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# The *Artemisia annua* FLOWERING LOCUS T Homolog 2, AaFT2, Is a Key Regulator of Flowering Time

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## Abstract

*Artemisia annua* is a typical short-day photoperiod (SD) and belongs to Asteraceae family. FLOWERING LOCUS T Homolog genes play important roles in regulation of flowering time. Two FT-like genes *AaFT1* and *AaFT2* were isolated from cDNA libraries constructed from leaves of *A. annua*. The gene expression analysis revealed that *AaFT1* and *AaFT2* mRNAs were expressed predominantly in leaves and could be induced by GA3. Subcellular localization results indicated that AaFT1 and AaFT2 proteins were both localized to the nuclei and cytoplasm. The Y2H assay results indicated that AaFT1 and AaFT2 could interact with a bZIP transcription factor, AaFD. *AaFT2* was induced by SDs condition and may play an important role in modulation of flowering. In the present study, we performed RNA interference (RNAi) experiments to inhibit the mRNA level of *AaFT2* in *A. annua* for detecting *AaFT2* gene function. Our findings suggest that inhibition of *AaFT2* may delay the flowering time and increase the accumulation of artemisinin (ART) in transgenic *A. annua*. Collectively, these results indicate that AaFT2 is a key regulator of regulation flowering time and can be used for genetic engineering to improve artemisinin yield.



## Key words

*Artemisia annua*; artemisinin; FT

Abbreviation

ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; ART, artemisinin; BFS, β-farnesene synthase; CPS, β-caryophyllene synthase; Cb, carbenicillin; CYP71AV1, amorphadiene 12-hydroxylase; DBR2, artemisinic aldehyde Δ11(13) reductase; DMADP, dimethylallyl diphosphate; DHAA, dihydroartemisinic acid; FDS, fanesyl diphosphate synthase; FT, FLOWERING LOCUS T; GA3, gibberellin; GAS, germacrene A synthase; HMGR, 3-hydroxy-3-methylglutary-CoA reductase; Ka, Kanamycin; NAA, Naphth-alene-I-acetic acid; 6-BA, N6-benzoyladenine; qRT-PCR, quantitative real-time PCR; SD, short-day photoperiod; RNAi, RNA interference; SQS, squalene synthase; Y2H, Yeast two hybrid

## 1.Introduction

Malaria is an infectious disease with more than 429 000 deaths in 2016 (World Malaria Report 2016). Artemisinin is produced by the Chinese traditional medicine plant *Artemisia annua*. The artemisinin combination therapie (ACT) was recommended by WHO to prevent parasite resistance found in some places of Cambodia (Dondorp et al., 2009). In recent years, it has been found that artemisinin has the potential to cure tuberculosis and diabetes (Li et al., 2016; Zheng et al., 2016). In addition, artemisinin may have potential as a therapeutic for the treatment of cancer (Chen et al., 2014). Therefore, there is growing concern about high-quality of artemisinin that needs to be supplied with consistently.

There are many ways to improve artemisinin content, such as overexpression of artemisinin biosynthetic pathway genes, inhibition of artemisinin biosynthesis competition branch pathway genes, transcript regulation of artemisinin biosynthesis genes and modulation of transportation of artemisinin and so on. To improve artemisinin content, three of artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1* and *CPR* were co-overexpressed in *A. annua.* The contents of artemisinin in *A. annua* transgenic plant was increased to 2.4-fold when contrasted with the control plants (Lu et al., 2013). Inhibition of the artemisinin biosynthesis competition branch pathway genes germacrene synthase (*GAS*), β-farnesene synthase (*BFS*), β-caryophyllene synthase (*CPS*) and squalene synthase (*SQS*) may redirect the metabolic flux to artemisinin biosynthetic pathway and increase artemisinin content (Lv et al., 2016a). Transcription factors play important roles in regulation of gene expression in artemisinin biosynthetic pathway. A NAC transcription factor AaNAC1 in *A. annua* has been characterized that can regulate the expression of the *ADS* in vivo and increase artemisinin content (Lv et al., 2016b). Terpene transporters are

important for terpenes transfer. Transporter Pleiotropic Drug Resistance 2 (AaPDR2) from *A. annua* increases accumulation of artemisinin and DHAA in the apoplast of *N. benthamiana* leaves and has the potential for increasing artemisinin content in *A. annua* (Wang et al., 2016).

Besides the above-mentioned methods, modulation of flowering time may be a promising way to regulate biomass or improve quality. Flowering is closely related to the photoperiods. The *A. annua* is typical short-day (SD) plant induced by photoperiods and belongs to the Asteraceae (Compositae) family. Photoperiod response is very important to modulate the FLOWERING LOCUS T (FT) expression level. The FT gene encodes about 170 amino acids (aa) and belongs to phosphatidylethanolamine-binding protein (PEBP) family (Liu et al., 2016). Recently studies indicate that FT is universally conserved and synthesized in the phloem, act as a florigen in higher plants. It can be transported to the apex to play its function. Previously, it was shown that FLOWERING LOCUS T (FT) functioned as a potent factor for modulation of flowering (Abe et al. 2005; TeperBamnolker and Samach 2005). In Arabidopsis, FT interacted with FD, which is a basic leucine zipper (bZIP) transcription factor, to form a transcriptional complex in the shoot apical meristem (SAM) modulation of floral regulator genes such as FRUITFULL (FUL) and APETALA 1 (AP1), leading to flowering promotion (Turck et al. 2008; Huang et al. 2005). However, the expression level of FT is induced by CONSTANS (CO), which is an important protein in response to photoperiod (Valverde et al. 2004). The mRNA level of CO is regulated by a circadian clock. The CO/FT module in the Arabidopsis is considered as placed at the core of the pathway to regulate flowering in response to changes in photoperiods. previous studies indicated that the mRNA abundance of CO was regulated by GIGANTEA (GI) and FLAVIN BINDING, KELCH REPEAT, F BOX protein 1 (FKF1)(Turck et al. 2008; Sawa et al. 2007). The GI and FKF1 can form a complex, which

is crucial for the CO/FT module to mediate the degradation of CYCLING DOF FACTOR 1 (CDF1), a key CO repressor (Sawa et al. 2007).

Flowering is an important regulator for transition of vegetative into reproductive growth. Delaying of plant growth stage or inhibition of plant flowering may increase plant biomass. Previous study indicated that cutting off initiated flower stalks of *Hesperaloe funifera* may divert energy to leaf production and increase fiber yields (McLaughlin 2003). In Brassica species, early flowering may penalty yield or product quality. To avoid losing in yield and reducing of quality, a flowering regulation gene FLC was overexpressed in Chinese cabbage (Kim et al. 2007). Extended vegetative stage by delaying flowering was observed in the transgenic plants (Curtis et al. 2002). In forage grass, the high-quality forage grass is non-flowering and more digestible with higher feeding value. A *TFL1* gene was overexpressed in the red fescue, the transgenic plants showed delayed flowering (Jensen et al. 2004). Switchgrass has been evaluated as a bioenergy crop plants. To improve its biomass, the *Corngrass1* gene (encoded a microRNA) was overexpressed in switchgrass. The results indicated that the transgenic plants flowered approximately 2 to 3 wk later while starch content was increased to 250% and biomass yield was enhanced to 58%–101% when compared with the control (Chuck et al., 2011). Consistent with other evidence, delayed flowering time enhancing biomass production had been observed in the tobacco (Salehi et al., 2005).

Delayed flowering can increase the accumulation of primary metabolism in plant (Salehi et al. 2005). However, delayed flowering time affecting the secondary metabolism is still unknown and needs to be elucidated in medicine plants. Therefore, in this study, we set out to isolate *AaFT1* and *AaFT2* to identify the gene function in *A. annua*. The expression patterns showed AaFT2 has

the potential to modulate flowering in *A. annua*. We show that the artemisinin accumulation is due to the alteration of the flowering time caused by inhibition of *AaFT2* expression level in transgenic *A. annua* plants.

## 2. **Materials and Methods**

### 2.1 Bioinformatics Analysis

The theoretical isoelectric point and molecular weight of protein AaFT1 and AaFT1 were calculated by the expasy (http://www.expasy.ch/tools/pi_tool.html). The neighbor-joining method of MEGA 7 was used to construct the phylogenetic tree. Bootstrap analyses were carried out using 1000 replicates.

The sequences reported in this paper have been deposited in the GenBank database with the following accession numbers: *AaFD*(MF438125), *AaFT1*(MF438126), *AaFT2*(MF438127).

### 2.2 Plant materials and hormonal treatments

The seeds of *A. annua* named 'Huhao 1' were collected from Shanghai in China and grown on the soil in the greenhouse with a light/dark cycle 16/8 h and light of 7500 lux at 26℃ (Shen et al., 2016). Four-week-old wild type plants of *A. annua* were sprayed with 100 μM GA3 (Sigma-Aldrich) solution. Water was used as the mock treatment. Samples were analyzed at 0, 1, 2, 4, 6, 8, 12 and 24 h after the treatment.

### 2.3 Isolation of gene and vector construction

A full-length enriched RNA sequencing (RNA-Seq) library in our lab was constructed from developing *A. annua* seedlings (Lv et al., 2016b). The gene sequence of *AaFT1, AaFT2* and *AaFD* were isolated from the cDNA library in *A. annua*. All the PCR products were cloned into the pMD18T-simple vector and sequenced. The 525 bp fragment of the *AaFT1* and *AaFT2* were

amplified from the *AaFT1*-18T and *AaFT2*-18T and then digested with restriction enzymes HindIII and PstI, fused with T4 DNA ligase to generate new transformation vector AaFT1-YFP and AaFT2-YFP. The PCR products *AaFT1* and *AaFT2* were digested with restriction enzymes Eco*R*I and Bam*H*I, fused with T4 DNA ligase to generate new transformation vectors AaFT1-GBKT7 and AaFT2-GBKT7. All the primers sequences are shown in Supplementary. DNA fragment was cloned into unique Eco*R*I and Bam*H*I site from the AaFD gene coding sequences to generate vector constructs AaFD-GADT7. The first 200 bp fragment of the *AaFT2* was fused into expression vector PHELLSGATE to generate RNAi vector *AaFT2*-RNAi (Helliwell and Waterhouse, 2003).

## 2.4 Yeast two hybrid (Y2H) assay

The yeast strain AH109 was transformed with the constructs (containing bait vector AaFD-GADT7, prey vectors AaFT1-GBKT7 and AaFT2-GBKT7) by the lithium acetate method (Gietz and Schiestl, 2007) and then incubated at 30°C for 4 d. Four single transformations (diluted with sterilized water) were picked out and spotted on the plates SD-Leu-Trp, SD-Leu-Trp-His and SD-Leu-Trp-His-Ade (Clontech), respectively.

## 2.5 Subcellular localization assay

To determine the subcellular localization of AaFT1 and AaFT2, AaFT1-YFP and AaFT2-YFP fusions were introduced into *A. tumefaciens* strain EHA105 and transiently expressed in the five-month-old *N. benthamiana* leaves. After incubation of overnight at 30°C, the culture centrifugated at 6000 rpm for 8 min and diluted to an OD600 nm of 0.6 in MS medium (containing 10 mM MES and 150 mM acetosyringone). Three hours later, the solution was injected into the *N. benthamiana* leaves. The *N. benthamiana* leaves were checked by confocal laser scanning microscopy after 48 hours. The *A. tumefaciens* strain EHA105 harboring vector

35S::PHB-YFP was taken as the negative control.

## 2.6 Transformation of *A. annua*

The constructs of AaFT2-RNAi was transferred into *A. tumefaciens* strain EHA105 by a conventional freezing-and-melting method and the strains were used in transformating *A. annua*. The leaves of 2 weeks old *A. annua* seedlings were infected by *A. tumefaciens* and then cultured in the dark at 28℃ for 3 day. After the dark culture, the explants were transferred into the selective medium (MS0+2.5 mg $L^{-1}$ 6-BA+0.25mg $L^{-1}$ NAA +50 mg $L^{-1}$ Ka+250 mg $L^{-1}$ Cb) for 4 wk (Zhang et al., 2009). The regenerated plantlets were transferred into rooting medium (1/2 MS). The rooted seedlings were grown in the greenhouse for further growth.

## 2.7 Isolation of total RNA and Q-PCR analysis

The method of extraction of total RNA of roots, stems, leaves and buds for Q-PCR is based on previous work for detection of gene expression (Lv et al., 2016b). The samples were collected from five-month-old *A. annua* seedlings at afternoon. The total RNA was extracted from mature leaves and then reversed transcribed into the first cDNA using One-Step PrimeScript™ RT-PCR Kit (Takara, Dalian, China). The Q-PCR primers of *AaFT1* and *AaFT1* were designed by primer premier 5 software. To confirm the relative expression levels, the housekeeping gene *β-actin* was used as the endogenous reference gene. The Q-PCR was performed by SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa Code: DRR420A) with BioRad MJ (Bio-Rad, Hercules, CA, USA). The Q-PCR cycling conditions were 95°C for 30s, 40 cycles of 95°C for 10 sec, 55°C for 30 s, 72°C for 25 s.

## 2.8 Detection of artemisinin

The leaves of *A. annua* were cut off and dried at 50°C in a dry oven for 24h, then grounded into fine powder. The definite methods for extracting and detecting of artemisinin were referenced to

Zhang (Zhang et al., 2009).

## 3. Results

### 3.1 Molecular characterization of *AaFT1* and *AaFT2*

To know the characterization of the *AaFT1 and AaFT2*, the full length cDNA clone was obtained from the *A. annua* RNA sequencing (RNA-Seq) data (Lv et al., 2016b). We used Roche-454 pyrosequenceing sequencing on the glandular trichomes transcriptome of *A. annua* to obtain the expressed sequence tag (EST) sequences for identifying genes. The *AaFT1* has full-length ORF while *AaFT2* has partial sequence in the EST sequences. The *AaFT2* full-length ORF was amplified by 3′ RACE (SMART™ RACE cDNA Amplification Kit, Clontech, 634923, USA) according to the manuscription's instructions. The primers are 3RACE-F1 and 3RACE-F2 (Supplement). Both *AaFT1* and *AaFT2* are 525 bp in length and encode 175-aa residues. The predicted molecular mass and the calculated isoelectric point of *AaFT1* and *AaFT2* are 19.6 kDa, 6.90 and 19.7 kDa, 6.83 respectively.

To elucidate the evolutionary relationship of *AaFT1 and AaFT2* with other genes, a phylogenetic tree was built. The phylogenetic tree (Fig. 1A) demonstrated that AaFT1 is closely related to *CsFTL2* (AB679271) and *AaFT2* is closely related to *CsFTL3* (AB679272) in chrysanthemum. The results indicated that both of AaFT1, AaFT2 and CsFTL2, CsFTL3 are derived from a common ancestor, respectively. Since CsFTL3 is an important positive regulator in modulation of photoperiodic flowering in chrysanthemums, *AaFT2* may play a positive role in promoting flowering in *A. annua*. Previous studies indicated that all PEBP proteins have two regions with highly conserved residues, the amino acid residue Tyr85(Y) and Gln140 (Q) might play critical role in determining positive effects on flowering (Ahn et al., 2006; Hanzawa et al., 2005). All the

FT-like proteins have the residue Tyr85(Y) and Gln140 (Q) (Fig. 1B), the same as the MtFTa1 and CsFTL3, reflecting these FT-like genes might play critical roles in regulation of flowering time.

### 3.2 The expression pattern characteristics of *AaFT1*, *AaFT2 and AaFD*

To examine the different tissue specific expression patterns of the *AaFT1, AaFT2 and AaFD* genes in *A. annua*, Q-PCR analysis was employed. *AaFT1, AaFT2 and AaFD* mRNAs were detected in roots, stems, young leaves, mature leaves and buds. In seedlings, *AaFT1* has 15-fold expression levels in the mature leaves and 3-fold expression levels in the roots and young leaves (Fig. 2A,B). *AaFT2* was mainly expressed in mature leaves, transcript levels are very low in young leaves and roots (Fig. 2C). Therefore, *AaFT1* and *AaFT2* were mainly expressed in the mature leaves and hardly detected in the young leaves (Fig. 2B and D). *AaFD* was mainly expressed in buds and mature leaves (Fig. 2E). The transcript level of *AaFD* little was changes from leaf0 to leaf7 (Fig. 2F).

### 3.3 Expression patterns of *AaFT1* and *AaFT2* in *A. annua* under different light conditions and GA3 treatment

To study the effect of different light on *AaFT1* and *AaFT2* in *A. annua*, the relationship between the gene expression patterns and photoperiod conditions was investigated. The three-month-old wild type plants were treated with different light and the mature leaves were used for RNA extracting. After 10 d of growth under SD conditions, the expression level of *AaFT1* was decreased when contrasted with control (Fig. 3A). With the increase of SD length, the expression level of *AaFT1* was gradually increased. While the expression level of *AaFT2* was gradually increased and peaking at the 10 d of treatment under SD conditions (Fig. 3B). Under the treatment of long-day photoperiod conditions, the mRNA level of *AaFT1* was increased while

*AaFT2* transcripts level was decreased (Fig. 3C and D).

GA is an important plant hormone for regulation of plant flowering (Kumar et al., 2012). It can be used to manipulate flowering and increase crops yield. To elucidate the relationship between FT and GA3, we investigated the expression level of *AaFT1* and *AaFT2* under the treatment of GA3. *AaFT2* was strongly induced by GA3 at 10-fold while *AaFT1* was induced by GA3 only 2-fold (Fig. 3E and F).

### 3.4 Both AaFT1 and AaFT2 subcellular localization of nuclei and the cytoplasm

To confirm the subcellular localization of the AaFT1 and AaFT2 proteins, AaFT1 and AaFT2 were subcloned into YFP-PHB and then introduced into *A. tumefaciens* strain EHA105. The *A. tumefaciens harboring* AaFT1-YFP and AaFT2-YFP constructs were infiltrated into the leaves of *N. benthamiana*. As shown in Fig.4, confocal microscopic examination showed that both AaFT1-YFP and AaFT2-YFP fusion proteins were located on the nuclear and the plasma membrane.

### 3.5 Interaction of proteins of the AaFT1/AaFT2-AaFD by yeast two-hybrid assays

The FT-FD protein complex plays an important role in the procession of floral induction (Abe et al., 2005). Previous studies have shown that FT-FD protein complex positively regulates flowering while TFL1–FD protein complex negatively modulates flowering in Arabidopsis (Abe et al., 2005; Higuchi et al., 2013). To gain clues to the molecular basis of AaFT1 and AaFT2 during floral induction, the interactions between AaFT1/AaFT2 and AaFD were detected by Y2H. The yeast strain AH109 co-transformed with AaFT1-GBKT7/AaFT2-GBKT7 and AaFD-GADT7 grew on SD-Leu-Trp-His-Ade selective medium confirming that both AaFT1 and AaFT2 can interact with AaFD (Fig. 5).

### 3.6 The phenotype of the transgenic plants

According to the results of phylogenetic tree assays and SD (floral-inductive) induction, AaFT2 may play a crucial role in regulation of flowering. Therefore, the mRNA level of AaFT2 was inhibited by RNAi technique. According to the expression levels of AaFT2, we selected six transgenic lines for further study.

To demonstrate whether the phenotype of the transgenic plants changed or not, the height, flower time and trichome number were observed. Compared with the control plants, all the transgenic plants were not changed significantly in the trichome number (data not show). *AaFT2* may not have direct roles in growth and trichome development in *A. annua*. However, the transgenic plants shown late flowering (Fig. 6) with different level (Fig. 7). It is indicated that *AaFT2* is involved in flowering regulation.

### 3.7 Inhibition of *AaFT2* improves artemisinin accumulation in *A. annua*

To detect the gene expression levels of the *AaFT2* in the transgenic plants, Q-PCR assay was employed in this study. The transcription level of *AaFT2* was suppressed by 90% in most of the transgenic plants (Fig. 8). Four-month-old plants were used for detecting the content of artemisinin. When down-regulated the *AaFT2* expression by RNAi, the content of the artemisinin was increased significantly as compared with the untransformed control, with an increase by 64% in some transgenic lines (Fig.9).

## 4. Discussion

Flowering time is regulated by many kinds of signaling pathways, including photoperiod, temperature, plant age, gibberellins, shading, and other factors (Song et al., 2013). These signaling pathways are integrated to modulate the transcript level of a major floral regulator FT, which is able to bind to FD and promote flowering (Wigge, 2011). Changes plant flowering time is

very important to seed production (Liu et al., 2017). At the same time, increase of the vegetative growth time may important to the biomass production (Murphy et al., 2014). Previous studies suggest that FT plays important roles in regulation of flowering time (Oda et al., 2012). In this study, we investigated the biology function of *AaFT2* in *A. annua* by knocking down the mRNA levels of *AaFT2*.

### *4.1* Identification of FT genes in *A. annua*

The mRNA of *LsFT*, a paralog of FT, was isolated and identified to play important roles in the induction of flowering in lettuce (Fukuda et al., 2011). A recent study indicates that sunflower carries at least four additional FT paralogs (Blackman et al., 2010). In *A. annua*, two FT genes encoded 175-aa residues with highly conserved amino acid residue Tyr85(Y) and Gln140 (Q) were cloned from the mature leaves (Fig.1B). Phylogenetic tree analysis indicated that both *AaFT1* and *AaFT2* were clustered into the composite family (Fig.1A). The gene expression level of *AaFT2* was induced by SDs, suggesting that *AaFT2* might play a key role in modulation of flowering.

### *4.2 AaFT2* induces flowering in *A. annua*

FT is the important gene of the photoperiod pathway to promote flowering in plants (Abe et al., 2005). Inhibition of the transcription level of *AaFT2*, the flowering time was delayed by 5-29 days in transgenic plants (Fig.7). The results are consistent with previous evidences that repression of FT may play a decisive role in modulation of flowering time (Lazakis et al., 2011). However, *AaFT2* could not rescue Arabidopsis *ft-1* mutant (data did not shown). It is indicated that the functions of FT paralogs are diversifications in plants (Oda et al., 2012).

The mRNA level of *AaFT2* in transgenic plants was found to be inversely proportional to the artemisinin content (Fig.9). Results indicate that the expression level of *AaFT2* was connected

with the artemisinin content. However, the expression levels observed for *AaFT2* were not always correlated with delayed flowering time according to the RNAi-21 and RNAi-38 transgenic lines (Fig.8, 9). *CsFTL3* is a positive inducer of flowering in chrysanthemun. Previous reports have suggested that high *CsFTL3* transcript levels did not perform the early flowering phenotype, indicated that the mRNA expression levels may depend on post-transcriptional control (Oda et al. 2012).

However, gene expression levels were not always correlated with artemisinin content according to the RNAi-38 transgenic line (Fig.7, 8). There may be caused by some unknown reasons. The relative expression of *AaFT2* in RNAi-13 transgenic lines was the lowest while no significant change in the growth period and the content of artemisinin. The reason may be that post-transcriptional regulation modulates the protein production of AaFT2, leading to RNAi-13 transgenic lines did not change in the growth period and artemisinin content.

### *4.3 AaFT2* interacted with FD to regulate flowering time

FT is a major component of florigen signal transduction pathway for accelerating flowering and it mRNA levels can be modulated by various factors. Many transcriptional repressors, such as TEMPRANILLO 1(TEM1), FLOWERING LOCUS C (FLC) and PHYTOCHROME INTERACTING FACTOR4 (PIF4), all can regulate FT by binding to its promoter specially (Kumar et al., 2012). The phytohormone gibberellin 3 (GA3) plays important role in regulation of flowering. GA3 application enhances *AaFT2* expression level about 10-fold (Fig. 3F), these results are consistent with previous work in Arabidopsis (Hisamatsu and King, 2008). The reason for GA3 induces flowering is probably that GA3 indirectly regulation of PIF4 protein activity causes PIF4 to activate FT (Kumar et al., 2012).

FT interaction with FD is involved in the regulation of floral by transcriptional activation of *APETALA1*, which determines the flower organ (sepal and petal) development in Arabidopsis (Abe et al., 2005). Consistent with previous work, FT interacts with the transcription factor FD in *A. annua* (Fig. 5). But we did not find a significant difference about flower organ between transgenic group and control in *A. annua* (Fig. 6).

### 4.4 Delay in flowering time and increase in accumulation of artemisinin

Previously, light plays important role in improving artemisinin yield (Wang et al., 2001). Increase the time of illumination may affect sesquiterpene and triterpene biosynthesis (Korth et al., 2000). To strengthen illumination, an *Arabidopsis* blue light receptor gene *CRY1* was overexpressed in *A. annua* and artemisinin accumulation was enhanced (Hong et al., 2009). Therefore, delay in flowering is an effective way to prolong vegetative growth and increases artemisinin accumulation.

Consistent with previous work, our study has shown that transgenic plants are late flowering for 5-29 days when compared with the non-transformed plants (Fig. 7). There is a positive correlation between late-flowering and artemisinin content (Fig.S1). However, it is indicated that there may be no direct linkage between flowering and artemisinin biosynthesis in *A. annua* (Wang et al., 2007). The reason may be that late flowering improves the availability of total solar radiation. Firstly, delayed flowering (greater solar radiation is available) may promote vegetative growth and accumulate much substrate (such as pyruvic acid, acetyl-CoA and glyceraldehyde 3-phosphate) for producing artemisinin. Secondly, delayed flowering time may increase the amount of $^{1}O2$ (produced by UV radiation), which is the activator for modulation of artemisinin biosynthesis (Zeng et al., 2011).

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**Figure legends**

**Fig.1.** Cloning of FT-like genes from *A. annua*. (A) Phylogenetic tree based on the nucleic acid

sequences of the PEBP gene family. The tree was constructed by the Neighbor-Joining method. (B) Partial amino acid alignment of PEBP proteins. Asterisks showing Tyr85(Y) and Gln140(Q) residues are conserved in PEBP family.

**Fig.2.** Expression patterns of *AaFT1*, *AaFT2* and *AaFD* in different tissues of *A. annua*. Expression patterns of *AaFT1* (A), *AaFT2* (C) and *AaFD* (E) in B (Buds), YL (young leaves), ML (mature leaves), S (stems), R (roots) , *AaFT1* (B), *AaFT2* (D) and *AaFD* (F) in L0 (leaf0), L1(leaf1), L2 (leaf2), L3 (leaf3), L4 (leaf4), L5 (leaf5), L6 (leaf6) and L7 (leaf7). QRT-PCR was employed to determine the expression level. Error bars are SE (n= 3).

**Fig.3.** Expression patterns of *AaFT1* and *AaFT2* were induced by SD, LD conditions or GA3 treatments. *AaFT1* (A) and *AaFT2* (B) under SD treatment, *AaFT1* (C) and *AaFT2* (D) under LD treatment, *AaFT1* (E) and *AaFT2* (F) under GA3 treatment. Error bars are SE (n= 3).

**Fig.4.** Subcellular localization of AaFT1 and AaFT2 was tested in tobacco epidermal cells. The constructed vectors 35S::*AaFT1*-YFP and 35S::*AaFT2*-YFP were induced into tobacco (*Nicotiana benthamiana*) epidermal cells by infiltration.

**Fig.5.** AaFT1 and AaFT2 Physically Interact with AaFD. Yeast two-hybrid assays examining the interactions between AaFT1, AaFT2 and AaFD proteins. Yeast expressinged empty vector GADT7 and GBKT7 were used as negative controls.

**Fig.6.** The phenotypes of transgenic *A. annua*. (A) The amplificatory picture of flower buds of the WT *A. annua.* (B) Delayed flowering of transgenic *A. annua*.

**Fig.7.** Delayed flowering time in transgenic plants.

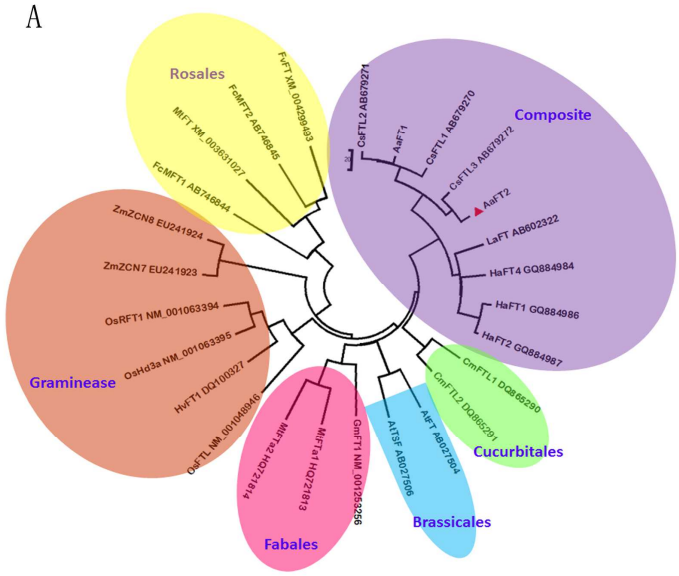
**Fig.8.** The expression levels of *AaFT2* in the transgenic plants were determined by qRT-PCR analysis. Error bars are SE (n= 3).

**Fig.9.** The content of artemisinin (ART) was determined in transgenic plants. Each sample was measured by HPLC for the ART. Data represent means ± SE from three replicates. Statistical significance is determined by Student's t-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between CK and transgenic *A. annua* plants.

**Fig.S1.** The relationship between the delayed time and the artemisinin content. The Y-axis is delayed time and the x-axis is artemisinin content.

**Supplement.** The primers used in the study.

A

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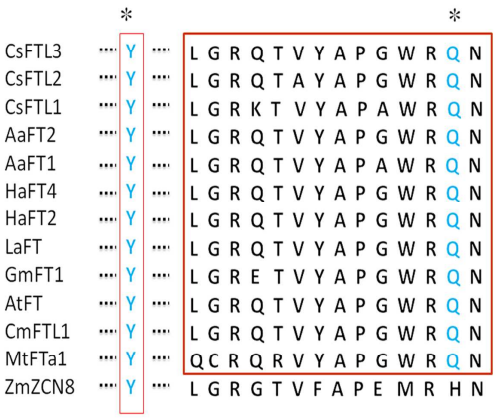

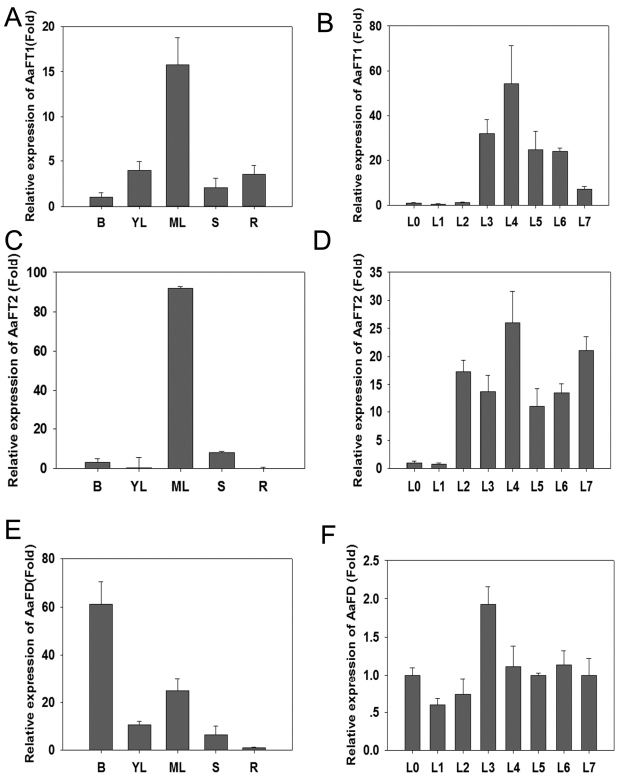

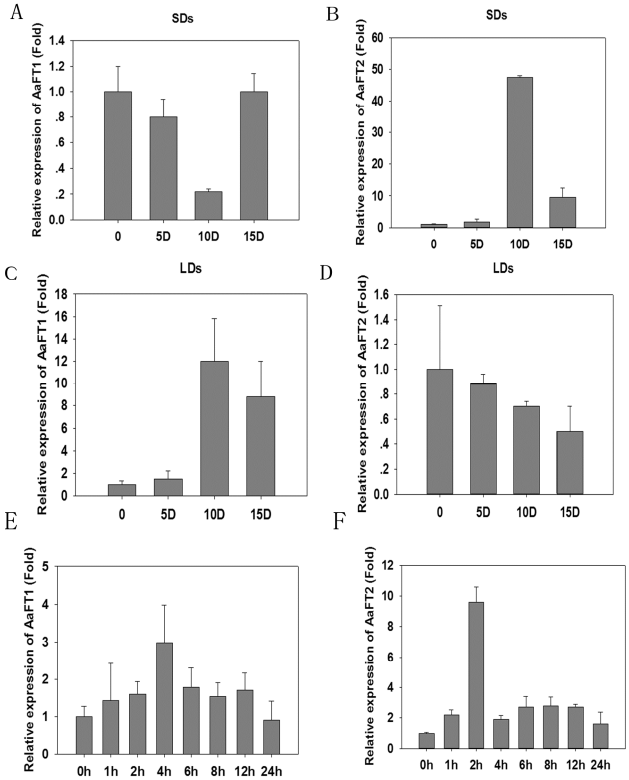

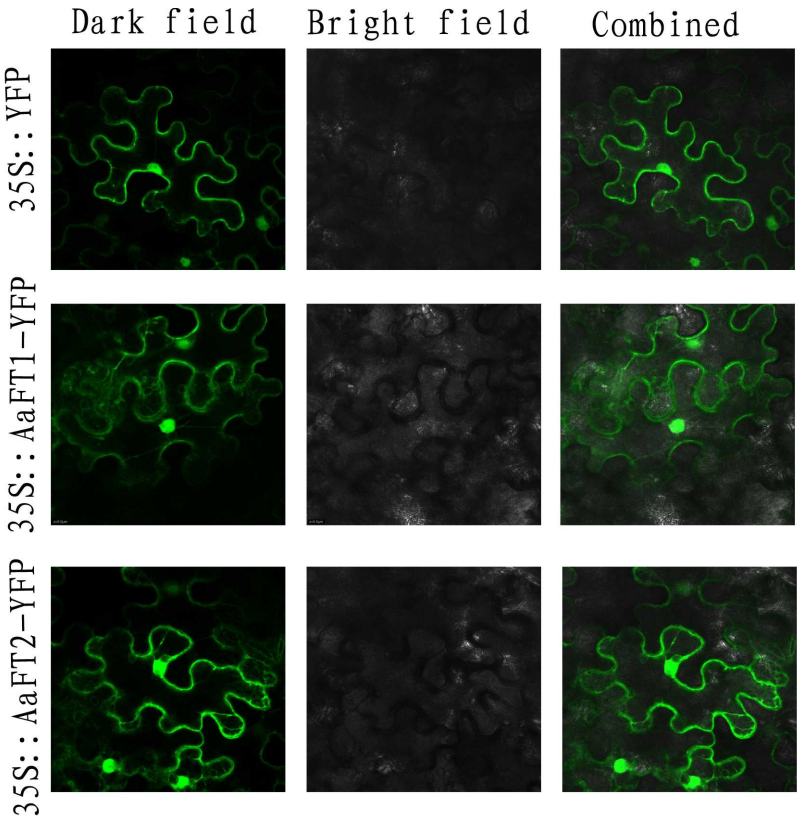

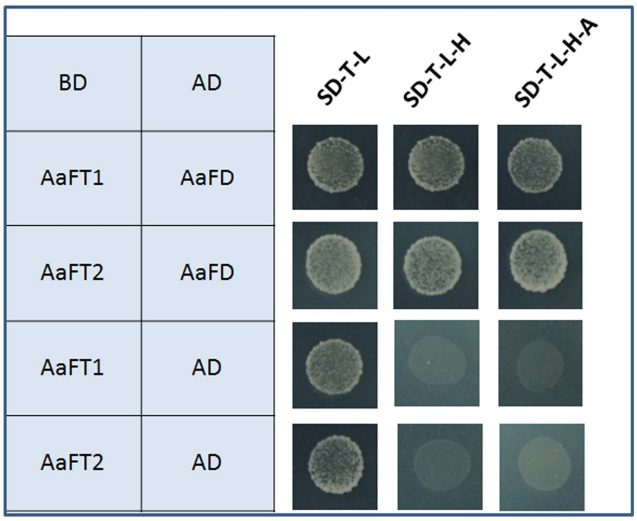

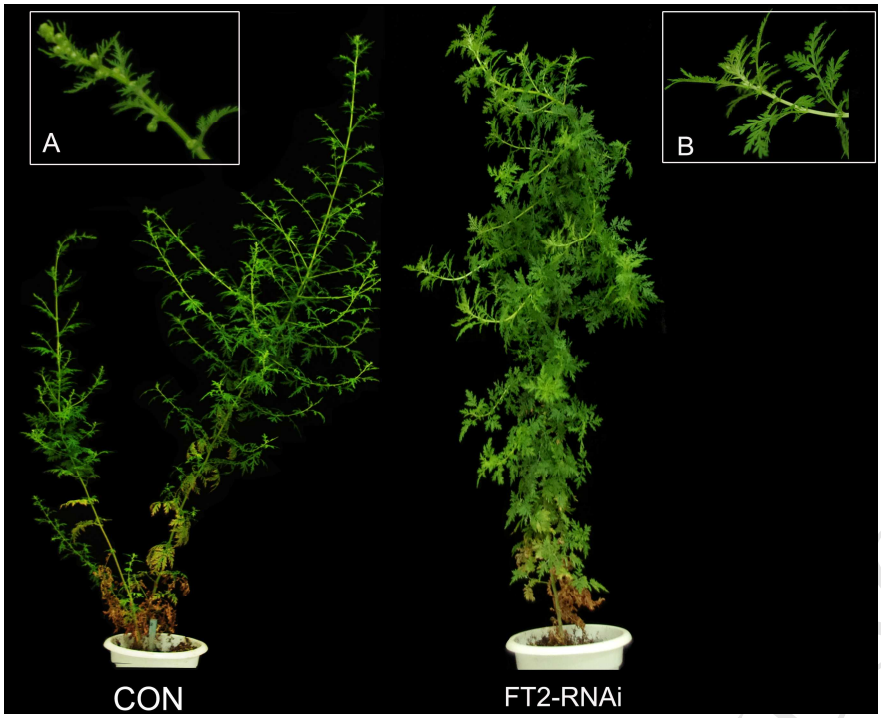

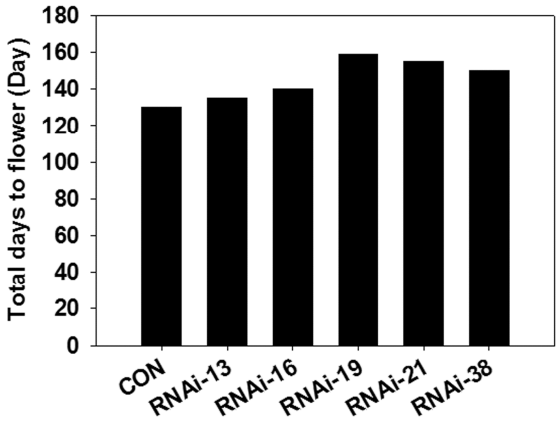

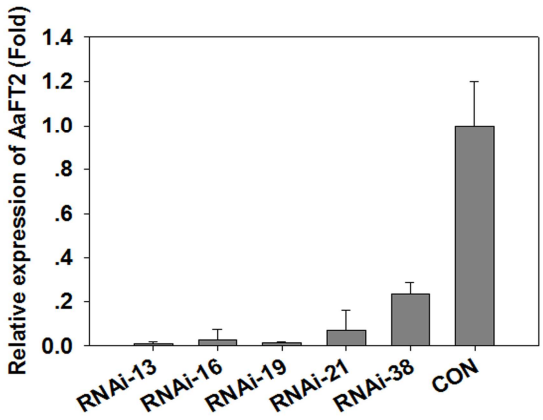

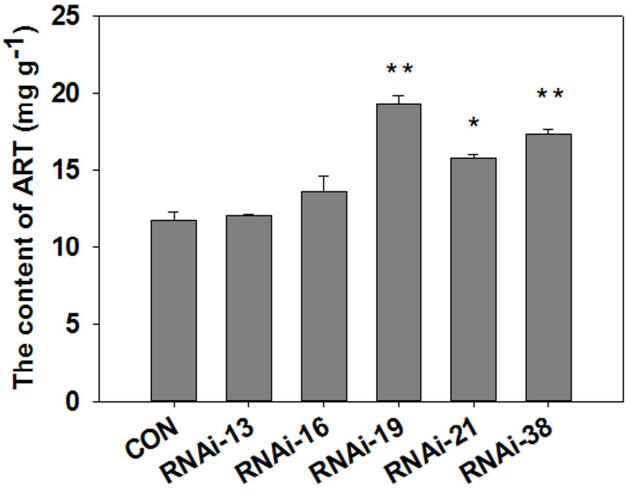

Highlights

1 *AaFT1was* inhibited by short days (SDs) (floral-inductive) while *AaFT2* was induced under SDs

2 Inhibition of *AaFT2* delayed flowering time

3 Inhibition of *AaFT2* accumulated artemisinin (ART)

ZL conducted transgenic plant seedlings analysis in *Artemisia annua*. L, LZ assayed gene expression and Y2H in *Artemisia annua*. L, LC, FZ and KT were responsible for project design, and all authors contributed to writing the manuscript.