Supporting Information

Fujimoto et al. 10.1073/pnas.1204464109

SI Materials and Methods

Plant Materials. Columbia-0 (Col), C24, and Landsberg erecta (Ler) ecotypes were used for analysis of the heterosis phenotype and the transcriptome. *flc-a* (SALK-092716), *flc-b* (SALK-003346), and *flc-3* mutants in Col and *flc-20* in C24 were used for examining the heterosis phenotype and flowering time. Plants were grown in a controlled environment (22 °C) under fluorescent lights (150–180 μmol photons·m^{-2}·s^{-1}) and a 16-h/8-h (day/night) photoperiod. Plants were grown in plastic dishes containing Murashige and Skoog (MS) (1) agar medium supplemented with 1.0% sucrose (pH 5.7), and at 18 d after sowing (DAS) they were transferred to soil. Flowering time was evaluated by counting rosette leaf numbers. For the inhibitor studies, seedlings were grown for 3 d on MS plates and transferred to MS plates with 0.5 μM or 1.0 μM norflurazon (Sigma-Aldrich).

It is important to have well-controlled conditions, because there are differences in growth and level of heterosis under different light intensities and temperature regimes. The parents and hybrids derived by reciprocal crosses between the parents were all grown on the same agar plate divided into two or four regions and compared on one plate using one line as a control.

The cotyledons in hybrids (3-DAS) open earlier than in the parents (4-DAS), but leaf numbers of the hybrids are the same as that of the parents at 7, 10, and 14 DAS, indicating that the developmental stage of each of the lines was similar following cotyledon opening.

Gene Expression Analysis. Total RNA was isolated from cotyledons, true leaves, and whole seedlings using the SV Total RNA Isolation System (Promega). From 500 ng total RNA, first-strand cDNA was synthesized using random primers by SuperScript III Reverse Transcriptase (Invitrogen). For tiling array analysis, total RNAs were isolated from more than 50 whole plants (hybrids at 3 DAS and parents at 4 DAS). For microarray analysis, total RNAs were isolated from three bulked whole plants without roots for both hybrids and parents at 10 DAS. Quantitative real-time (q) PCR was performed using a Rotor-Gene 3000 Real-Time Cycler (Qiagen). The cDNA was amplified using Platinum Taq DNA polymerase (Invitrogen). PCR conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Expression levels of genes were calculated relative to *FDH* or *IPP2* genes using the comparative quantification analysis method with Rotor-Gene 6 (Qiagen). Data presented are the average and SE from biological and experimental replications. Primer sequences are shown in Dataset S1, Table S13.

Tiling Array and Microarray Analyses. *Arabidopsis* Tiling 1.0R (Affymetrix) was used for whole-genome transcriptome analysis. Total RNA (1 μg) from whole plants of the F_1 hybrid between C24 and Col at 3 DAS and Col and C24 at 4 DAS was used for probe synthesis. Biotinylated cRNAs were synthesized using the GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix). Hybridization and scanning were performed according to the manufacturer's instructions. Two independent biological replicates were performed.

Arabidopsis ATH1 Genome Array (Affymetrix) was also used for transcriptome analysis. Total RNA (100 ng) from seedlings without roots at 10 DAS from Col, C24, and the F_1 hybrid between C24 and Col was used for probe synthesis. Biotinylated cRNAs were synthesized using the IVT Labeling Kit (Affymetrix). Hybridization and scanning were performed according to the manufacturer's instructions. Three independent biological replicates were performed.

Data were analyzed using R (http://cran.r-project.org) and Bioconductor packages affy, affxparser, and limma (http://www.bioconductor.org). Briefly, raw probe measures were background-subtracted using the fifth percentile of array intensities and log2-transformed. Data were normalized between samples using quantile normalization, and the mean gene expression value between replicates was used in subsequent fold-change comparisons. The midparent value (MPV) was calculated as the mean of parental expression for each probe in the case of the tiling array and for each probe set in the case of the genome expression array.

The tiling array analysis identified a total of 18,368 annotated genes showing above-background expression in any of the parent or hybrid samples. Between parental samples, 92,173 genic and 82,622 nongenic probes showed fold changes (≥1.5), whereas 170,693 genic and 131,578 nongenic probes showed fold changes between hybrid samples and MPVs. The *Arabidopsis* TAIR9 genome sequence (www.arabidopsis.org) and annotation was used to physically locate and annotate probe locations, and gene expression values were calculated as the median of all matching probes. Genes highlighted for further analysis were required to have at least 20 matching probes showing ≥1.5-fold change. The ≥1.5-fold–change threshold was chosen based on simulations that randomly sampled 10,000 sets of 20 consecutively mapped probes and their observed expression levels. The distribution of fold-change values between parent and hybrid for these sets suggested that a fold change of ≥1.5 could occur randomly in 2.6% of comparisons and a twofold change in 0.2% of comparisons. From the genome expression array, probe sets were analyzed for statistically significant differences in expression using the limma Bioconductor package and a threshold of $P \leq 0.01$ without correction for multiple testing.

Analysis for enrichment of gene functional ontology terms was completed using the gene ontology (GO) tool agriGO (2). The background reference for both the tiling and genome expression array analyses was the list of genes that displayed expression above-background in either the parental or hybrid samples from each platform. Statistical tests for enrichment of functional terms used the hypergeometric test and false discovery rate (FDR) correction for multiple testing to a level of 5% FDR.

Measurement of CO_2 Uptake Rates. Photosynthetic CO_2 fixation rate was examined by the light-dependent consumption of CO_2 (3). Plants were grown in plastic dishes containing MS agar medium with or without 1.0% sucrose (pH 5.7). One first or second rosette leaf at 10 and 14 DAS and several cotyledons at 7 DAS were used. A leaf was exposed to light at 200 or 1,000 μmol photons·m^{-2}·s^{-1} in 3.5% CO_2 at 25 °C. Leaf area was measured using ImageJ software (National Institutes of Health).

Chlorophyll Extraction and Quantification. Cotyledons at 7 DAS and first and second rosette leaves at 10 or 14 DAS were ground in 80% (vol/vol) acetone. Absorbance of the supernatants was measured at 646.6 and 663.6 nm, and concentrations of chlorophyll *a* and *b* were calculated using the following formulae: chlorophyll *a* (μg/mL) = 12.25 × A663.6 − 2.55 × A646.6; chlorophyll *b* (μg/mL) = 20.31 × A646.6 − 4.91 × A663.6.

Counting of Chloroplast Numbers and Determination of Palisade Cell Size. The number of chloroplasts in leaf cells from cotyledons at 7 DAS and first and second rosette leaves at 10 and 14 DAS were counted using Nomarski optics. Leaves were fixed with 3.5%

(vol/vol) glutaraldehyde and then incubated in 0.1 M Na_2-EDTA (pH 9.0) for 15 min at 50 °C (4). Cell area was measured using ImageJ software.

Cotyledons at 7 DAS, first or second rosette leaves at 10 or 14 DAS, third or fourth rosette leaves at 14 or 21 DAS, and fifth or sixth rosette leaves at 21 DAS were fixed in a formalin/acetic acid/alcohol solution and cleared in a chloral hydrate:water:glycerol (8:2:1) solution overnight. The samples were photographed under Nomarski optics and the palisade cell number per fixed unit area in the subepidermal layer of the center of the leaf blade between the midvein and the leaf margin was counted. The image of the whole cotyledon or leaf area was photographed under a stereoscopic microscope, and sizes were determined with ImageJ software. The images of vertical leaf sections obtained using a Microslicer (Nippon Medical and Chemical Instruments Co., Ltd.) were photographed under Nomarski optics. The thickness was determined with ImageJ software and thickness relative to Col calculated.

1. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15:473–479.
2. Du Z, Zhou X, Ling Y, Zhang Z, Su Z (2010) agriGO: A GO analysis toolkit for the agricultural community. *Nucleic Acids Res* 38(Web Server issue):W64–W70.
3. Takahashi S, Milward SE, Fan DY, Chow WS, Badger MR (2009) How does cyclic electron flow alleviate photoinhibition in *Arabidopsis*? *Plant Physiol* 149:1560–1567.
4. Okazaki K, et al. (2009) The PLASTID DIVISION1 and 2 components of the chloroplast division machinery determine the rate of chloroplast division in land plant cell differentiation. *Plant Cell* 21:1769–1780.

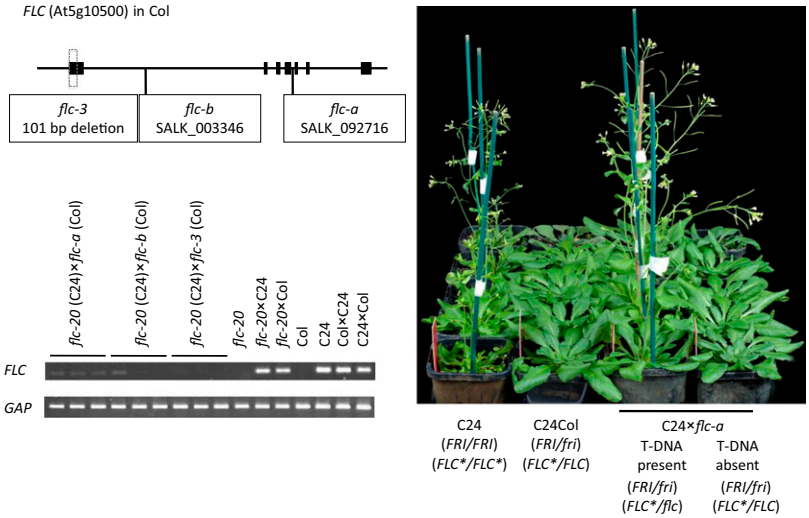

Fig. S1. The effect of the *flc* mutation on rosette diameter. A schematic representation of the positions of T-DNA insertions or deletions in the different *flc* mutants in Col is shown (*Upper Left*). Transcription level (RT-PCR) in the *flc* mutants is shown (*Lower Left*). Repression of *FLC* expression in *flc-20*, *flc-20* x *flc-a*, *flc-20* x *flc-b*, and *flc-20* x *flc-3* was confirmed. C24 x *flc-a* has an earlier flowering time than C24 x Col but similar biomass (*Right*).

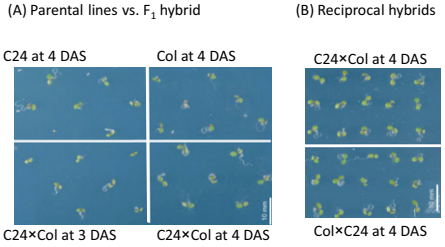

Fig. S2. Phenotypes of samples used in microarrays. (*A*) The parental lines are at 4 DAS and the F_1 hybrids are at 3 or 4 DAS. (*B*) The reciprocal hybrids, C24 x Col and Col x C24, are at 4 DAS.

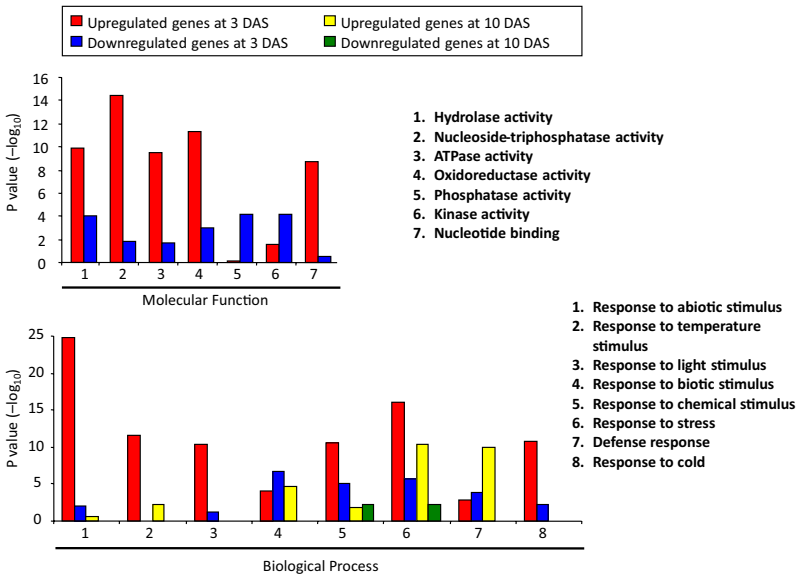

Fig. S3. GO classification of differentially expressed genes. Categories overrepresented in up- and down-regulated genes at 3/4 DAS (*Upper*) and in differentially expressed genes at 3/4 and 10 DAS (*Lower*) are shown. *x* axes are *P* value (−log10). The bar graphs were made from Dataset S1, Tables S5 and S6.

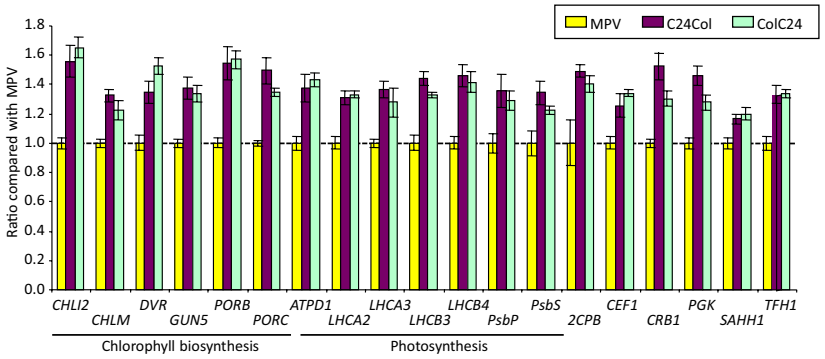

Fig. S4. Both reciprocal F_1 hybrids showed up-regulation of chloroplast-located genes at 3/4 DAS. Relative expression levels divided by the MPV of parental lines are shown. Expression levels of genes relative to the *FDH* gene were calculated using a comparative quantification analysis. Data presented are the average and SE from three biological and experimental replications. RNA samples for qPCR were different from those used for the tiling array analysis. RNAs for qPCR were isolated from ~50 bulked whole plants of Col and C24 at 4 DAS and Col x C24 and C24 x Col at 3 DAS. Error bars represent SE.

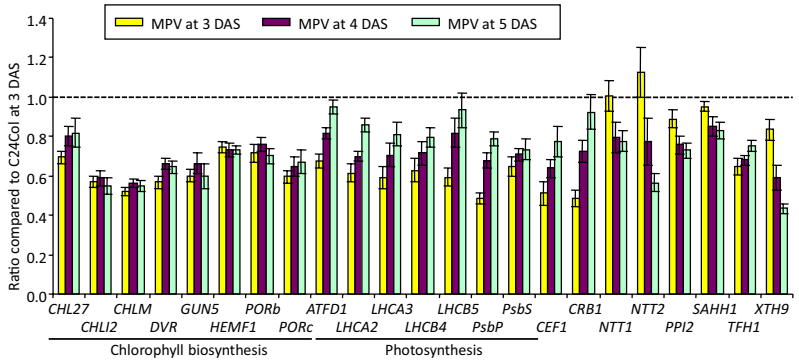

Fig. S5. One day's difference in growth did not affect the differential expression of genes. Expression levels were measured by qPCR. Relative expression levels divided by the expression level in the F_1 hybrid (C24 x Col) at 3 DAS are shown. Expression levels of genes relative to the *FDH* gene were calculated using a comparative quantification analysis. Data presented are the average and SE from three independent experiments with three biological and experimental replications. RNAs for qPCR were isolated from ~50, 30, and 15 bulked whole plants at 3, 4, and 5 DAS, respectively. Error bars represent SE.

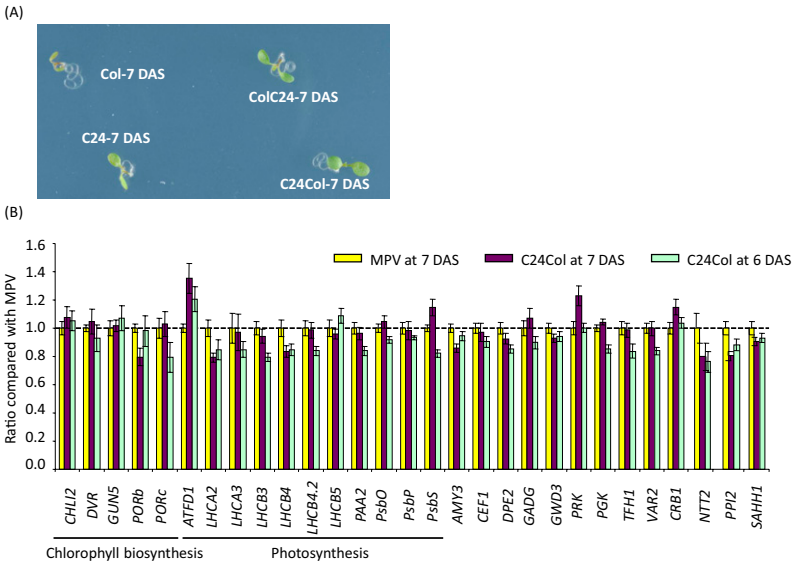

Fig. S6. Similar expression levels in parental lines and the F_1 hybrid at 7 DAS in genes differentially expressed at 3/4 DAS. (*A*) The plants at 7 DAS. (*B*) Expression levels were examined by qPCR. Relative expression levels were divided by the MPV. Expression levels of genes relative to the *FDH* gene were calculated using a comparative quantification analysis. Data presented are the average and SE from three biological and experimental replications. RNAs for qPCR were isolated from five bulked seedlings without roots in Col and C24 at 7 DAS and C24 x Col at 6 and 7 DAS. Error bars represent SE.

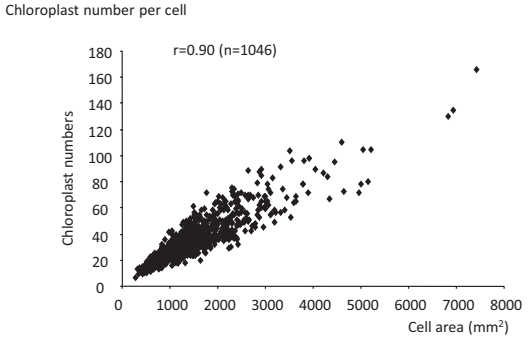

Fig. S7. The relationship between chloroplast number per cell and palisade mesophyll cell area. Chloroplast numbers per cell in Col, C24, and C24 x Col were counted from cotyledon cells at 7 DAS and first and second leaves at 10 and 14 DAS with five independent experiments with more than 10 cells from three independent leaves. All data at 7, 10, and 14 DAS in Col, C24, and C24 x Col were pooled. (r = 0.90, n = 1,046). Each correlation coefficient is as follows: 7 DAS: total, r = 0.92 (n = 229); Col, r = 0.85 (n = 73); C24, r = 0.92 (n = 80); C24 x Col, r = 0.95 (n = 76); 10 DAS: total, r = 0.89 (n = 428); Col, r = 0.95 (n = 134); C24, r = 0.90 (n = 137); C24 x Col, r = 0.87 (n = 157); 14 DAS: total, r = 0.91 (n = 389); Col, r = 0.93 (n = 122); C24, r = 0.88 (n = 131); C24 x Col, r = 0.93 (n = 136).

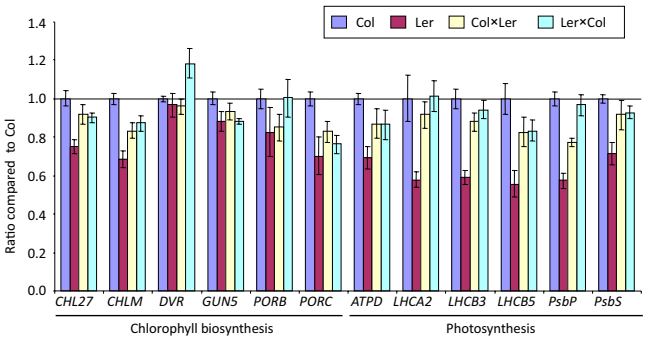

Fig. S8. Chlorophyll biosynthesis and photosynthesis genes are not up-regulated in the Col x L*er* or L*er* x Col hybrids. RNA samples from 10 bulked plants at 4 DAS (Col x L*er*) or 3-DAS Col x L*er* and L*er* x Col were analyzed for levels of gene expression using qPCR. Data presented are the average and SE from three biological and experimental replications.

Other Supporting Information Files

Dataset S1 (XLSX)