Arabidopsis FHY3/CPD45 regulates far-red light signaling and chloroplast division in parallel

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Plants adapt their growth according to changing environmental conditions, and one of the most important environmental inputs is light. Light not only provides energy for plants via photosynthesis, but also functions as a signal that regulates plant growth and development. Five types of light receptors have been reported in plants, namely phytochromes (phy)1, cryptochromes2, phototropins3, Zeitlupe4 and UVR85. Five phy species exist in Arabidopsis thaliana, phyA to F1–7. PhyA, a red light (620–700 nm) and far-red light (700–800 nm) sensor, is imported into the nucleus upon light illumination, with the help of FHY1 (far-red elongated hypocotyl3). FHY1 is a key factor in the far-red light signaling pathway in Arabidopsis. We previously showed that FHY3/CPD45 also regulates chloroplast division. Because light is also a regulator of chloroplast development and division, we sought to clarify the relationship between far-red light signaling and chloroplast division pathways. We found that the chloroplast division mutant arc5-3 had no defect in far-red light sensing, and that constitutive overexpression of ARC5 rescued the chloroplast division defect, but not the defect in far-red light signaling, of cpd45. thy1, which is defective in far-red light signaling, exhibited normal chloroplast division. Constitutive overexpression of FHY1 rescued the far-red light signaling defect, but not the chloroplast division defect, of cpd45. Moreover, ARC5 and FHY1 expression were not affected in thy1 and arc5-3, respectively. Based on these results, we propose that FHY3/CPD45 regulates far-red light signaling and chloroplast division in parallel by activating the expression of FHY1 and ARC5 independently. This work demonstrates how relationships between different pathways in a gene regulatory network can be explored.
FHY3/CPD45 regulates chloroplast division and far-red light signaling by activating the expression of ARC5 and FHY1 in parallel.

**Results**

Constitutive overexpression of FHY1 rescued the far-red light sensing defect, but not the chloroplast division defect, of cpd45. To establish whether the effect of FHY3/CPD45 on chloroplast division is mediated by FHY1 and the far-red light signaling pathway, we constitutively overexpressed 35S-FHY1 in the cpd45 mutant background (35S-FHY1;cpd45). After incubating seeds of various genetic backgrounds for four days under far-red light, we measured the hypocotyl lengths of the young seedlings. The hypocotyls of 35S-FHY1;cpd45 transgenic plants (2.625 mm) were significantly shorter than those of the cpd45 mutant (13.336 mm), but very close to those of the wild type (2.772 mm; Figures 1a, b, c and 2a). However, as observed in the cpd45 mutant, the chloroplasts of 40-day-old 35S-FHY1;cpd45 plants grown under white light were larger in size and reduced in number relative to the wild type (Figure 3a, b and c). We quantified the severity of the chloroplast phenotype using a best-fit linear plot of chloroplasts per cell versus cell area (Figure 4). The 35S-FHY1;cpd45 plants have a slope of 0.0048, which is similar to that of cpd45 (0.0059) and very different from that of the wild type (0.0127; Figure 4). These results suggest that the constitutive overexpression of FHY1 can rescue the far-red light sensing defect of the cpd45, but not the chloroplast division defect.

Interestingly, seedlings of 35S-FHY1;cpd45 were stronger than the others both in far-red light or the dark (Figure 1 and S1). Their cotyledon size was approximately twice as large as other plants. We measured the cotyledon size of the seedlings of various genetic backgrounds grown in the dark or far-red light. However, the response to far-red light of the seedlings of 35S-FHY1;cpd45 was similar to that of other plants and the cotyledon size was induced more than two times by far-red light (Figure S1).

Constitutive overexpression of ARC5 rescues the chloroplast division defect, but not the far-red light signaling defect, of cpd45. To investigate whether the far-red light signaling defect of cpd45 is caused by the chloroplast division defect, we transformed 35S-GFP-ARC5 into the cpd45 mutant. After 40 days of growth under white light, we observed the chloroplast division phenotype of leaf mesophyll cells using a microscope. We found that constitutive overexpression of GFP-ARC5 rescued the chloroplast defects of cpd45 (Figure 3d), because the chloroplast phenotype of GFP-ARC5;cpd45 transgenic plants was very similar to that of the wild type. In our best-fit linear plot of chloroplasts per cell against cell area, the slope of GFP-ARC5;cpd45 (0.0115) was higher than that of cpd45 (0.0059), and close to that of the wild type (0.0127; Figure 4). In contrast, the hypocotyls of GFP-ARC5;cpd45 transgenic plants grown under continuous far-red light was 12.190 mm long, more...
than four times as long as those of the wild type (2.772 mm) and close in length to those of cpd45 (13.336 mm; Figures 1a, b, d and 2a). Thus, 35S-GFP-ARC5 rescues the chloroplast division defect of cpd45, but not the far-red light signaling defect.

We next performed an immunoblot analysis to determine the expression level of the constitutively overexpressed GFP-ARC5 in the GFP-ARC5;cpd45 transgenic line and to compare this value with that of ARC5 in the wild type (Figure 5). We detected a band of around 100 kD in size in the wild type. This band was absent in arc5-3 and very faint in cpd45, suggesting that it corresponded with ARC5. GFP-ARC5 yielded a band of 130 kD, as expected. Based on our rough estimation, the expression level of GFP-ARC5 was at least five times greater than that of ARC5 in the wild type.

Mutations in FHY1 and ARC5 do not affect the expression of each other. We next performed semi-quantitative RT-PCR experiments to further characterize the relationship between FHY1 and ARC5 (Figure 6). To avoid saturation of the PCR amplification and for better comparison of the expression levels of the genes, the cDNA used in the experiment was serially diluted three times with a dilution ratio of 4. HTA9, which is a constitutively expressed gene, was used as a control. The expression level of FHY1 in arc5-3 was similar to that of the wild type.

![Figure 3](image3.png)

**Figure 3** Chloroplast division phenotypes of mesophyll cells with different genetic backgrounds. (a) Wild type. (b) cpd45 mutant. (c) cpd45 mutant harboring the 35S-FHY1 transgene. (d) cpd45 mutant harboring the 35S-GFP-ARC5 transgene. (e) fhy1 mutant. (f) arc5-3 mutant. Leaf tissue was sampled from 40-day-old plants. Scale bar represents 10 μm.

![Figure 4](image4.png)

**Figure 4** Graph of chloroplast number relative to cell area of 40-day-old plants. The best-fit lines have slopes of 0.0127 ($R^2 = 0.8701$), 0.0141 ($R^2 = 0.8069$), 0.0115 ($R^2 = 0.7408$), 0.0059 ($R^2 = 0.2894$), 0.0048 ($R^2 = 0.4459$), and 0.0003 ($R^2 = 0.0554$) for the wild type (WT), fhy1, GFP-ARC5;cpd45, cpd45, 35S-FHY1;cpd45, and arc5-3, respectively.
in the wild type, both in four-day-old seedlings grown under far-red light and in the leaves of 40-day-old plants grown under white light. Similarly, the expression level of ARC5 in fhy1 was similar to that of the wild type under the above two conditions. The fhy1 mutant used here (Salk_076131) has a T-DNA insertion in the first intron of FHY1 and disrupted gene expression (Figure S2). Thus, FHY1 and ARC5 do not regulate each other’s expression.

fhy1 is defective in far-red light signaling but has a normal chloroplast division phenotype. We measured the hypocotyl lengths of fhy1 seedlings grown under far-red light or in the dark for 4 days and analyzed the chloroplasts of fhy1 plants grown under white light for 40 days. Our results showed that the hypocotyl length of fhy1 under far-red light was 8.432 mm, a little more than three times that of the wild type (2.772 mm) (Figures 1a, e and 2). When grown in the dark, the hypocotyl length of fhy1 was 12.72 mm (Figure 2a). fhy1 had a chloroplast division phenotype similar to that of the wild type (Figure 3a, e). In the best-fit linear plot of chloroplasts per cell against cell area, the slope of fhy1 was 0.0141, quite close to that of the wild type (0.0127; Figure 4). This suggests that FHY1 plays an important role in the far-red light signaling pathway, but not in chloroplast division or in the regulation of this process.

arc5-3 has an abnormal chloroplast division phenotype and a normal hypocotyl phenotype in far-red light. We measured the hypocotyl lengths and analyzed the chloroplast phenotype of the null allele, arc5-3, under the same growth conditions as above. arc5-3 exhibited a strikingly abnormal chloroplast phenotype, with very large and very few chloroplasts (Figure 3f). The arc5-3 mutant yielded a slope of 0.0003 in our best-fit linear plot of chloroplasts per cell against cell area (Figure 4), which was much smaller than that of cpd45 (0.0059) (Figure 4). By contrast, the hypocotyls of the arc5-3 mutants were of normal length (2.817 mm, versus 2.772 mm in the wild type; Figure 1a, f and Figure 2a). Thus, ARC5 is an important factor in the process of chloroplast division, but not in the far-red light signaling pathway.

Discussion

FHY3/CPD45 is required for the expression of both FHY1 and ARC5. Previous studies showed that FHY1 is an important factor in the far-red light signaling pathway. If FHY3/CPD45 regulated ARC5 and chloroplast division through FHY1 and the far-red light signaling pathway, the chloroplast division defect of cpd45 would be rescued when FHY1 expression was recovered. However, our results showed that the constitutive expression of FHY1 only rescued the far-red light signaling defect of cpd45, but not the chloroplast division defect (Figures 1c and 3c). This suggests that FHY1 does not function in the chloroplast division pathway. Furthermore, our study showed that fhy1 had elongated hypocotyls under far-red light conditions, but that its mature leaves had a normal chloroplast division phenotype (Figures 1e and 3e), also suggesting that FHY1 does not function upstream of ARC5. Thus, FHY3/CPD45 regulates chloroplast division via a pathway that is distinct from far-red light signaling.

Furthermore, it was also unclear whether FHY3/CPD45 regulated far-red light signaling through the chloroplast division pathway or whether ARC5 and chloroplast division would affect far-red light signaling. The null allele of ARC5, arc5-3, exhibited a very severe chloroplast division phenotype (Figures 3f and 4), but its response to far-red light was similar to that of the wild type (Figures 1f and 2). This indicates that ARC5 is an important factor in chloroplast division, but not in the far-red light signaling pathway. Moreover, a transgene of GFP-ARC5 driven by the 35S promoter only rescued the chloroplast division defect of cpd45 (Figure 3d), but not the far-red light signaling defect (Figure 1d). These results suggest that ARC5 does not function upstream of FHY1.

FHY1 has been shown to be required for the nuclear import of phyA and thus the far-red light signaling. As shown in Figures 1 and 2, the fhy1 mutant had a defect of far-red light sensing like cpd45. However, fhy1 seemed to be not completely blind to far-red light like cpd45, because the elongation of its hypocotyls was slightly inhibited by far-red light (Figure 2). FHL had been shown to have some redundancy with FHY1 for the nuclear import of phyA, but with a minor role. So, a small part of phyA could be imported into the nucleus with the assistance of FHL for far-red light sensing. Alternatively but unlikely, the T-DNA in fhy1 was inserted into an intron of FHY1 gene (Figure S2), so that there might be still a very small amount of correctly spliced mRNA.

Seeds of 35S-FHY1;cpd45 appeared to be stronger than the others both in far-red light or the dark (Figure 1 and S1). Especially, the cotyledon size appeared to be larger. 35S promoter is a constitutive and very strong promoter. It could cause an over-expression of FHY1. This might increase the nuclear import efficiency of phyA or other proteins and affect the development of plants.
In contrast to the complete absence of ARC5 in arc5-3, our immunoblot analysis suggested that there was still a low level of ARC5 in cpd45 (Figure 5). This is probably because the EMS-induced point mutation in cpd45 only caused an amino acid substitution in the encoded protein, resulting in a leaky mutation25. Therefore, there is still some trace activity of GFP-ARC5 and a very slight activation of ARC5 expression in cpd45. Alternatively, cpd45 may have totally lost its activity and the ARC5 expression may be due only to basal expression. The lack of ARC5 in arc5-3 could be due to the incorrect splicing of the pre-mRNA, which introduced a premature stop codon25. Regardless, the low level, but not the absence, of ARC5 in cpd45 can explain why its chloroplast division defect is not as severe as that of arc5-3.

Our results also suggest that the constitutive overexpression of GFP-ARC5 can rescue the chloroplast division defect in cpd45 (Figure 3d). The level GFP-ARC5 in the overexpressing plants is at least five times greater than the level of ARC5 in the wild type (Figure 5). However, previous studies have shown that overexpression of a chloroplast division protein, such as PtoZ, MinD, or MinE, could inhibit chloroplast division30-32. This is probably because these proteins form a complex with a stringent stoichiometry and overexpression of one component causes misassembly of the complex and then faulty chloroplast division. The working mechanism of ARC5 may be different from that of other chloroplast division proteins.

Our gene expression analysis showed that expression of FHY1 or ARC5 was not substantially affected in the alternative mutant under both white light and far-red light conditions (Figure 6). This further suggests that these two genes do not function in the same pathway, i.e., that they do not function upstream or downstream of each other. Instead, they are both regulated by FHY3/CPD45 in parallel.

Many lines of evidence suggest that FHY3/CPD45 regulates circadian rhythm in Arabidopsis30-35. For instance, FHY3/CPD45 regulates the expression of ELF4 (Early Flower 4) and plays a dominant role in the CCA1/LHY-TOC1 circadian clock feedback circuit36-37, by binding to the ELF4 promoter, and thereby regulates the biological clock of plants35.

Collectively, the information presented in this and previous studies suggest that FHY3/CPD45 is a crnoode in the gene regulatory network, which links pathways of photomorphogenesis, circadian rhythm, chloroplast division and others. Therefore, we propose a genetic model of FHY3/CPD45-mediated gene regulatory pathways (Figure 7). In this model, FHY3/CPD45 is an important regulator of many genes that controls chloroplast division, far-red light signaling, and the circadian rhythm through regulating the expression of ARC5, FHY1, and ELF4, respectively. As an important transcription factor, FHY3/CPD45 may also regulate the expression of many other genes and govern many other biological pathways.

This work has finally clarified that the relationship between FHY1 and ARC5 in the gene regulatory network is parallel. It is well known that light signals are essential regulators of circadian rhythm and many other biological processes. Therefore, future studies should investigate the relationship between the far-red light signaling pathway and circadian rhythm and other pathways. Such studies may reveal a complex gene regulatory network mediated by FHY3/CPD45.

**Methods**

**Plant materials.** All Arabidopsis thaliana plants used in this study were in the Columbia-0 etymology background. arc5-3 is a mutant allele of ARC5, and cpd45 is a mutant allele of FHY335. phyA (Salk_076131) is a T-DNA insertion mutant of PHY1.

**Growth conditions.** Sterilized seeds were plated on 1/2 MS medium with 1% sucrose and 0.8% agar. After stratification at 4°C for 2 days, the plates were placed vertically under continuous far-red light (720 – 740 nm, 10 μmol·m⁻²·s⁻¹) or in the dark for 4 days without photoperiod at 22°C as before Ref. 25. Soil-grown plants were grown from seed in a growth chamber under white light (90 – 120 μmol·m⁻²·s⁻¹) with 40 – 60% relative humidity at 22°C and a photoperiod of 16-h-light/8-h-dark.

**Measurement of hypocotyl length and cotyledon size.** Plates were removed from the far-red light or dark box after 4 days of growth. Images were first taken with a dissection microscope and a USBS2.0 digital camera (Changheng, Beijing) and then the hypocotyl length and cotyledon size (area) were measured with the software Image Analysis System 10.0 (Changheng, http://www.crisoptical.com/lm2_41_344.htm). The sample size was 30.

**Leaf fixation and chloroplast phenotype analysis.** To characterize the chloroplast phenotype, leaf tissues from 40-day-old plants were fixed in an Eppendorf tube with 3.5% glutaraldehyde for 1 h in darkness, the glutaraldehyde was then replaced with 1 M Na₂EDTA (PH 7.4) and the tube was placed in a water bath of 55°C for 2 h. Images were taken with a USBS2.0 digital camera (Changheng, Beijing) coupled to an Olympus CX21 microscope (Olympus, Tokyo). The chloroplast division phenotype was quantified as described previously35. Thirty mesophyll cells of each group were used for the quantification.

**Identification of an fhy1 T-DNA insertion mutant, Salk_076131.** The fhy1 T-DNA insertion mutant Salk_076131 was obtained from the Arabidopsis Biological Resource Center. The sequence flanking the T-DNA insertion site was amplified using primers LBa1 (5’- TGG TTC ACG TAG TGG GCC ATC G -3’) and RP (5’- TGG TAG GCT TCT TTG TCT CAT G -3’) and then inserted into 3302Y3 (Figure 7). A simplified genetic model of FHY3/CPD45-mediated gene regulatory pathways. CPD45 (also known as FHY3) regulates the expression of many genes and is an important regulator of chloroplast division, the far red-light signaling pathway, circadian rhythm, and other biological processes. Arrows indicate genes that are directly regulated by FHY3/CPD45 and the pathways in which they are involved, dotted arrows indicate possible pathways that remain to be confirmed, and dotted lines represent unknown components or factors and steps not indicated.

**Plasmid construction.** FHY1 were amplified from the genomic DNA of wild-type plants using primers 5’- GCC ATC TAT GCC TGA AGT G-3’ and 5’-CGA CCA TGG GAT ACT CTT GAA CAC AAG ATT GG -3’ and then inserted into 3302Y3 using primers LBa1 (5’- TGG TTC ACG TAG TGG GCC ATC G -3’) and RP (5’- TGG TAG GCT TCT TG TCT CAT G -3’) with 40% 3’- 5’-GCC CAT GAC ACC GAA TGCC ATG G -3’ and 5’-GCC TTC GCA ACT GCT AT A CA C -3’.

**RNA extraction and semi-quantitative RT-PCR analysis.** RNA was isolated from seedlings grown on plates under far-red light for 4 days or from the leaves of 40-day-old plants grown in soil under white light, using an RNApure Total RNA Isolation Kit (Aidlab, Beijing) and reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). Semi-quantitative RT-PCR analysis was performed as before Ref. 38. The cDNA templates used for PCR were serially diluted three times at a dilution ratio of 4. FHY1 was amplified with the primers 5’-GCC ATC TAT GCC TGA AGT G-3’ and 5’-GAG TAG TAC AT TGG TCT GAG TCC GAG CCG TGG AGT GCT CAG TAC G -3’ and 5’-CCA CTC GAC GTA CAA CTT GGA TTC GAC ACC GAA TGCC ATG G -3’ and 5’-GCC TTC GCA ACT GCT AT A CA C -3’.
Protein purification, antibody preparation and immuno-blots analysis. The pET30a-ARCS plasmid was isolated and transformed into the Rosetta strain of *E. coli* for protein expression. Bacterial cultures were incubated at 37 °C until the OD600 reached 0.4–0.6. Then, 1 mM IPTG (isopropyl β-D-thiogalactoside) was added to the culture to induce the expression of ARCS. Bacterial cells were lysed by ultra-sonication and the induced ARCS protein was purified using Ni Sepharose 6 Fast Flow (GE Healthcare). The polyclonal and monoclonal antibodies against ARCS were raised in mouse. For immuno-blots analysis, proteins were separated by SDS-PAGE and transferred to Immob-Blot PVDF membrane (Bio-Rad). Blots were probed with anti-ARCS mouse monoclonal antibodies at a dilution of 1: 1000 for 1 h. Secondary antibodies of the Goat anti-Mouse IgG-conjugated HRP (1: 5000 dilution) were then used.

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Author contributions

N.C., Y.G. and X.L. performed the experiments. N.C., Y.G. and X.L. prepared the manuscript. H.G. provided advice on the experiments and improved the manuscript.

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