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Studies on the Effects of *fpf1* Gene on *Artemisia annua* Flowering Time and on the Linkage between Flowering and Artemisinin Biosynthesis

Abstract

The flowering promoting factor1 (*fpf1*) from *Arabidopsis thaliana* was transferred into *Artemisia annua* L. via *Agrobacterium tumefaciens*. The *fpf1* gene was firstly inserted in the binary vector pBI121 under the control of CaMV 35S promoter to construct the plant expression vector pBIfpf1, then leaf explants of *A. annua* were infected with *A. tumefaciens* LBA4404 containing pBIfpf1, and induced shoots. Transgenic plants were obtained through the selection with kanamycin. PCR, PCR-Southern and Southern blot analyses confirmed that the foreign *fpf1* gene had been integrated into the *A. annua* genome. RT-PCR and RT-PCR-Southern analyses suggested that the foreign *fpf1* gene had expressed at the transcriptional level. Under short-day conditions, the flowering time of *fpf1* transgenic plants was about 20 days earlier than the non-transformed plants; however, no significant differences were detected in artemisinin content between the flowering transgenic plants and the non-flowering non-transgenic plants. These results showed that flowering is not a necessary factor for increasing the artemisinin content, furthermore, there may be no direct linkage between flowering and artemisinin biosynthesis.

Key words
Artemisia annua L. · Asteraceae · Artemisinin · Flower · flowering promoting factor1 (*fpf1*)

Introduction

Artemisia annua L. (Asteraceae), an annual herbaceous plant, has been used in Chinese medicine for more than 2000 years in the treatment of fever. Analysis of the active compounds responsible for these pharmacological activities led to the discovery of artemisinin, a new compound with antimalarial activity [1]. Artemisinin possesses a different mode of action in comparison with existing antimalarial drugs and is active against chloroquine-resistant forms of *Plasmodium falsiparum* [2]. So far, the plant *A. annua* is the only commercially feasible source of artemisinin for drug formulations, and the yield of artemisinin from the plant is generally low (between 0.01 and 0.6% dry weight) [3]. This makes artemisinin a very expensive drug. Total synthesis of artemisinin and its derivatives on a commercial scale has yet to be achieved [4]. Therefore, all efforts in the field have been focused on how to increase the production of artemisinin [5].

Artemisinin mainly accumulated in leaves and flowering tops of the plant, and its content varied with the plant developmental stages [6], [7], [8]. In the literature, there are mainly two different views on the occurrence stage of the highest content of artemisinin in plant development. The one is that the highest content of artemisinin is reached just before plant flowering [7], [9], [10], the other is that it occurred at the full flowering period [8], [11]. Both views suggested a possible linkage between flowering and artemisinin biosynthesis. In this study, we investigate whether such a linkage does indeed exist by altering the plant flowering time through transgenic means. More specifically, in this study, the flowering promoting factor1 (*fpf1*) from *Arabidopsis thaliana*

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Received August 5, 2003 · **Accepted** January 25, 2004

Bibliography
Planta Med 2004; 70: 347–352 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943 · DOI 10.1055/s-2004-818947

[12] was transferred into *Artemisia annua* L., and the effects of *fpf1* on *Artemisia annua* flowering time as well as on the possible linkage between flowering and artemisinin biosynthesis were investigated.

Materials and Methods

Plant material and culture conditions

The seeds of the high artemisinin yielding strain 001 were kindly provided by the Beijing Holley Cotec Pharmaceuticals Co. Ltd. The seeds were surface sterilized with 0.1% mercuric chloride for 10 min. After that, the seeds were washed thoroughly with sterile distilled water, and then germinated on MS medium [13] supplemented with 3.0% sucrose and solidified with 0.7% agar. The pH was adjusted to 5.8 before the addition of agar. The medium was autoclaved at 1 bar for 20 min. The cultures were incubated at 26 °C and exposed to photoperiods of 16 h. A voucher specimen (A-annua-001) of the plants is deposited at The In Vitro Plant Germplasm Collection (IVPGC), Institute of Botany, Chinese Academy of Sciences, Beijing, China.

Construction of plasmids

The plasmid pBSKfpf1 containing *fpf1* gene, kindly provided by Dr. S. Melzer (Swiss Federal Institute of Technology, Institute for Plant Science, Switzerland), was digested with *Kpn*I and blunted with T4 DNA polymerase, then digested with *Xba*I and the 700 bp fragment was collected. This 700 bp fragment was then inserted into the *Sma*I and *Xba*I sites of plasmid pBI221 to form intermediate vector pBI221-fpf1. The intermediate vector pBI221-fpf1 was digested with *Sac*I and *Xba*I and the 2.5 kb fragment was collected, then inserted into the *Sac*I-*Xba*I sites of the binary vector pBI121 to generate the recombinant binary vector pBIfpf1 (Fig. **1**). The recombinant binary vector pBIfpf1 contained the *fpf1-gus* fusion gene, driven by cauliflower mosaic virus (CaMV) 35S promoter, and the *npt*II gene of kanamycin resistance driven by the NOS promoter. Kanamycin was used for the selection of the transformants due to the good sensitivity of *A. annua* to it.

Introduction of pBIfpf1 to *Agrobacterium tumefaciens*

The recombinant plasmid pBIfpf1 was first amplified in DH5α and then introduced to the disarmed *A. tumefaciens* strain LBA4404 by the freeze-thaw method. Transformants were selected on YEB medium (yeast extract 1.0 g, beef extract 5.0 g, pepton 5.0 g, $MgSO_4$ 7 H_2O 0.49 g, pH 7.0) supplemented with 50 mg/L kanamycin, 100 mg/L rifampicin and 50 mg/L streptomycin. *A. tumefaciens* harboring the recombinant vector pBIfpf1 was used for *A. annua* transformation.

Transformation of *A. annua*

A. annua transformation was performed essentially according to the method described by Chen et al. [14]. For the transformation experiments, young leaves from 3-week-old seedlings were used as explants. For inoculation, *A. tumefaciens* was grown overnight at 28 °C, under shaking, in liquid YEB medium supplemented with 100 mg/L rifampicin, 50 mg/L streptomycin and 50 mg/L kanamycin. The overnight culture was pelleted by centrifugation and resuspended in YEB liquid medium (without antibiotics) till OD_{600} reached to 0.6 – 0.8. After being diluted 10-fold with MS liquid medium, the bacterium suspension was used for *A. annua* transformation. The leaves were gently shaken in the diluted bacterial suspension for 20 min, dry-blotted on sterile filter paper and then cultured on solid MS medium in the dark at 28 °C. After 3 days co-cultivation, the leaves were transferred to shoot inducing medium (MS basal medium supplemented with 2.5 mg/L 6-BA, 0.05 mg/L NAA, 500 mg/L carbenicillinum and 15 mg/L kanamycin) to induce shoots. Carbenicillinum was used to eliminate surplus *Agrobacterium*, kanamycin was used to select the transformants, and the media without kanamycin were considered as controls. The explants were transferred to fresh shoot inducing medium weekly during the first month. Afterward, subculture was made every 2 weeks. Carbenicillinum was reduced to 100 mg/L after 8 weeks, and completely stopped 12 weeks later. When the induced shoots had grown to about 2 cm in height, the shoots were separated from the explants and transferred to root-inducing medium (MS basal medium supplemented with 0.05 mg/L NAA) to induce roots. A voucher specimen (A-annua-fpf1) of the plants is deposited at The In Vitro Plant Germplasm Collection (IVPGC), Institute of Botany, Chinese Academy of Sciences, Beijing, China.

PCR analysis

Putatively transformed plants were initially analyzed by PCR assays. In the plant expression vector pBI121, the *fpf1* gene and the *gus* gene share the CaMV 35S promoter, so we detected the expression of the *fpf1* gene as well as the *fpf1-gus* fusion gene by PCR. The PCR to amplify *fpf1* gene was carried out in 50 μL volumes, each containing 0.2 mM of each dNTP, 0.2 μM of each oligonucleotide primer, 2.5 U *Taq* polymerase and 50 ng of genomic DNA. The reaction consists of 30 cycles: a 40 s denaturation at 94 °C, a 40 s annealing at 68 °C, and a 1.5 min elongation at 72 °C. The last cycle was with a final 10 min extension step at 72 C. The primers for amplifying the *fpf1* gene were designed according to the sequences at the 5′- and 3′-terminal of the *fpf1* gene of *Arabidopsis thaliana*. These sequences were: forward 5′-GCA CGA GTC ATG TCA GGC GTG T-3′ and reward 5′-AAT GGG AGT CTC GGA CAT GGA A-3′, and a 300 bp band was amplified. In order to amplify the *fpf1-gus* fusion gene, the *fpf1* forward primer and the *gus* reward primer were used. The used *gus* reward primer was: 5′-CAA AGC CAG TAA AGT AGA ACG GT-3′, the forward primer was the same as that for the *fpf1* gene. The PCR system to amplify the *fpf1-gus* fusion gene was the same as that for the *fpf1* gene. The *fpf1-gus* fragment between the two primers was 1.5 kb. The reaction also consists of 30 cycles: a 1 min denaturation at

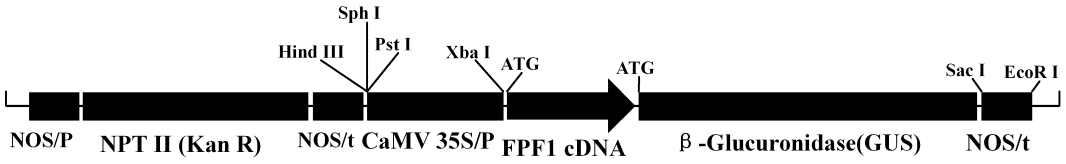

Fig. **1** Structure and diagram of recombinant plasmid pBIfpf1

94 °C, a 40 s annealing at 65 °C, and a 2 min elongation at 72 °C. The last cycle was with a final 10 min extension step at 72 °C. In the above two PCRs, DNA from plasmid pBIfpf1 and that from the non-transformed plants were used as the positive control and the negative control respectively.

The amplified *fpf1* and *fpf1-gus* fragments were electrophoresed on 0.8% agarose gel and photographed using the BioCaptMW software in a VILBER LOURMAT gel imaging system. After that, the fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim), and PCR-Southern analyses were carried out by using [α-^{32}P]-radioactively labeled *fpf1* and *fpf1-gus* fragments as probes respectively.

Southern blot analysis

For DNA hybridization analysis, total genomic DNA from putatively transformed plants and that from non-transformed plants were both digested with *Eco*RI and *Xba*I; DNA from plasmid pBIfpf1 was also digested with the same enzymes as a positive control. The digested DNA was separated by 0.7% agarose gel electrophoresis, and then blotted onto a positively charged nylon membrane (Boehringer Mannheim). Southern hybridization was performed using [α-^{32}P]-radioactively labeled *fpf1-gus* fragment as a probe. The membrane was washed at high-stringency washing conditions in a buffer consisting of 0.1 X SSC, 0.5% SDS at 65 °C. The hybridization and washing membrane were performed according to Sambrook et al. [15].

RT-PCR and RT-PCR-Southern analyses

Total RNA from the leaves of transgenic plants and that from the control were isolated by a one-step method using Trizol extract buffer (GIBCOBRL). First-stand cDNA was synthesized by using a TaKaRa RNA PCR Kit (TaKaRa), the protocol for reverse transcription was: 42 °C for 30 min, 99 °C for 5 min and 5 °C for 5 min. The resultant first-stand cDNA was used as the template to amplify the *fpf1* gene as well as the *fpf1-gus* fusion gene, the primers used here were the same as those in the PCR analyses.

The conserved tubulin gene was used as the internal control for gel loading. The primers used for tubulin gene amplification were: forward 5′-AAC AAC TGG GCC AAG GG-3′, backward 5′-TCC TGG TAC TGC TGG TAC TC-3′. The fragment between the two primes was 1.0 kb. The PCR amplification was performed by adding the two prime pairs (the *fpf1* gene primer pair and the tubulin gene primer pair) in the same PCR tube. The PCR consisted of 30 cycles: 1 min at 94 °C, 40 s at 65 °C, and 1 min at 72 °C. The last cycle was with a final 10 min extension step at 72 °C.

The RT-PCR products were separated on 0.8% agarose gel and photographed. The RT-PCR-Southern analyses of *fpf1* and *fpf1-gus* were performed using [α-^{32}P]-radioactively labeled *fpf1* and *fpf1-gus* as probes respectively. The RT-PCR-Southern analysis of tubulin was carried out by using [α-^{32}P]-radioactively labeled tubulin gene as a probe.

Induction of flowering

The rooted germ-free transgenic plants and control were transplanted to a greenhouse at 20 °C. During the first 3 months, all the plants were under a 16-h photoperiod (fluorescent lamps, ca. 3000 lux, were used to counter the shortage of natural lighting). After 3 months growing, some transgenic plants and non-transgenic plants were treated at an 8-h photoperiod to induce flowering, and those still grown at the 16-h photoperiod were used as control for flowering.

Determination of artemisinin

The detection of artemisinin was performed according to the method of Zhao et al. [16]. The leaves of transformed and non-transformed plants were collected and dried to constant weight in an oven at 50 °C. Then the dried leaves were ground to powder. Some 100 mg of the exactly weighed powder were added to an extraction bottle containing 20 mL petroleum ether (boiling range 30 – 60 °C) and extracted overnight. After treatment in a supersonic bath for 5 min, the extraction mixture was filtered under vacuum. The filtrates were collected and evaporated to dryness. The residue was dissolved in 1 mL methanol and centrifuged at 12,000 rpm for 10 min to precipitate the undissolved components. The supernatant was subjected to artemisinin extraction and used for HPLC detection.

200 μL of the above prepared artemisinin extraction was placed in a 10-mL tube, added 800 μL methanol and 4 mL 0.2% sodium hydroxide, mixed and maintained in a 50 °C water bath for 30 min. After being cooled to room temperature, 0.5 mL of the reaction mixture was placed in a 1.5-ml Eppendorf tube and 100 μL methanol and 400 μL 0.05 mol/L acetic acid were added, mixed, then purified by an NC filter (40 μm). The artemisinin standard (Sigma, MO) solution with concentrations of 4, 8, 12, 16, 20, 24 μg/mL were prepared in the same way as the sample.

The C_{18} reverse column used was 4.6 × 250 mm, 5 Micron. The mobile phase was 0.01 mol/L sodium phosphate buffer: methanol (55 : 45), pH 7.0 with flow rate of 1 mL/min. The injection volume was 10 μL. The retention time was 6 – 8 min and artemisinin standard appeared at about 4 min under the above mentioned conditions (Fig. **2**).

Results and Discussion

Genetic transformation and regeneration

In 1996, Vergauwe et al. reported the first *A. tumefaciens*-mediated transformation of *A. annua* and the recovery of transgenic plants [17]. However, *A. annua* is a rather recalcitrant plant, the transformation frequency was significantly affected by such factors like explants type, age of the plant for explants, *A. tumefaciens* strain and type of binary vector [18]. We had also established *A. tumefaciens*-mediated transformation of *A. annua* in our laboratory [14], [19]. In this paper, we have further optimized some influencing factors for the transformation frequency.

For the *A. annua* 001 strain, the *Agrobacterium*-infected leaves were cultured on the shoot-inducing medium to induce shoots. Kanamycin was used to select the transformants due to the good sensitivity of *A. annua* to it. Both the transformed leaves and the control were cultured on the shoot-inducing medium containing 15 mg/L kanamycin to induce shoots, the leaves of the control did not generate shoots in the wounds, the leaves gradually filemotted and finally died. However, for the transformed

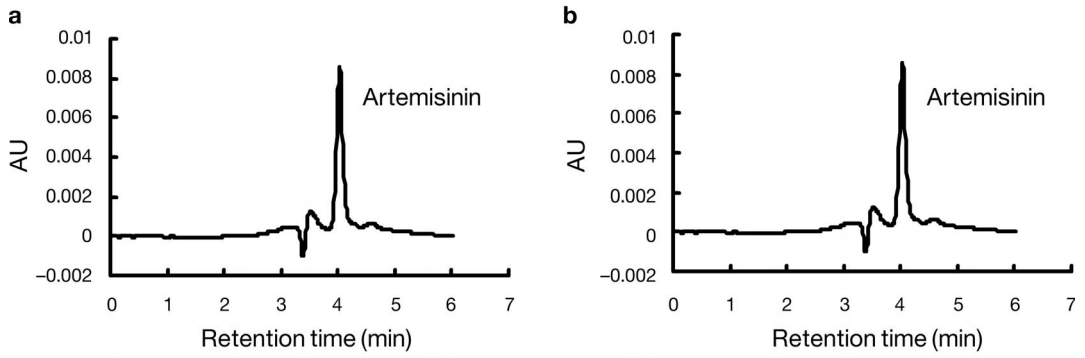

Fig. **2** HPLC detection of artemisinin. **a** Artemisinin standard. **b** Extract from transgenic plants.

leaves, first, the wounds of the leaves generated some "green shooting-point", later, the shooting-point propagated, and generated a shoot cluster. Most of the shoots were directly generated from the wounds of the leaves, however there were some leaves where calli were first formed in the wounds. When the calli were separated from the explants and transferred on the modified hormone component medium to induce shoots, a small portion of these calli generated shoots. Hence it seems the best way to induce shoots is from explants directly rather than from induced calli. This is in agreement with the results by Vergauwe et al [17]. The well-grown kanamycin resistant shoots were separated from the explants and transferred to root-inducing medium to induce root, about 2 weeks later the shoots rooted in the root-inducing medium.

PCR analysis

PCR was performed on those lines that were putatively transformed with the *fpf1* gene, as evidenced by their resistance to kanamycin. The PCR using *fpf1* gene primers showed a 300 bp band, the size of the band was the same as that of pBIfpf1 as positive control, and no DNA band was detected in the non-transformed control (Fig. **3**). PCR-Southern analysis confirmed that the 300 bp band was indeed the product amplified by *fpf1* gene (data not shown). The PCR using *fpf1* forward and *gus* reward primers showed a 1.5 kb band, the size of the band was the same as that of pBIfpf1 as positive control (data not shown), and the results of PCR-Southern analysis confirmed that the 1.5 kb band was the product amplified by *fpf1-gus* fusion gene (data not shown).

Southern blot analysis

Genomic DNA digested with *Eco*RI and *Xba*I and hybridized with the *fpf1-gus* probe produced bands representing the DNA fragment containing the *fpf1-gus* fusion gene. No hybridization bands were detected in the untransformed plants (Fig. **4**). The results of Southern blot indicated that the foreign *fpf1* gene had been integrated into the *A. annua* genome.

RT-PCR and RT-PCR-Southern analyses

The expression of the *fpf1* gene and the *gus* gene at the transcriptional level was confirmed by the appearance of *fpf1* gene and *fpf1-gus* fusion gene separately by RT-PCR. The RT-PCR analysis using *fpf1* gene primers showed a 300 bp band in the transgenic plants, but no bands were detected in the non-transformed control (Fig. **5**). The results of RT-PCR-Southern analysis using *fpf1* as a probe confirmed that the 300 bp band was indeed the product amplified by *fpf1* gene (data not shown). The RT-PCR using *fpf1* forward and *gus* reward primers showed a 1.5 kb band (data not shown), and the results of RT-PCR southern confirmed that the 1.5 kb band was the product amplified by *fpf1-gus* fusion gene (data not shown). These results indicated that the *fpf1* gene as well as the *gus* gene had expressed at the transcriptional level.

By the RT-PCR using *fpf1* gene primer pair and tubulin gene primer pair, the transgenic plants showed two bands: one 300 bp band of *fpf1* gene, and one 1.0 kb band of tubulin gene. But in the control, only 1.0 kb tubulin gene band can be seen (Fig. **6**). The results of RT-PCR-Southern analysis indicated that the 1.0 kb band was for the conserved tubulin gene, which served as an endogenous control (data not shown).

The effects of *fpf1* gene on *A. annua* flowering time

In short day conditions, the flowering time of the *fpf1* transgenic plants was about 20 days earlier than the non-transformed control. One example is shown in Fig. **7**. The results showed that *fpf1* gene can promote flowering in *A. annua*, which was in consistent with the results of *A. thaliana*[12], [20].

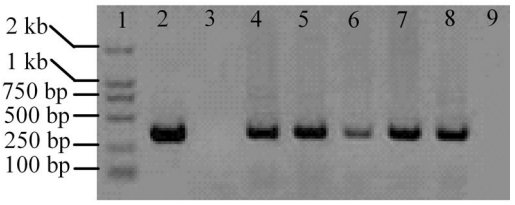

Fig. **3** Identification of transgenic plants by PCR for *fpf1* gene sequence. Lane 1: DL 2000 marker. Lane 2: Positive control, pBIfpf1 as template. Lane 3: DNA from non-transformed plant. Lanes 4 – 8: DNA from transgenic plants. Lane 9: Negative control, water as template.

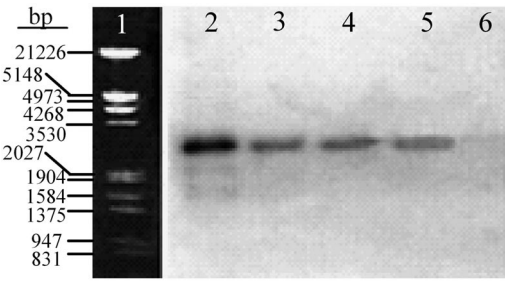

Fig. **4** Southern blot analysis of *fpf1* transgenic plants. Lane 1: λ-DNA (*Eco*RI + *Hind* III) marker. Lane 2: Positive control, pBIfpf1 plasmid digested with *Eco*RI and *Xba*I. Lanes 3 – 5: DNA isolated from *fpf1* transgenic plants. Lane 6: DNA isolated from non-transformed plant. The plant DNA was also digested with *Eco*RI and *Xba*I.

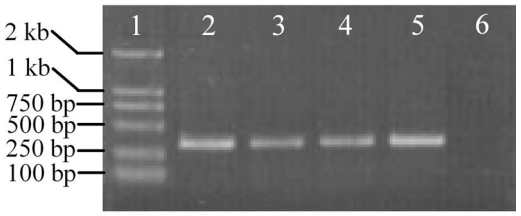

Fig. **5** Identification of transgenic plants by RT-PCR for *fpf1* gene sequence. Lane 1: DL 2000 marker. Lane 2: Positive control, PCR product of pBIfpf1. Lanes 3 – 5: *fpf1* transgenic plants. Lane 6: Non-transformed plant.

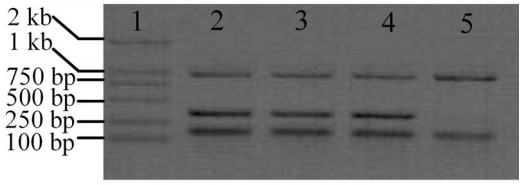

Fig. **6** Identification of transgenic plants by RT-PCR for *fpf1* gene sequence and *tubulin* gene sequence. Lane 1: DL 2000 marker. Lanes 2 – 4: *fpf1* transgenic plants. Lane 5: Non- transformed plant. The bands of 1.0 kb were corresponding to *tubulin* gene, which was used as an internal control for gel loading and transferring; and the ones of 300 bp to *fpf1* gene.

The linkage between flowering and artemisinin biosynthesis

The introduction of *fpf1* gene to *A. annua* successfully induced the early flowering of the *fpf1* transgenic plants. In order to detect whether the artemisinin content of the *fpf1* transgenic plants varied with the plants' flowering time, 3-month-old transformed and non-transformed *A. annua* plants were treated with an 8-h photoperiod to induce flowering, and those still grown at the 16-h photoperiod were used as controls for flowering. The leaves of both short-day-treated and non-treated plants were collected to determine the artemisinin content in different developmental stages (vegetative growth period, before flowering, full flowering and post flowering), data are shown in Fig. **8** (each value represents mean of three replicates with five samples each).

It was observed that after short-day treatment for 20 days, the *fpf1* transgenic plants generated flower buds, but the non-transformed plants were still in vegetative growth at this time. Both the transformed and non-transformed plants maintained under long days were in vegetative growth during the whole experimental periods, which was consistent with the fact that *A. annua* is a short-day plant [11], [21].

The artemisinin contents of the transgenic and non-transgenic plants were shown in Fig. **8**. From Fig. **8**, we know that before flowering, for the short-day-treated *fpf1* transgenic plants, the short-day-treated non-transgenic plants, and the non-short-day-treated ones, their artemisinin contents all increased with the plant development. The artemisinin content of the short-day-treated *fpf1* transgenic plants reached its maximum before the emergence of flower buds, the artemisinin content of the short-day-treated non-transformed plants is close to this maximum value at the same time. However, for the non-short-day-treated transformed and non-transformed plants, although remaining vegetative, their respective artemisinin contents showed no significant difference in comparison with those of the short-day-treated ones. After flowering, the artemisinin content of *fpf1* transgenic plant began to decrease significantly, but for the non-short-day-treated *fpf1* transgenic plants and control plants, being still in vegetative growth, their artemisinin contents still kept at a high level.

The above results showed that the foreign *fpf1* gene can indeed promote *A. annua* to flower earlier, but since the artemisinin content of the flowering transformed plants and that of the non-transformed control plants did not manifest any significant differences, we can conclude that the increase of artemisinin content before flowering is not induced by flowering itself. Furthermore, perhaps there is no direct linkage between flowering and artemisinin biosynthesis.

Fig. **7** Comparison of flowering time in *fpf1* transgenic plant (left) and non-transgenic plant (right).

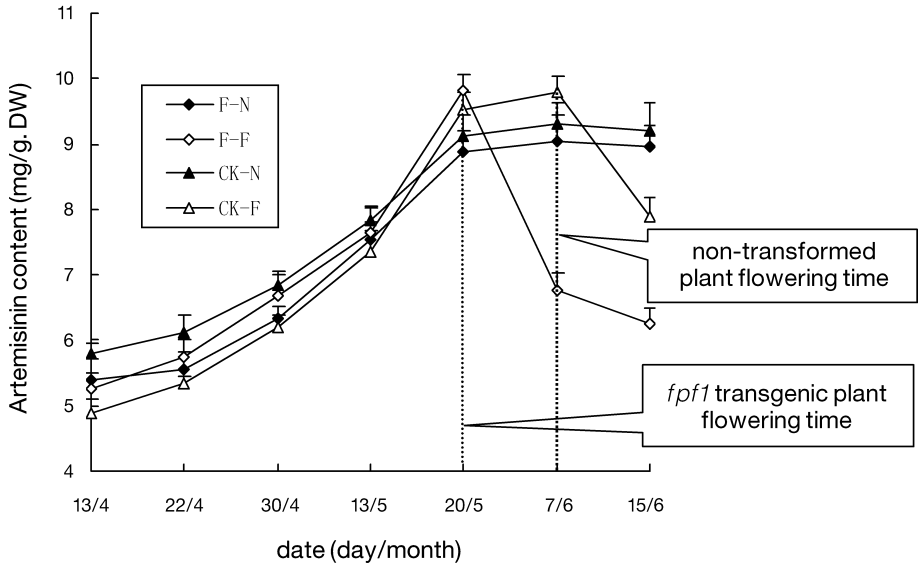

Fig. **8** Comparison of artemisinin content in flowering and non-flowering *fpf1* transgenic and CK plants. F-F: Flowering *fpf1* transgenic plants; F-N: Non-flowering *fpf1* transgenic plants; CK-F: Flowering CK (non-transformed) plants; CK-N: Non-flowering CK (non-transformed) plants.

After flowering, on the one hand, the artemisinin contents in the leaves began to decrease, and on the other hand, the plant began to senesce, and the leaves gradually wither and abscise, so the biomass of the leaves decreased, thus the production of artemisinin decreased significantly after flowering. In the period between the later vegetative growth stage and before the occurrence of flowering bud, both the artemisinin content and the biomass of the leaves reached their maximums, so in order to gain the highest artemisinin production, *A. annua* should be harvested in this period.

Acknowledgements

The authors would like to thank Dr. S. Melzer, Swiss Federal Institute of Technology, Institute for Plant Science, Switzerland, for the gift of the plasmid pBSKfpf1.

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