein levels oscillate in LD condition in *Arabidopsis* [10]. The further work to study the relationship between GmZTL3 protein and photoperiod would help us to understand the mechanism of circadian clock in soybean.

In *Arabidopsis*, *ZTL* is expressed throughout the plant and in almost all cell types [21]. In this study, we found that *GmZTL3* was constitutively expressed throughout the plant. This result is consistent with the argument that GmZTL3 acted as a photoreceptor to participate in the whole plant developmental progress. In previous reports, *ZTL* plays a role in the process of seedling photomorphogenesis (hypocotyl expansion) in *Arabidopsis* [25]. The higher expression of *GmZTL3* in the root and hypocotyl at the time of the unifoliolates fully opened indicated the GmZTL3 may play a role in morphogenesis in soybean. GmZTL3 may be involved in seed filling due to its higher expression in the mature seed. The expression of *GmZTL3* in unifoliolates and different trifoliolates was increased before flowering and then concentrated in the stem and petioles at the time of flowering, inferring that GmZTL3 may function in the control of flowering.

The subcellular localization of transcript regulators contributes to the generation and maintenance of the

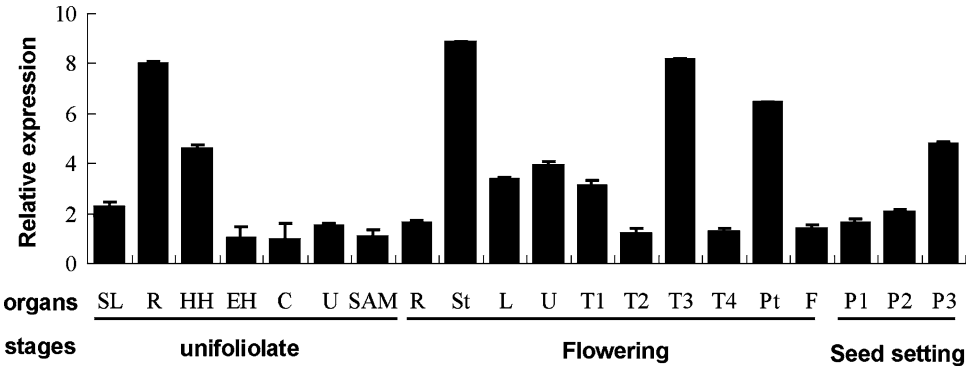

Fig. 4 The expression of *GmZTL3* in developmental stages of different tissues/organs. Notes for tissue/organs: *SL* seedlings, *R* roots, *HH* hypocotyls, *EH* epicotyls, *C* cotyledons, *U* unifoliolates, *SAM* shoot apex (including the apical meristem and immature leaves), *St* stems, *L* lateral leaves, *T1 to T4* the 1st to 4th trifoliolates, *Pt* petioles, *F* flower buds, *P1 to P3* pod walls at 7, 14, 21 days after flowering, respectively. Notes for developmental stages: Unifoliolates, unifoliolates *fully opened*; flowering, onset of flowering; seed setting, seed growth. *Error bars* represents standard deviation of three independent experiments

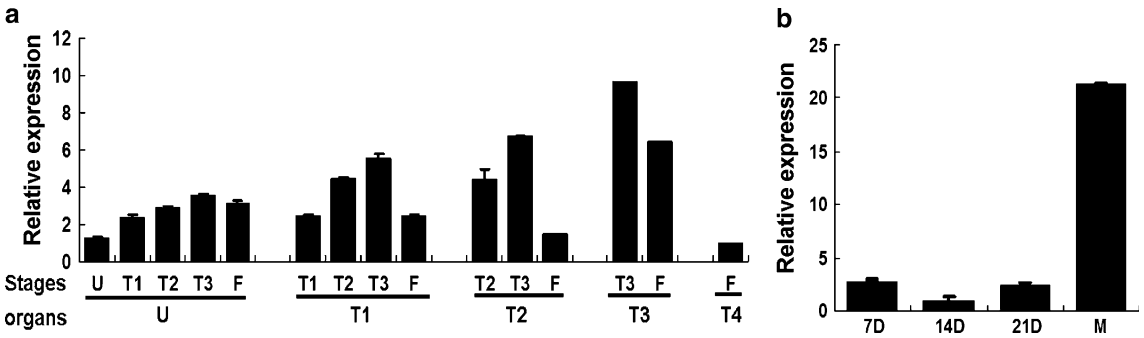

Fig. 5 The specific expression of *GmZTL3* in leaves (**a**) and seeds (**b**) during development. *Organs* U for unifoliolates; T1 to T4 for the 1st to 4th trifoliolates. *Stages* at the different leaves (U and T1 to T4) *opened fully*. Seeds were harvested at 7, 14 and 21 days (7D, 14D and 21D) after flowering or at mature (M). *Error bars* represent standard deviation of three independent experiments

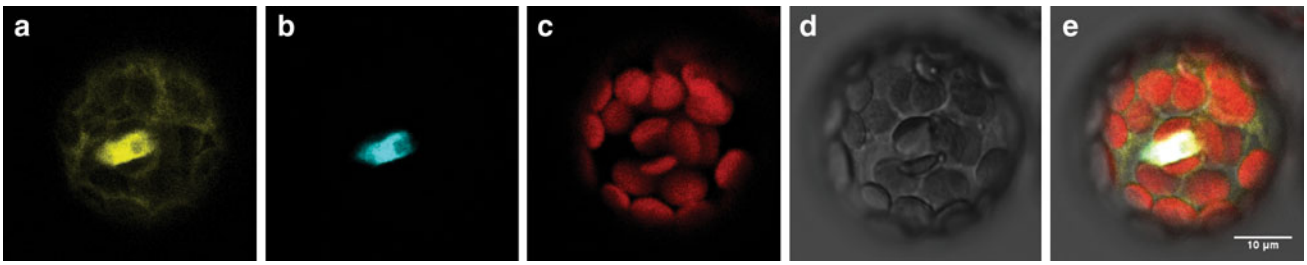

Fig. 6 Subcellular localization of GmZTL3 proteins in *Arabidopsis* mesophyll protoplasts. **a** Yellow fluorescent signals for GmZTL3 proteins. **b** Cyan fluorescent signals for the nuclear marker, AHL22 proteins. **c** Chloroplast auto-fluorescence, **d** Bright field, **e** Merged of **a**, **b**, **c**, and **d**. *Scale bar*, 10 μm

cellular oscillator in *Arabidopsis* [20, 21], and the AtZTL protein mobilizes between the nucleus and cytoplasm in circadian-dependent mode [22]. Transient expression analysis revealed the GmZTL3 was expressed in both the cytoplasm and the nucleus, indicating that GmZTL3 played a role as a similar mode as AtZTL does. To examine the effects of clock regulation on GmZTL3 localization would facilitate to elucidate the mechanism of GmZTL function.

In *Arabidopsis*, *ZTL* overexpression significantly delays flowering under long-day conditions. Here, the ectopic expression of *GmZTL3* in *Arabidopsis* also delayed flowering independent of plant background, suggesting that *GmZTL3* had a similar function in controlling the flowering and that there was no much difference of ZTL function between the short-day plant (soybean) and the long-day one (*Arabidopsis*). Therefore, the function of *ZTL* was

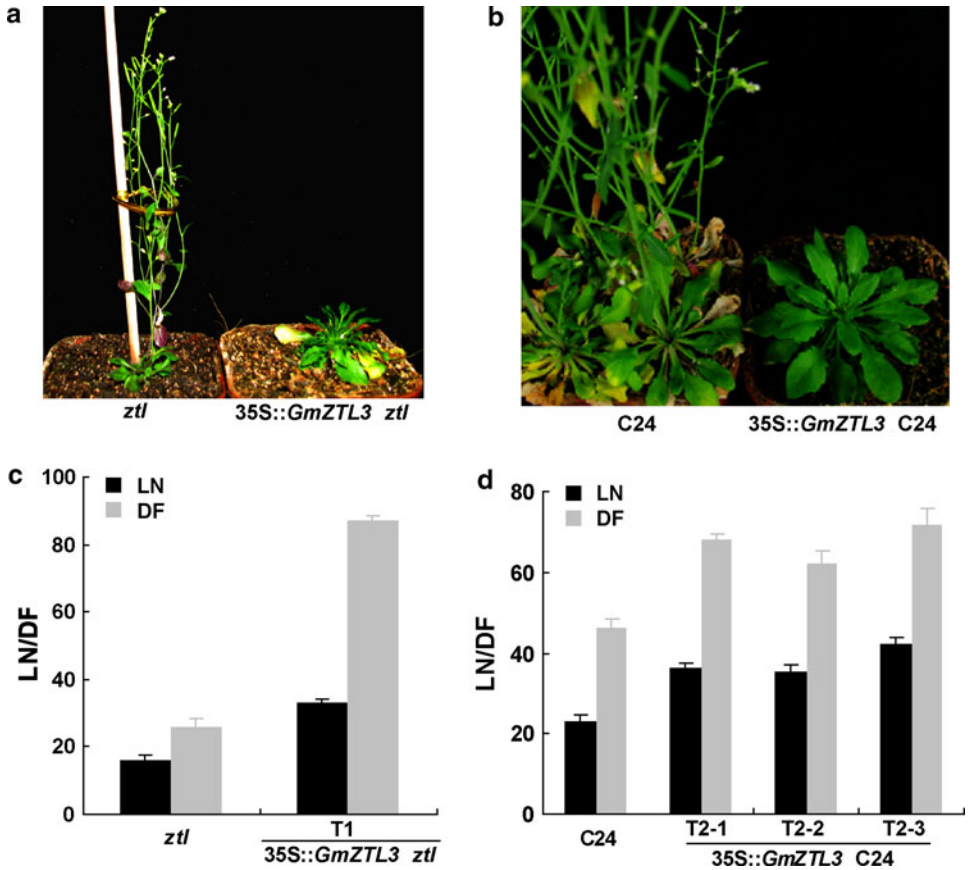

Fig. 7 Effect of ectopic expression of *GmZTL3* on *Arabidopsis* flowering. **a** T1 plants carrying *35S::GmZTL3* in *ztl* mutant, **b** T2 plants carrying *35S::GmZTL3* in C24 wild type, **c** the total number of rosette leaves (LN) and days to flowering (DF) corresponding to **a**, **d** the total number of rosette leaves (LN) and days to flowering (DF) corresponding to **b**. *Error bars* denote the standard deviation, which were from ten *ztl* plants and three independent T1 plants or ten C24 plants and ten T2 plants derived from three independent T1 plants

evolutionally conserved as a flowering inhibitor regardless of the photoperiod response of various plants.

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