

Loss of Function of Ferredoxin2 Can Promote Flowering in *Arabidopsis thaliana*

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Abstract: In *Arabidopsis thaliana*, the red and far-red light photoreceptors phytochromes (PHYs) act to involve in regulating flowering. Phytychromobilin synthase (HY2) synthesizes the open chain tetrapyrrole chromophore which is essential to the light-sensing function of phytochromes, and it is a member of the ferredoxin-dependent bilin reductases (FDBRs). Here, we found a Ds-T-DNA insertion line of *Arabidopsis thaliana* for the gene encoding the most major ferredoxin (*Fd2*, At1g60950), which can promote flowering in the process of growth both under long-day and short-day conditions. In this report we show that loss of AtF_{d2} can promote flowering, AtF_{d2} interacts with AtHY2 in the chloroplast, and *Fd2-KO* mutants are impaired in the responses mediated by phytochromes. Together, these results implicate that loss of AtF_{d2} may promote flowering by impairing the physiology function of phytochromes.

Key words: ferredoxin 2, *Arabidopsis thaliana*, flowering

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拟南芥铁氧还蛋白基因缺失促进花期提前的初步研究*

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摘 要: 拟南芥的红光/远红光受体光敏色素 (PHYs) 参与花期调节过程, 而铁氧还蛋白色素还原酶 (FDBRs) 的一种——植物色素合成酶 (HY2) 对于光敏色素的合成是必不可少的。研究发现拟南芥铁氧还蛋白——AtF_{d2} 的基因缺失突变体 (*Fd2-KO* 突变体) 在长日照与短日照培养条件下, 较其野生型而言均表现出花期提前的表型, 而且显示 AtF_{d2} 与 AtHY2 在叶绿体中发生互作, 并且 F_{d2} 突变体对光敏色素的反应受到抑制。推测 AtF_{d2} 基因的缺失可能通过影响光敏色素介导的相关生理功能进而对植株的花期进行调节。

关键词: 铁氧还蛋白2; 拟南芥; 开花

Flowering is the turning point from the vegetative stage to the reproductive stage in the growth process of plants. In *Arabidopsis thaliana*, the flowering time is regulated by many different pathways: gibberellin, autonomous, vernalization, and light-dependent pathways^[1-2].

Plants utilize light as the signal to determine the timing of flowering. In *Arabidopsis thaliana*, there are a lot of various photoreceptors to monitor light quality and quantity. These photoreceptors can perceive light to regulate the flowering time^[3]. In *Arabidopsis*, these

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photoreceptors include the red/far-red light-absorbing phytochromes (PHYs) (PHYA to PHYE) [3-5] and the blue/UV-A light-absorbing cryptochromes (CRYs) (CRY1 and CRY2) [6-9], phytochromes and cryptochromes work together to involve in regulating flowering [5,10]. In the previous studies, we have known the far-red light can promote flowering [5, 11, 12] and the far-red light photoreceptor mutant—*phyA* mutant displays a late-flowering phenotype when grown both under long-day and short-day conditions [10]. In contrast, red light can delay flowering [5, 11-13] and the red light photoreceptor mutant—*phyB* mutant displays an early-flowering phenotype when grown both under long-day and short-day conditions [10]. Moreover, the *phyA phyB* double mutant also displays an early-flowering phenotype when grown under long-day and short-day conditions [10]. So it could be concluded that the impairment in the physiology function of phytochromes can promote flowering in the overall effect.

In plants, to sense and harvest the light signals, the photoreceptors must maintain some structures under the participation of the chromophores. In plants, open chain tetrapyrroles can function as the chromophores of light-sensing phytochromes [14]. To synthesize tetrapyrrole chromophores, the heme is first linearized by heme oxygenase to form the open-chain tetrapyrrole intermediate—biliverdin IX α (BV). BV is subsequently reduced by phytychromobilin (P Φ B) synthase—HY2, which is a ferredoxin-dependent bilin reductase to produce the P Φ B chromophore for light-sensing phytochromes [15-16]. Therefore HY2 plays very important role in the formation of phytochromes and the physiology function of phytochromes. Moreover, the *hy2* mutant also displays the early-flowering phenotype [17], this result further supports the conclusion that the impairment of the physiology function of phytochromes can promote flowering in the overall effect.

Ferredoxins (Fds) are the major donor systems for electrons to many various receptor systems in plastids. In photosynthetic organisms, Fds play roles not only in electron transfer system of photosynthesis but also in many redox reactions mediated by several oxidoreductases, such as sulfite reductase, nitrite reductase, glutamate synthase, and ferredoxin: thioredoxin reductase [18]. In *Arabidopsis thaliana*, the P Φ B syn-

thase—HY2 has been shown to require ferredoxins (Fds) as the electron donors for reduction reaction [15-16].

Here, we found that a Ds-T-DNA insertion line of *Arabidopsis thaliana* for the gene encoding the most major ferredoxin (Fd2, At1g60950) can promote flowering in the process of growth both under long-day and short-day conditions. We show in this report that the loss of AtFd2 function can promote flowering, AtFd2 can interact with AtHY2 in the chloroplast, and the loss of AtFd2 function can impair the responses mediated by phytochromes. These results implicate that the loss of AtFd2 can promote flowering by impairing the physiology function of phytochromes.

1 Materials and methods

1.1 Plant materials

For all experiments shown in this work, *Arabidopsis thaliana* plants of the ecotype Noessen WT and *Fd2-KO* were sent by Renate Scheibe and determined as described [17].

1.2 Flowering phenotype studies

The Noessen WT and *Fd2-KO Arabidopsis thaliana* seeds were germinated on soil and grown under long-day conditions (16h light/8h dark) or short-day conditions (8 h light/16 h dark) illuminated by white light at a fluence rate of 120 μ mol/m²/s. The number of days from germination to bolting, the number of rosette leaves when bolting, and the number of cauline leaves at maturity were scored.

1.3 BIFC study

Plasmid HY2-YN was made by cloning the *AtHY2* fragment without the stop codon from *Arabidopsis* cDNA with the primers 5'-AGTCGACATGGCTTTATCAATGAGT-3' and 5'-TTACTCGAGGCCGATAAATTGTCCT-3' and inserting into the pUC-SPYNE (YN) multiple cloning sites (MCS) with *Sal* I and *Xho* I.

Plasmid Fd2-YC was made by cloning the *AtFd2* fragment without the stop codon from *Arabidopsis* cDNA with the primers 5'-TAGGATCCATGGCTTCCACTGCTCT-3' and 5'-GCCCTCGAGAACAATGTCTTCTTCT-3' and inserting into the pUC-SPYCE (YC) multiple cloning sites (MCS) with *Bam*H I and *Xho* I.

Plasmids bZIP63-YN and bZIP63-YC were made

by cloning the *bZIP63* fragment without the stop codon from *Arabidopsis* cDNA and inserting into the pUC-SPYNE and pUC-SPYCE multiple cloning sites (MCS) with *Bam*H I and *Xho* I.

Construct pairs of bZIP63-YN and bZIP63-YC, HY2-YN and Fd2-YC, pUC-SPYCE (YC) and HY2-YN, pUC-SPYNE (YN) and Fd2-YC, YN and YC were transiently expressed in rice protoplasts isolated from 9-day-old rice seedlings. Fluorescence was observed using an Olympus fluorescent microscope and visualized with Olympus DP2-BSW software.

1.4 Hypocotyl Measurements

Arabidopsis thaliana seeds were surface-sterilized in 50% bleach for 15 min and washed five times with sterile ddH₂O before plating on a 1? 2 MS plate with 0.8% (*w*) Agar. The seeds were vernalized in the dark at 4 °C for 4 days. Germination was induced with a 4 hr white light treatment. The seeds were followed grown at 23 °C in a LED chamber (Percival Scientific, Perry, IA) under dark and certain light conditions (red or far-red light) for 6 days. For both photograph and hypocotyl length measurements, 6-day-old seedlings were photographed using a camera, and hypocotyl length was measured using ruler.

2 Results

2.1 Loss of AtFd2 can promote flowering in *Arabidopsis thaliana*

We found that the *Fd2-KO* mutant flowered earlier than wild type both under long-day (16h light/8h dark) and short-day (8 h light/16h dark) conditions (Figure 1A and 2A). In order to further support the phenotype, we chose three most common physiological indexes (the number of days from sowing to bolting, the number of rosette leaves when bolting, and the number of cauline leaves at maturity) to score. These physiological indexes could reflect the period of flowering, generally speaking, the numerical number was smaller, the flowering was earlier. The results showed that the number of days from sowing to bolting, the number of rosette leaves, and cauline leaves of *Fd2-KO* mutant were all smaller than that of wild type (Figure 1B-D and Figure 2B-D). So we confirmed that the loss of AtFd2 can promote flowering in *Arabidopsis thaliana*, and the loss of AtFd2 may involve in the reg-

ulation of flowering in *Arabidopsis thaliana* through some pathway.

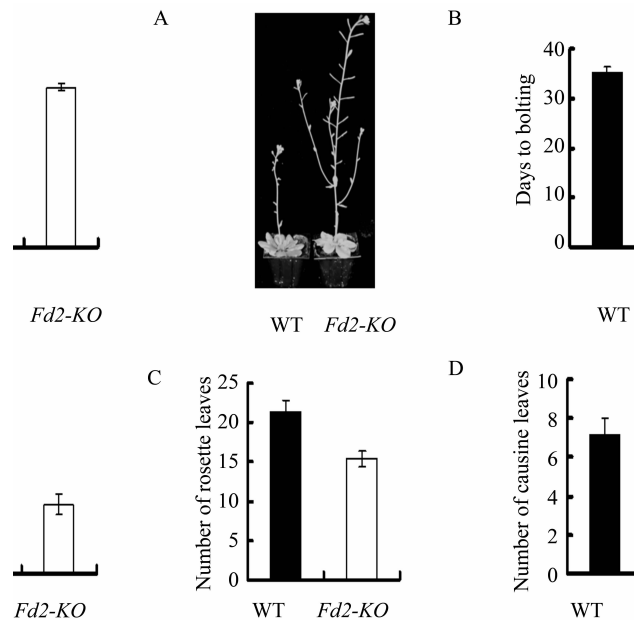


Fig. 1 Phenotypes of the Noessen WT and *Fd2-KO* *Arabidopsis* plants under long-day conditions

A: Phenotypes of the Noessen WT and *Fd2-KO* *Arabidopsis* plants under long-day conditions; B: The number of days from sowing to bolting of the Noessen WT and *Fd2-KO* *Arabidopsis* plants under long-day conditions; C: The number of rosette leaves when bolting of the Noessen WT and *Fd2-KO* *Arabidopsis* plants under long-day conditions; D: The number of cauline leaves when bolting of the Noessen WT and *Fd2-KO* *Arabidopsis* plants under long-day conditions (Thirty plants of Noessen WT or *Fd2-KO* *Arabidopsis thaliana* were calculated, three independent experiments)

2.2 AtFd2 interacts with AtHY2 in the chloroplast

In order to further confirm whether AtFd2 interacts with AtHY2, we created the plasmid for expression of *AtHY2* fused to the N-terminal yellow fluorescence protein (YN) under control of the 35S-CaMV promoter and the plasmid for expression of *AtFd2* fused to the C-terminal yellow fluorescence protein (YC) under control of the 35S-CaMV promoter. And we transiently expressed HY2-YN and Fd2-YC together in the rice protoplasts. The fluorescence emission of YFP was monitored in rice protoplasts using an Olympus fluorescence microscope. We found that co-expression of the HY2-YN and Fd2-YC fusion proteins in rice green tissue protoplasts produced obvious YFP signals in the chloro-

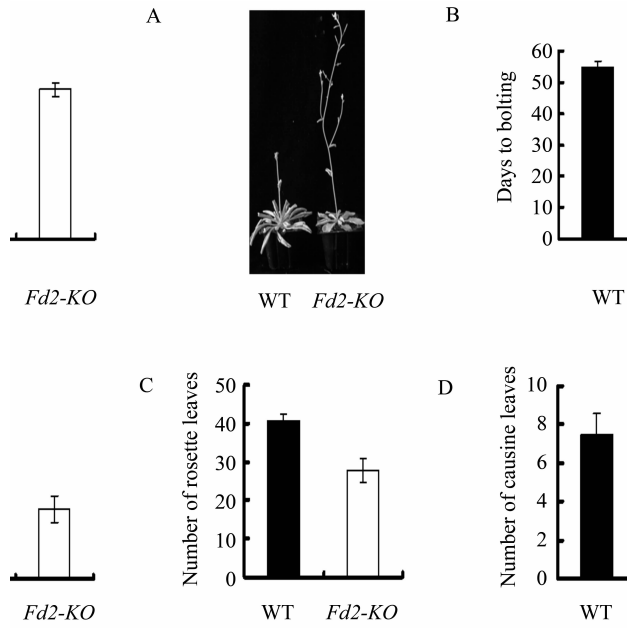


Fig. 2 Phenotypes of the Noessen WT and *Fd2-KO Arabidopsis* plants under short-day conditions

A: Phenotypes of the Noessen WT and *Fd2-KO Arabidopsis* plants under short-day conditions; B: The number of days from sowing to bolting of the Noessen WT and *Fd2-KO Arabidopsis* plants under short-day conditions; C: The number of rosette leaves when bolting of the Noessen WT and *Fd2-KO Arabidopsis* plants under short-day conditions; D: The number of cauline leaves when bolting of the Noessen WT and *Fd2-KO Arabidopsis* plants under short-day conditions

(Thirty plants of Noessen WT or *Fd2-KO Arabidopsis thaliana* were calculated, three independent experiments)

plast (Figure 3D). This result is in agreement with several previous studies that AtHY2 and AtFd2 are localized to the chloroplast. And the result confirms that AtHY2 physically interacts with AtFd2. As the negative controls, co-expression of HY2-YN with empty pUC-SPYCE (YC) vectors, empty pUC-SPYNE (YN) with *Fd2*-YC vectors, or two empty vectors pUC-SPYNE (YN) with pUC-SPYCE (YC) produced no BiFC fluorescence (Figure 3A-C). As a positive control, the bZIP63-YN and bZIP63-YC fusion proteins in rice green tissue protoplasts produced obvious YFP signals in the nucleus (Figure 3E).

2.3 *Fd2-KO* mutants are impaired in the responses mediated by phytochromes

In order to study whether *Fd2-KO* Mutants promote flowering by impairing the responses to both red light and far-red light mediated by phytochromes, we

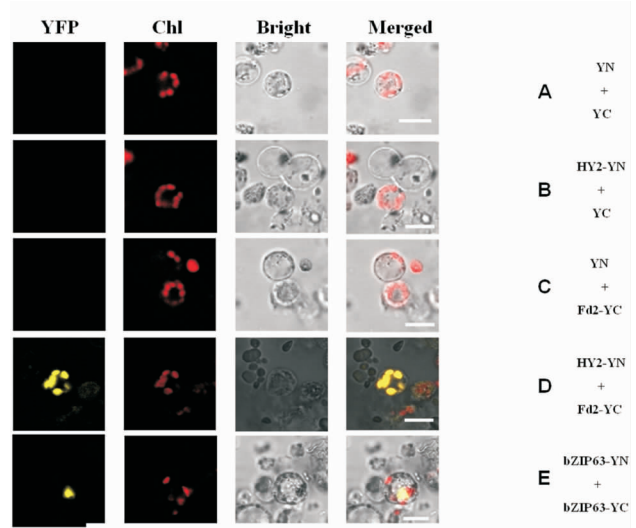


Fig. 3 AtFd2 interacts with AtHY2 in the chloroplast

Construct pairs of bZIP63-YN and bZIP63-YC, HY2-YN and *Fd2*-YC, pUC-SPYCE (YC) and HY2-YN, pUC-SPYNE (YN) and *Fd2*-YC, YN and YC were transiently co-expressed in rice green tissue protoplasts. BiFC fluorescence was indicated by the YFP signal. Individual and merged images of YFP and chlorophyll autofluorescence (Chl) as well as bright field images of protoplasts were shown. Scale bars = 10 μ m

first examined the response to red light mediated by phytochromes in *Fd2-KO* seedlings by measurement of hypocotyl lengths of 6-day-old seedlings compared to wild type grown under continuous red light. In dark conditions, *Fd2-KO* seedlings had shorter hypocotyl lengths than wild type since the loss of AtFd2 inhibited the normal growth of seedling. But the gap of the hypocotyl length between *Fd2-KO* seedlings and wild type seedlings reduced with the increase of the red light intensity (Figure 4A). Moreover, *Fd2-KO* seedlings had longer hypocotyl length than wild type seedlings under high red light intensity (Figure 4B).

The response to far-red light mediated by phytochromes was also examined in *Fd2-KO* seedlings by measurement of hypocotyl lengths of 6-day-old seedlings grown under continuous far-red light compared to wild type. The result was similar with the result of red light (Figure 5A and 5B). These results indicate that *Fd2-KO* Mutants are impaired in the responses mediated by phytochromes and implicate that the loss of AtFd2 may promote flowering by impairing the physiology function of phytochromes.

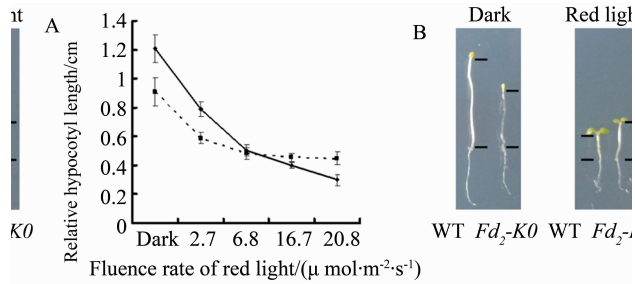


Fig. 4 Phenotypes of the Noessen WT and *Fd2-KO* *Arabidopsis* seedlings under red light conditions.

A: Relative hypocotyl length of 6-day-old grown Noessen WT (solid line), *Fd2-KO* (dotted line) *Arabidopsis* seedlings under different fluence of red light and dark conditions; B: Phenotypes of the 6-day-old grown Noessen WT and *Fd2-KO* *Arabidopsis* seedlings under red conditions ($20.8 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and dark conditions

(Thirty plants of Noessen WT or *Fd2-KO* *Arabidopsis thaliana* were calculated, three independent experiments)

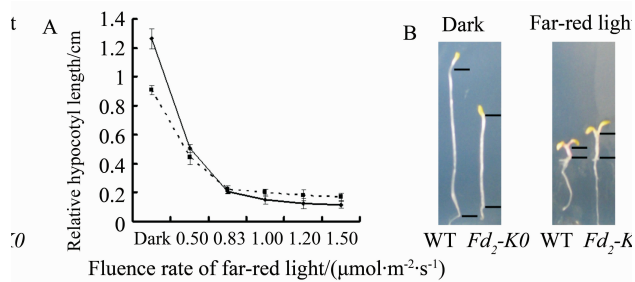


Fig. 5 Phenotypes of the Noessen WT and *Fd2-KO* *Arabidopsis* seedlings under far-red light conditions.

A: Relative hypocotyl length of 6-day-old grown Noessen WT (solid line), *Fd2-KO* (dotted line) *Arabidopsis* seedlings under different fluence of far-red light and dark conditions; B: Phenotypes of the 6-day-old grown Noessen WT and *Fd2-KO* *Arabidopsis* seedlings under far-red conditions ($1.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and dark condition

(Thirty plants of Noessen WT or *Fd2-KO* *Arabidopsis thaliana* were calculated, three independent experiments)

3 Discussion

Based on the phenotype of *Fd2-KO* mutants, which flowered earlier than wild type both under long-day and short-day conditions, we proposed the possible pathway AtFd2 regulates in flowering.

The previous studies have shown that phytochromes are involved in the regulation of flowering. The far-red light photoreceptor-PHYA and the red light photoreceptor-PHYB are the most important phytochromes in *Arabidopsis thaliana*. Based on the effect of

far-red/red light and the phenotype of *phyA*, *phyB*, *phyAphyB* mutants^[5, 10-13], we may come to the conclusion that the impairment of the physiology function of phytochromes can promote flowering in the overall effect.

And we learned that one of the most important enzymes in the synthesis of chromophore for light-sensing phytochromes —HY2, which is ferredoxin-dependent and requires ferredoxins (Fds) as the electron donors to perform its normal physiological function^[15-16]. Moreover, the *hy2* mutant also flowered early^[18], this result further supported the conclusion that the impairment of the physiology function of phytochromes can promote flowering in the overall effect.

AtFd2 is the most important ferredoxin in *Arabidopsis thaliana*, so we guessed that the loss of AtFd2 may promote flowering by impairing the physiology function of phytochromes.

We demonstrated that AtFd2 can interact with AtHY2 in chloroplast. Though the previous studies have proven that AtHY2 requires AtFd2 as the electron donors through the biochemical and structural studies, our results first prove that AtFd2 can interact with AtHY2 in vivo through BIFC. The results further support that AtHY2 is ferredoxin-dependent and may require AtFd2 as the electron donors for double bond reductions.

In conclusion, all the results implicated that the loss of AtFd2 may promote flowering by impairing the physiology function of phytochromes. However, because of the difference between the physiology progress mediated by phytochromes and the flowering progress, we can identify the genetic regulation relationship between AtFd2 and phytochromes to further confirm our conclusions in the following research.

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