The Reduced Plastid-Encoded Polymerase-Dependent Plastid Gene Expression Leads to the Delayed Greening of the Arabidopsis fln2 Mutant

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Abstract

In Arabidopsis leaf coloration mutants, the delayed greening phenomenon is common. Nonetheless, the mechanism remains largely elusive. Here, a delayed greening mutant fln2–4 of FLN2 (Fructokinase-Like Protein2) was studied. FLN2 is one component of Transcriptionally Active Chromosome (TAC) complex which is thought to contain the complete plastid-encoded polymerase (PEP). fln2–4 displayed albino phenotype on medium without sucrose. The PEP-dependent plastid gene expression and chloroplast development were inhibited in fln2–4. Besides interacting with thioredoxin z (TRX z), we identified that FLN2 interacted with another two members of TAC complex in yeast including its homologous protein FLN1 (Fructokinase-Like Protein1) and ptAC5. This indicates that FLN2 functions in regulation of PEP activity associated with these TAC components. fln2–4 exhibited delayed greening on sucrose-containing medium. Comparison of the PEP-dependent gene expression among two complete albino mutants (trz z and ptac14), two yellow mutants (ecb2–2 and ysl1) and the fln2–4 showed that fln2–4 remains partial PEP activity. FLN2 and FLN1 are the target proteins of TRX z involved in affecting the PEP activity. Together with the data that FLN1 could interact with itself in yeast, FLN1 may form a homodimer to replace FLN1–FLN2 as the TRX z target in redox pathway for maintaining partial PEP activity in fln2–4. We proposed the partial PEP activity in the fln2 mutant allowed plastids to develop into fully functional chloroplasts when exogenous sucrose was supplied, and finally the mutants exhibited green phenotype.

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Introduction

The first step in the life cycle of a plant is seedling establishment after seed germination. When seedlings are exposed to light, they synthesize chlorophyll; the cotyledons expand and turn green [1]. During this process, the etioplasts of cotyledons develop into photosynthetically functional chloroplasts, which allow seedlings to become photoautotrophic and no longer depend on energy stored photosynthetically functional chloroplasts, which allow seedlings to become photoautotrophic and no longer depend on energy stored in the seed [2,3]. Disruption of chloroplast function often results in severe phenotype, such as embryo lethality, albinism, or pale-green plants [4]. Chloroplast development is regulated by both nuclear and plastid genomes, and recent investigations revealed that many factors are involved in the process [5,6].

The transcription of plastid genes in higher plants is dependent on two RNA polymerases, the nuclear-encoded plastid RNA polymerase (NEP) and plastid-encoded plastid RNA polymerase (PEP) [7]. NEP preferentially transcribes housekeeping genes, while PEP is responsible for transcribing genes involved in photosynthesis [8,9]. PEP and its associated proteins/DNA can be purified by different biochemical purification procedures [10,11]. This protein/DNA-complex, termed “transcriptionally active chromosomes” (TAC), can be separated from the plastid components by gel filtration [12–14]. In Arabidopsis and mustard (Sinapis alba), a total of 35 components have been identified in the TAC complex including the core subunits of PEP encoded by plastome-located rpo genes and nuclear encoded subunits [15]. In recent years, a group of Arabidopsis leaf coloration mutants defective in the TAC components have been isolated. The Fe-superoxide dismutase (FSD) double mutant fas2–1 fas3–1 exhibited low tolerance to oxidative stress and accumulated decreased amounts of mRNA for PEP-dependent plastid genes [16]. Studies on the knockout mutants of thioredoxin z (TRX z) and fructokinase-like protein (FLN) showed that TRX z interacts with FLN1 and FLN2 to regulate the PEP-dependent transcription and chloroplast development in redox signaling pathway [17–19]. Mutations in the ptac3, ptac7 and ptac14 genes also resulted in the expression defects of PEP-dependent genes [20–22]. Based on the
decreased expression of PEP-dependent genes, the PEP activity in these leaf coloration mutants was severely impaired which led to complete albino phenotype.

However, there exists another group of delayed greening mutants including fln2–4 [15] and some non-fln mutants, such as aos [23], dgl [24] and pisp1 [25]. These mutants initially showed an albino phenotype on medium without sucrose, but they could turn green when exogenous sucrose was provided. In addition, a group of yellow mutants were reported such as ys1 [26] and ecb2–2 [27]. These mutants shared a similar phenotype that their cotyledons and true leaves were initially very yellow, but then turned green gradually without supplementation with exogenous carbon. 15S1 encodes a chloroplast-localized pentatricopeptide repeat (PPR) protein, which is required for the editing of tpdB transcripts encoding the beta subunit of PEP in Arabidopsis [26]. ATECB2 also encodes a PPR protein with a C-terminal DYW domain. It regulates the editing of the plastid genes accD and ndhF [27]. The PEP activity has not been analyzed in pisp1 [25]. In all other delayed greening mutants and yellow mutants, PEP activity was defective [15,23,24,26,27]. Nonetheless, the mechanism for the greening of these PEP-related leaf coloration mutants is largely unknown.

During the seedling greening process, one of the most important events is the formation of chlorophyll, which allows plants to absorb energy from light [28]. The chlorophyll biosynthesis is strictly regulated by environmental and endogenous cues such as light signals [29], hormone signals [30], and plastid retrograde signals [31]. Previous studies revealed that several signaling-responsive transcription factors are required for chlorophyll biosynthesis. The transposase-derived transcription factors FLH3/FAR1 are responsive to light signals and directly activate the expression of the key gene HEMB1 in chlorophyll biosynthetic pathway [32]. The ethylene-stabilized transcription factors EIN3/EIL1 were found to promote chlorophyll synthesis in the ethylene-induced signaling pathway [33]. In addition, recent studies have demonstrated that the regulation by light, auxin/cytokinin and plastid-derived retrograde signals is dependent on the golden2-like transcription factors (GLKs), which is required for the expression of several chlorophyll biosynthesis genes [34,35]. Apart from the known chlorophyll biosynthesis, photosynthesis gene expression is crucial for the greening process by affecting the assembly of the photosynthetic apparatus [35]. PEP is the major machinery in regulating photosynthesis-related plastid gene expression. Thus, it is necessary for us to elucidate the relationship between PEP and seedling greening.

Here, we report the characterization of a delayed greening mutant fln2–4 in Arabidopsis, which displays albino phenotype but can develop greenish leaves on sucrose-containing medium. The PEP-dependent plastid gene expression and chloroplast development were inhibited in fln2–4. Comparison of the PEP-dependent gene expression among five leaf coloration mutants (tx z, pta114, fln2–4, ecb2–2 and ys1) indicates that the PEP activity is critical for the leaf color phenotypes. The different degrees of PEP activity often give rise to the different leaf colors. Based on our yeast two-hybrid assay, FLN1 may form a homodimer instead of the FLN1–FLN2 heterodimer in function in regulation of PEP activity, which supports that the PEP activity in fln2–4 is higher than that in the complete albino mutants (tx z and pta114). The relatively high PEP activity in fln2–4 allows the slow accumulation of the PEP-dependent gene transcripts for chloroplast development when supplemented with sucrose. With the formation of functional chloroplasts, the fln2–4 mutant shows green phenotype. All of these investigations should prove to be helpful to understand the mechanism of the greening phenomenon in many PEP-related Arabidopsis leaf coloration mutants.

Results

Identification and Characterization of the fln2 Mutant

To analyze the functional roles of FLN2 gene during plant growth and development, we obtained two T-DNA insertion lines, SALK_005734 and CS811853, from the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu/). In these two lines, the T-DNA was inserted in the 3rd exon and the 5th exon of the FLN2 gene, respectively (Figure 1A). Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the FLN2 transcript was absent in both mutants (Figure 1B). Due to the SALK_005734 line named as fln2–3 [19], the CS811853 line in this work was termed as fln2–4. Both fln2–3 and fln2–4 displayed albino cotyledons and were seedling lethal on MS medium without sucrose (Figure 1C). The phenotypes of the two allelic mutants were similar; thereby the fln2–4 mutant was chosen for further analysis. Transmission electron microscopy (TEM) observations revealed that the chloroplasts in the 7-day-old fln2–4 mutants had a visible change in ultrastructural organization with irregular morphology and lacked internal membrane structures (Figure 1D). To confirm that the knockout of FLN2 was responsible for the defects in the fln2–4 phenotype, a construct containing the genomic sequence of the FLN2 gene, as well as 1517-base pair (bp) upstream and the FLAG sequence was introduced into the heterozygous plant (FLN2/fln2–4). A total of 33 transgenic plants were obtained. Six of them were identified to be homozygous for the T-DNA insertion, and exhibited normal morphology as the wild type (WT) (Figure 1E). These results demonstrate that the FLN2 gene is responsible for the defective phenotype in fln2–4 mutant, and FLN2 is important for chloroplast development and seedling growth.

Loss of FLN2 Down-Regulates the Expression of PEP-Dependent Plastid Genes

FLN2 is one component of TAC, and many TAC members were reported to affect the plastid gene expression [15,17,20,22]. To investigate the effect of the knockout of the FLN2 gene on the plastid gene expression, we used Northern hybridization to examine the transcriptional levels of plastid genes in 7-day-old fln2–4 mutants grown on MS medium without sucrose. The plastid genes are categorized into three classes based on whether they are transcribed by PEP and/or NEP [36]. The class I and class III genes are exclusively transcribed by PEP and NEP, respectively. While the class II genes are both PEP- and NEP-dependent. Our results showed that the expression of the class I genes (psbA, psbB and rbcL) were strongly reduced in the fln2–4 mutant compared with that of the WT. By contrast, the transcript levels of the class-III genes (rnl16 and clpP) and the class-II genes (accD and tpdB) did not have any significant variation between fln2–4 mutant and WT (Figure 2). These results confirm that loss of FLN2 mainly affects the expression of PEP-dependent plastid genes.

FLN2 Interacts with FLN1 and pTAC5 in Yeast

FLN2 belongs to the components of TAC complex in Arabidopsis [15]. To establish the relationship between FLN2 and the other components of TAC, we performed a yeast two-hybrid screen. FLN2 was fused to the GAL4 DNA-binding domain (BD) as a bait to screen a pool composed of the thirty-five reported TAC components fused to the GAL4 activation domain (AD). After yeast transformation, we randomly selected clones with blue appearance in selective dropout (SD) medium lacking trypthphan.
(Trp), leucine (Leu), histidine (His) and adenine hemisulfate salt (Ade) with X-α-gal for PCR amplification. Sequencing the PCR products identified three genes including FLN1, pTAC5 and TRX z. Yeast two-hybrid experiments verified the interactions between FLN2 and the three proteins (Figure 3A). We subsequently performed pull-down assay to further confirm the interactions between FLN2 and FLN1 or pTAC5. Results showed that both the recombinant proteins GST-FLN1 and GST-pTAC5 were able to pull down FLN2-His (Figure 3B and C). FLN2 and FLN1 belong to the pkB family, and share high peptide similarity [37]. To examine whether the FLN1 and FLN2 proteins form homodimers, we analyzed the interactions between them by yeast two-hybrid assay. Results showed that FLN1 can interact with itself, but FLN2 can not (Figure 3D). RpoA, pTAC12 and pTAC14 are the essential subunits in the PEP complex [18]. Yeast two-hybrid assay showed that FLN2 did not interact with these essential subunits in yeast (Figure 3E). Based on previous investigations [21,22] and data in this study, a working model for partial TAC components is proposed (Figure 3F). In this model, pTAC7 interacts with FLN1, pTAC10, pTAC12 and pTAC14 while FLN2 interacts with FLN1, TRX z and pTAC5.

Figure 1. T-DNA insertion sites of fln2 and their phenotypes. (A) The sketch map of the FLN2 gene, the T-DNA insertion sites of fln2 mutants. Boxes, exons; lines, introns. (B) The expression of FLN2 in WT (Col-0), fln2–3 and fln2–4. (C) Photographs of the fln2–3 and fln2–4 seedlings grown on MS medium for 7 days. (D) Ultrastructural analysis of chloroplasts from 7-day-old WT and fln2–4 seedlings grown on MS medium. Scale bars: 1 μm. (E) Phenotypes of WT and the fln2–4 complemented seedlings grown in soil. doi:10.1371/journal.pone.0073092.g001

Figure 2. Expression analysis of the plastid encoded genes in fln2–4 seedlings. Northern blot was performed to detect the plastid gene transcript levels in the 7-day-old fln2–4 seedlings and WT grown on MS medium without sucrose. Three classes of genes were examined, psbA, psbB, and rbcL were selected as PEP-dependent genes, clpP and rm16 were selected as PEP- and NEP-dependent genes, accD and rpoA were selected as NEP-dependent genes. doi:10.1371/journal.pone.0073092.g002
The fln2–4 Mutant Exhibited a Delayed Greening Phenotype when Grown on Sucrose-Containing Medium and can Grow Autotrophically in Soil

The delayed greening fln2–4 mutant exhibits sugar-dependent for the survival of seedlings and growth of green true leaves. When supplemented with sucrose, the 7-day-old fln2–4 seedlings displayed yellow-tinted cotyledons, and then developed greenish true leaves in two weeks after germination (Figure 4A). After transplanting them into soil, all the mutant seedlings with greenish true leaves could flower and produce fertile seeds without sucrose supplementation (Figure 4B). To unveil the ultrastructural basis of fln2–4 seedlings during the delayed greening process, we examined dynamic changes in chloroplasts using TEM. In the leaves of 7-day-old WT grown with sucrose, chloroplasts were crescent-shaped and contained well-formed internal membrane structures including stroma thylakoids and stacked grana thylakoids (Figure 4C). In contrast, at the same stage, the chloroplasts in the 7-day-old fln2–4 mutants were still highly vacuolated, but the lamellar membrane structures appeared (Figure 4C). In the 14-day-old fln2–4 mutants grown under the same conditions, chloroplasts with well-organized thylakoid membrane could be observed (Figure 4C). This suggests the fln2–4 chloroplast can be gradually formed although its development is slower than that in WT. In addition, we measured the levels of chlorophyll a and b in both the mutant and WT seedlings during the greening process. The total chlorophyll content of fln2–4 mutant (93.58 ± 2.45 mg g⁻¹ fresh weight for the fln2–4 mutant type) was about 16.9% of WT (554.94 ± 15.51 mg g⁻¹ fresh weight for the wild type) grown with sucrose for 7 days, and the chlorophyll a/b ratio of fln2–4 was about half of WT (Figure 4D; Table 1). In 14-day-old mutant seedlings grown under the same conditions, the chlorophyll content (292.16 ± 24.91 mg g⁻¹ fresh weight for the fln2–4 mutant type) was about 42% compared to that of the WT (695.19 ± 24.14 mg g⁻¹ fresh weight for the wild type), and the chlorophyll a/b ratio was close to that of WT (Figure 4D; Table 1). These observations indicated that the chlorophyll biosynthesis in
fln2–4 was partially recovered. Furthermore, we investigated the plastid ultrastructure development of the WT and fln2–4 grown on sucrose-containing medium during de-etiolation. In the WT and fln2–4 seedlings grown in darkness for 5 days, the etioplasts contained a large prolamellar body (Figure 5, left panels). When etiolated seedlings were exposed to light for 6 hours, the prolamellar in WT developed into stromal lamellae (Figure 5, middle panels). In contrast, plastids in fln2–4 contained less stromal lamellae (Figure 5, middle panels). After de-etiolation for 24 hours, plastids in WT contained well-developed thylakoid membrane system and starch granules, while loose thylakoid lamellae and grana thylakoids were detected in fln2–4 (Figure 5, right panels). When grown on MS medium without sucrose in darkness for 5 days, the etioplasts of fln2–4 were similar with that of the WT. After de-etiolation for 24 hours, only a few thylakoid lamellae could be observed in fln2–4 (Figure 5). These results revealed that plastid development in fln2–4 grown on sucrose-containing medium proceeds slowly, but it can gradually form well-structured chloroplast during the de-etiolation process.

PEP-Dependent Plastid Gene Expression in fln2–4 Is Higher than that in the Complete Albino Mutants, but Lower than that in the Yellow Mutants

The most distinct characteristic of fln2–4 mutant is its delayed greening phenotype when grown on sucrose-containing medium. We assayed the expression of PEP-dependent plastid genes in fln2–4 during its greening process. Results showed that the transcript levels of the three selected genes (rbcL, psbA, psbB) in 7-day-old fln2–4 mutant were still significantly lower than that in WT. However, after 14 days of growth on sucrose-containing medium, the rbcL mRNA accumulated to similar levels in WT, while the psbB transcription was slightly enhanced (Figure 6). These results indicated that the transcripts of several PEP-dependent genes were able to gradually accumulate during the greening of fln2–4. To disclose whether these differences in plastid gene expression were tightly linked with leaf phenotype, we chose four leaf coloration mutants for further research, which including two complete albino mutants trx z [17] and ptac14 [22], and two yellow mutants ecb2–2 [27] and ys1 [26]. When germinated on MS medium, trx z and ptac14 exhibited albino cotyledons and died before developing true leaves. While grown on MS medium containing 2% sucrose, they could produce pale yellow cotyledons and true leaves (Figure 7A). Nevertheless, these mutants subsequently died. In contrast, the

Figure 4. Alterations to seedling development in fln2–4 plants. (A) The phenotypes of WT and fln2–4 plant grown on 2% sucrose-containing MS medium for 7 days and 14 days. (B) The phenotypes of WT and fln2–4 plants grown in soil after growing on sucrose-containing medium for 14 days. Bars represent 1 cm. (C) Chloroplast ultrastructure in 7-day-old WT, 7-day-old fln2–4 plants, 14-day-old WT and 14-day-old fln2–4 plants. All of these plants grew on sucrose-containing medium. Scale bars: 1 μm. (D) The relative chlorophyll levels in WT and fln2–4 seedling during growth on sucrose-containing MS medium. The values presented are averages of three replicates ± SD.

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**Discussion**

**FLN2 is One Component of TAC to Regulate PEP-Dependent Plastid Gene Expression**

The knockouts of many TAC members in *Arabidopsis* have been reported to affect plastid gene expression and chloroplast development including *ptac6*, *ptac12* [15], *fds2*, *fds3* [16], *trx z* [17], *fln1* [17,18], *ptac14* [18,22], *ptac3* [20] and *ptac7* [21]. FLN2 is one component of TAC and is involved in the redox pathway mediated by TRX z [15,17]. The knockout lines *fln2–1* and *fln2–2*, exhibited pale green cotyledons and slow greening on growth medium and soil [19]. Here, we isolated another two knockout lines *fln2–3* and *fln2–4*. These two lines displayed albino phenotype when grown on MS medium (Figure 4C), but they could turn green when grown on sucrose-containing medium (Figure 4A and data not shown), which was slightly different from the reported phenotypes of *fln2–1* and *fln2–2* [19]. This difference was probably due to the different growth conditions. Nevertheless, the PEP-dependent plastid gene expression was down-regulated in the yellow mutants.

The expression levels of *rbcL*, *psbA* and *psbB* genes in 7-day-old and 14-day-old *fln2–4* mutants were determined by Northern blot as compared with WT, respectively. The experimental WT and *fln2–4* seedlings were grown on sucrose-containing MS medium.

| Table 1. Chlorophyll accumulation in the WT and *fln2–4* mutants. |
| --- |
| **Sample** | mg Chl (a+b)/g FW* | Relative amount | Chl a/b ratio |
| WT 7 d/MS+2%Suc | 554.94±15.51 | 100% | 2.03±0.02 |
| *fln2–4* 7 d/MS+2%Suc | 93.58±2.45 | 16.9% | 0.92±0.13 |
| WT 14 d/MS+2%Suc | 695.19±24.14 | 100% | 1.94±0.07 |
| *fln2–4* 14 d/MS+2%Suc | 292.16±24.91 | 42.03% | 1.53±0.18 |

**Figure 5. Ultrastructure development of WT and *fln2–4* cotyledon plastids during de-etiolation.** Seedlings were grown in darkness for 5 days, and subsequently illuminated for 0 h (left panels), 6 h (middle panels) and 24 h (right panels). Abbreviations used in the panels are listed: Pb, prolamellar body; S, starch granule; St, stromal thylakoids; Gt, grana thylakoids. Scale bars: 1 μm.

**Figure 6. Changes in the transcript levels of PEP-dependent genes during the greening process of *fln2–4* mutant.** The expression levels of *rbcL*, *psbA* and *psbB* genes in 7-day-old and 14-day-old *fln2–4* mutants were determined by Northern blot as compared with WT, respectively. The experimental WT and *fln2–4* seedlings were grown on sucrose-containing MS medium.

**Figure 7. *Arabidopsis* RNAi lines.*

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than that of fln2. In addition, silencing of FLN2 results in weaker effect on PEP-dependent plastid gene expression than does silencing of FLN1 [17]. Furthermore, the ptac5 mutant showed similar phenotype with WT under normal condition, but was sensitive to heat stress (Figure S1). Overall, FLN2 and pTAC5 may represent peripheral component rather than core subunit to function as a regulator for environmental adaptation in the TAC complex.

Figure 7. Comparison of PEP-dependent plastid gene expression between fln2–4 and four leaf coloration mutants. (A) The phenotypes of WT and four leaf coloration mutants grown with or without sucrose. (B) qRT-PCR analysis the transcript levels of four PEP-dependent plastid genes in fln2–4 seedlings and other four leaf coloration mutants grown on MS medium without sucrose for 7 days. These PEP-dependent genes refer to psbA, psbB, psaB, petD. Expression levels are presented as the percentage relative to WT. Data are means ± SD (n = 3). (C) The accumulation of psbA and psbB transcripts detected by Northern blot.

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PEP Activity is Critical for the Early Development of Chloroplast and Leaf Greening

Leaf coloration mutants are frequently observed in plant. In Arabidopsis, seedling leaf coloration mutants can be classified into several groups. For the yellow mutants such as ys1 [26] and ecb2–2 [27], they initially exhibited yellow cotyledons and true leaves, and subsequently turned green and grew photo-autotrophically under normal condition. For the complete albino mutants such as trx z [17] and ptac14 [22], they displayed albino cotyledons and could not turn green even on sucrose-containing medium (Figure 7A). These lines are seedling lethal and can not complete their life cycles. Apart from the complete albino mutants and the yellow mutants, there exists another group of delayed greening mutants such as ptac2 [15], wco [23], dg1 [24] and pisp1 [25]. These mutants showed albino phenotype initially, however, they could turn green on the sucrose-containing medium. The fln2–4 mutant is similar with the delayed greening mutants (Figure 4A). These different groups of leaf coloration mutants often showed different degrees of
defects in chloroplast development. During seed germination, the photosynthetically functional chloroplasts are quickly formed and seedlings turn green before the energy in the seed is exhausted. The rapid formation of mature chloroplast during seed germination is very important for seedling greening. Thus, it seems that the different degrees of chloroplast developmental status give rise to the different leaf colors of mutant seedlings.

The PEP-dependent plastid gene expression analysis in this work indicates the PEP activity in the delayed greening mutant is higher than that in the complete albino mutants, but lower than that in the yellow mutants (Figure 7B and C). The PEP activity in these leaf coloration mutants is in agreement with their phenotypes. The more seriously reduced PEP activity results in the defected chloroplast development. In the fln2–4 mutant, relatively high PEP activity allows the slow chloroplast development. However, the energy in seed is not enough to support the formation of mature chloroplast and therefore seedlings show albino phenotype. The exogenous sucrose in the medium supports the mutant plants to overcome energy deficiency and gradually accumulate enough photosynthesis gene transcripts for functional chloroplast formation. The development of fully functional chloroplast allows the mutant plants to show green phenotype.

A Homodimer Formed by FLN1 may Partially Compensate the Loss of FLN2–FLN1 Heterodimer to Maintain some PEP Activity

The cellular redox environment has a fundamental role in regulating most plastid processes, including secondary metabolism, gene transcription, and protein synthesis as well as import [39,40]. In Arabidopsis, Arsova et al. identified that a novel thioredoxin TRX z which may be involved in the redox pathway to regulate PEP-dependent plastid gene expression and chloroplast development through interacting with FLN1 and FLN2 [17]. Here, our data showed that there exist an interaction between FLN1 and FLN2, which indicated that the FLNs may form a heterodimer as the TRX z target in the TAC complex. The homologs of FLN1 and TRX z are present in moss, pteridophyta and angiosperm, while FLN2 homologs are only present in angiosperm, based on the available genomic data. This suggests that FLN1 might be an ancient protein, while FLN2 is a novel protein evolved from FLN1 after the emergence of the pteridophyta during the course of evolution. In this work, FLN1 was found to interact with itself in yeast (Figure 3D). This suggests that FLN1 might form a homodimer in the TAC complex to regulate the PEP activity and chloroplast development in moss. With the presence of FLN2, the FLN1 may interact with FLN2 rather than itself. However, as the essential protein in the redox pathway, FLN1 remains the ability to interact with itself. In Arabidopsis, both the TRX z and FLN1–FLN2 heterodimer are the essential components of redox pathway in regulation of PEP activity. The PEP activity in the knockout of TRX z was severely impaired and therefore the trz z mutant showed complete albino phenotype. In the fln1 mutant, the redox pathway may be blocked due to the absence of the FLN1–FLN2 heterodimer, which leads to albinism phenotype. While in the fln2–4 mutant, FLN1 may form a homodimer to replace the FLN1–FLN2 heterodimer, and thus the mutant remains partial signaling of the redox pathway in regulation of PEP activity. This supports the slow accumulation of the PEP-dependent plastid gene transcripts for chloroplast development and the delayed greening of this mutant.

Materials and Methods

Plant Materials and Growth Conditions

WT and mutant Arabidopsis thaliana (Columbia) seeds were used in this study. The T-DNA insertion lines SALK_005734 (fln2–3), CS811853 (fln2–4), SALK_0028162 (trz z), SALK_005814 (ptac14), SALK_049133 (ptac5) were obtained from the ABRC. Plants were grown on Murashige and Skoog (MS) agar plates supplemented with or without 2% (w/v) sucrose. All plate-grown seedlings were vernalized under 4°C condition for 2 days and then were grown in a chamber at 22°C with a 16-h-light (120 mmol photon m–2 s–1)/8-h-dark cycle. The soil-grown plants were grown with a photon flux density of 120 mmol m–2 s–1 at 22°C.

Identification and Compensation of the fln2 Mutant

The primers used for PCR verification of the T-DNA insert in the mutant were AtLB1 (5’-TGGTTTCAGTATGGGCGCATCGG-3’) and the plant specific primers (fln2–3-LP, 5’-GAGATTTTCATGCCAAAGCTG-3’; fln2–2-3R, 5’-CAGCTTCTTCTGATTTGAGG-3’; fln2–4-LP, 5’-TTGGAACATTGAGTGGTCGG-3’; fln2–4-3R, 5’-TCATCGTCATCTGCAGTTTCACGAGAGAGTGGGCGCATCGG-3’). The abovementioned primers also were used to distinguish the etiolated WT and fln2–4 seedlings during de-etiolation process. A 3861-bp DNA fragment including the genomic sequence of the gene FLN2 (At1g69200) and 1517-bp upstream sequence, was amplified using KOD polymerase (Takara, Japan) with the two specific primers (FLN2-F: 5’-GGATCCATATGTAATTATTTCTGTTTTCGCAAGCT-3’; FLN2-R: 5’-GTCGACTAAACTACCATCTTCAACCATTTGAGCC-3’). The fragment was cloned into pCAMBIA1300-3×FLAG vector (modified from pCAMBIA1300) and then introduced into heterozygous FLN2/fln2–4 plants by Agrobacterium tumefaciens-mediated transformation. The genomic background of these independent hygromycin-resistant transgenic plants was verified by PCR analysis with a primer of AtLB1 and the plant-specific primers (FLN2-F: 5’-CAGCTTCTGAGGCTGACTAAACTACCATCTTCAACCATTTGAGCC-3’; FLN2-R: 5’-CCAGAGGATCCACTTGCTTGCAGTTTCGCAAGCT-3’). These transgenic lines were examined Hitachi H7650 transmission electron microscopy (http://www.hitachi.com).

Chlorophyll Determination and Chloroplast Ultrastructure

Total chlorophyll content was measured according to the record as described earlier [41]. Arabidopsis leaf segments were from the cotyledons of 7-day-old fln2–4 plants grown with or without sucrose and the true leaves of 14-day-old mutants grown on sucrose-containing medium. Transmission electron micrographs were obtained exactly as described earlier [42]. The specimens were examined Hitachi H7650 transmission electron microscopy (http://www.hitachi.com).

RNA Isolation, cDNA Synthesis, RT-PCR Analysis and Quantitative Real-Time RT-PCR

Procedures for the purification of total RNAs for cDNA synthesis, RT-PCR, and qRT-PCR were performed as previously described [41]. The specific primers used to quantify the expression of FLN2 were as follows: sense primer, 5’-ATGGCTGCTGGTAGGAAAG-3’; antisense primer, 5’-
TCATAA ACTACCATTCAAA-3'. The β-tubulin was used as the internal standard for qRT-PCR analysis.

**Northern Hybridization**

Approximately 10μg total RNAs of each sample were separated on a 1.5% formaldehyde-agarose gel, transfomed onto a nylon membrane (Fall, Mexico) and hybridized with the specific probes. The probes were synthesized with a PCR amplification-DIG-labeling kit. Sequence data for the PCR primers performed in this study can be found in previous report [22]. Chemiluminescent detection was carried out according to the Roche manual (Roche, http://www.roche.com).

**Yeast Two-Hybrid Assays**

Yeast two-hybrid techniques were performed as described [22]. The following gene-specific primers were used: the FLN1 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-CGCGGCGATGGCTTCTCCACTTAATGGGACGACG-3' and 5'-TCGACGATGACATGGCTGCTGGTAG-3', respectively. The FLN2 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-GGATCCTTATTACTTCAATATTGTTGCAGC-3' and 5'-GTCGACCTACCACATTGATGGAACATA-3', respectively. The FLN1 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-GGATCCTTAATGGGACGACG-3' and 5'-GTCGACCTACCACATTGATGGAACATA-3', respectively. The FLN2 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-GGATCGCC ATGGCTGCTGG-3' and 5'-GGATCCTTATAAGTTTTTTGCCGTCG-3', respectively. The FLN1 and FLN2 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-CATATGATGTGCTTCTCCACTCAAAATC-3' and 5'-GGATCCTTAATGGGACGACG-3', respectively. The FLN1 and FLN2 gene cloned into pGADT7 vector were amplified using primers 5'-CATATGATGTGCTTCTCCACTCAAAATC-3' and 5'-GGATCCTTAATGGGACGACG-3', respectively.

**Protein Expression and GST Pull-Down Experiment**

The full-length FLN1 and FLN2 lacking the N-terminal transit peptide sequence were cloned into pGEX-4T-1 (GE Healthcare, http://www.roche.com). The primers used to amplify for peptide sequence were cloned into pGEX-4T-1. The primers for isolation of FLN1 without transit sequence were as follows: 5'-CCGGGCGATGGCTTCTCCACTTAATGGGACGACG-3' and 5'-CTGACGATGACATGGCTGCTGGTAG-3', respectively. The FLN2 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-GGATCCTTAATGGGACGACG-3' and 5'-GTCGACCTACCACATTGATGGAACATA-3', respectively. The FLN2 gene cloned into pGADT7 and pGBK7 vector were amplified using primers 5'-GGATCCTTATAAGTTTTTTGCCGTCG-3' and 5'-GGATCCTTAATGGGACGACG-3', respectively. The primers for FLN1 and FLN2 can be found in previous report [22].

**Supporting Information**

**Figure S1** Loss of ptac5 causes a heat-sensitive phenotype. (A) Gene structure of At4g13670 showing the T-DNA insertion site of the SALK_049133. White boxes represent exons; thin lines indicate introns. Sequences of primers used for isolation of homozygous lines were indicated as follows: At4g13670-specific primer 5'-TTAAAGGAAGCTTGAATGGGGG-3' and 5'-TCTTTTCTTCTAGAAATATATGCC-3'. (B) The expression of ptac5 in wild type and ptac5 by semiquantitative reverse transcriptase (RT)-PCR analysis. The β-tubulin was used as control. Primers used for RT-PCR analysis were as follows: β-tubulin specific primers: 5'-GATTTCAGATGATGGGTAGA-3', 5'-GTTCTGAAGCAATTGTCATAGAG-3'; ptac5-specific primer 5'-CATATGATGTGCTTCTCCACTCAAATC-3' and 5'-GGATCCTTAATGGGACGACG-3'. Phenotype of ptac5 mutants. Top panels show growth phenotype of ptac5 mutants grown on MS medium for 7 days at 22°C compared with WT. Bottom panels show phenotypes of WT and ptac5 seedlings after 7 days at 28°C. (TIF)

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**Author Contributions**

Conceived and designed the experiments: CH QBY. Performed the experiments: CH QBY. Analyzed the data: CH QBY RHL QQY GYC. Contributed reagents/materials/analysis tools: CH QBY QQY ZNY. Wrote the paper: CH QBY ZNY.

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