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Supplemental Information

Heme Synthesis by Plastid Ferrochelatase I Regulates Nuclear Gene Expression in Plants

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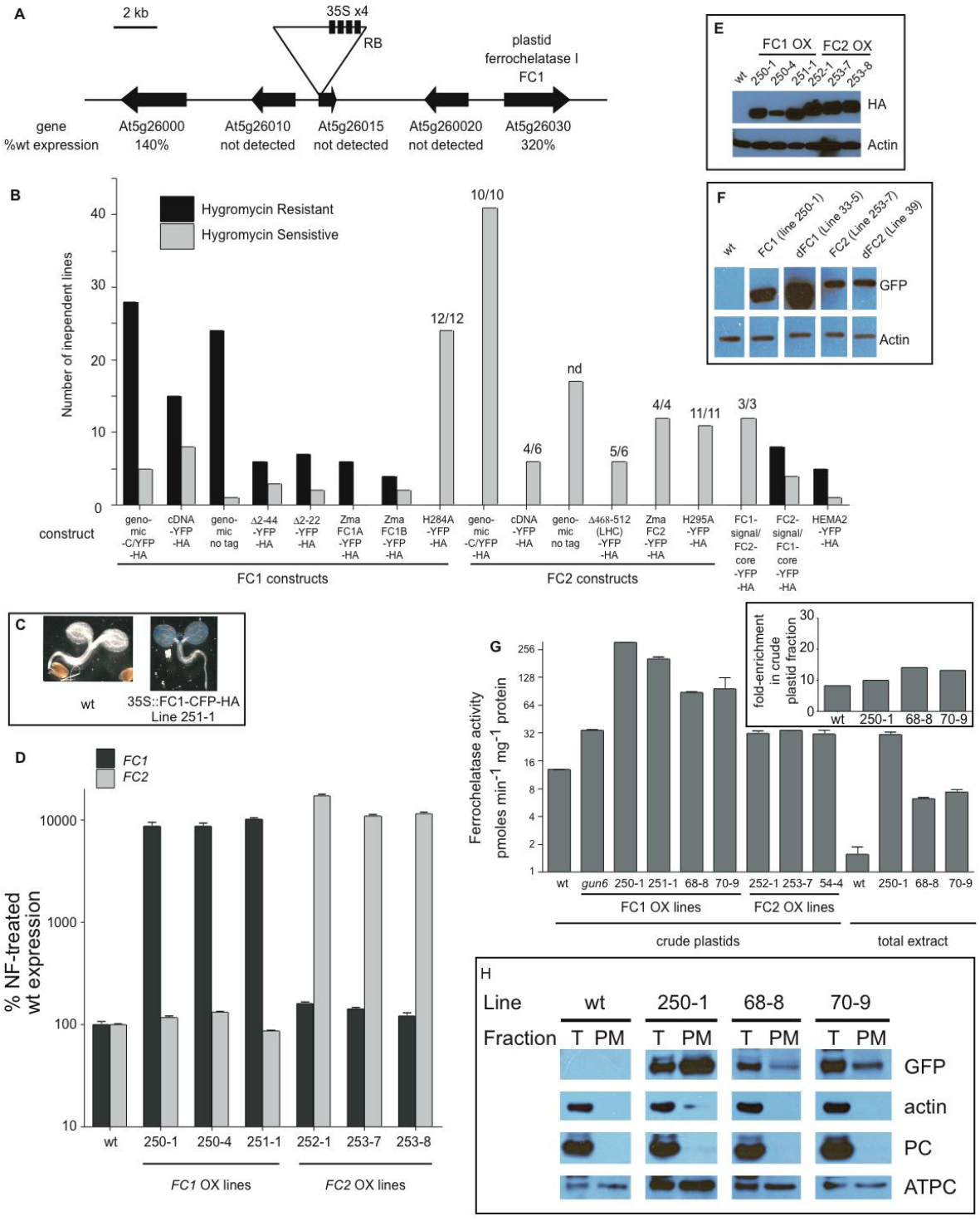
A
2 kb
35S x4
RB
plastid ferrochelatase I FC1
gene
%wt expression
At5g26000 140%
At5g26010 not detected
At5g26015 not detected
At5g260020 not detected
At5g26030 320%
B
Hygromycin Resistant
Hygromycin Sensistive
Number of inependent lines
construct
FC1 constructs
FC2 constructs
C
wt
35S::FC1-CFP-HA Line 251-1
D
FC1
FC2
% NF-treated wt expression
FC1 OX lines
FC2 OX lines
E
FC1 OX
FC2 OX
HA
Actin
F
GFP
Actin
G
Ferrochelatase activity pmoles min-1 mg-1 protein
fold-enrichment in crude plastid fraction
FC1 OX lines
FC2 OX lines
crude plastids
total extract
H
Line
Fraction
GFP
actin
PC
ATPC

Figure S1.

Figure S1. Phenotypes of *gun6-1D* and Other Ferrochelatase Transgenic Lines (Related to Figure 1)

(A) Schematic of the 24 kb region of chromosome five surrounding the causative T-DNA in *gun6-1D* mutants. Genes are indicated by arrow boxes that are labeled underneath. mRNA transcript levels (relative to wt) are indicated below. For genes At5g26010, At5g26015, and At5g26020 no transcript could be detected in either genotype. The right border of T-DNA (RB) still contained multimerized 35S enhancer elements.

(B) Shown is the distribution of *gun* mutants among the various stable transgenic *Arabidopsis* over-expression lines used in this study. The *gun* phenotype of these lines was assessed by scoring the growth phenotype of ~30 NF-treated T2 seedlings from independent T1 lines when challenged with hygromycin. All lines contain the pOCA107-2 [1] that contains a hygromycin resistance cassette under the control of a minimal *LHCB1.2* promoter. After seven days, a seedling with a *gun* phenotype will de-repress *LHCB,* induce hygromycin resistance, and will still be growing. Conversely, seedlings with a wt phenotype will have repressed *LHCB* expression, will not induce hygromycin resistance, and will die. Lines were scored as resistant (*gun* mutants) if at least 25% of the T2 seedlings were hygromycin resistant. The total number of lines is indicated on the y-axis. When constructs did not result in any lines with a *gun* phenotype, C/YFP expression was measured by confocal microscopy. The number of lines with protein accumulation (Lines with C/YFP expression/Total number of lines checked) are indicated above the bars. The use of *Zea mays* (Zma) genes is indicated.

(C) Histochemical staining of NF-treated seedlings with the *uidA* (*GUS*) gene under the control of the *LHCB1.2* minimal promoter. Blue color indicates tissue where LHCB promoter is de-repressed.

(D) Shown is the level of mRNA transcript accumulation in *FC1*- and *FC2*-OX lines. RT-qPCR analysis of steady-state mRNA transcript levels was measured in 7-day old NF-treated seedlings. Data shown are the mean +/- SEM of triplicate reactions of a representative experiment.

(E) Western blot analysis of ferrochelatase protein from overexpression lines and

(F) Catalytically inactive ferrochelatase protein. An equal amount of total protein was fractioned by SDS-PAGE and blotted. Filters were probed with antibodies specific for the proteins indicated to the right.

(G) Ferrochelatase activity in 5 day old NF-treated seedling (total and crude plastid) extracts. Activity was measured using the codeuteroporphyrin assay and calculated by determining the amount of substrate used min^{-1} mg^{-1} protein. Data shown are the mean +/- SEM of duplicate reactions of a representative experiment. The inset shows the fold-enrichment in crude plastid ferrochelatase activity compared to total extracts.

(H) Western blot analysis of enriched plastid membrane fractions from NF-treated seedlings; T, total protein; PM, plastid membrane fraction. Protein from Total (T) (15 ug) and Plastid Membrane (PM) (6 ug) fractions were fractioned by SDS-PAGE and blotted. Filters were probed with antibodies specific for the proteins indicated to the right. Plastocyanin (PC) and ATPC were used as controls for soluble and membrane-bound plastid protein content, respectively.

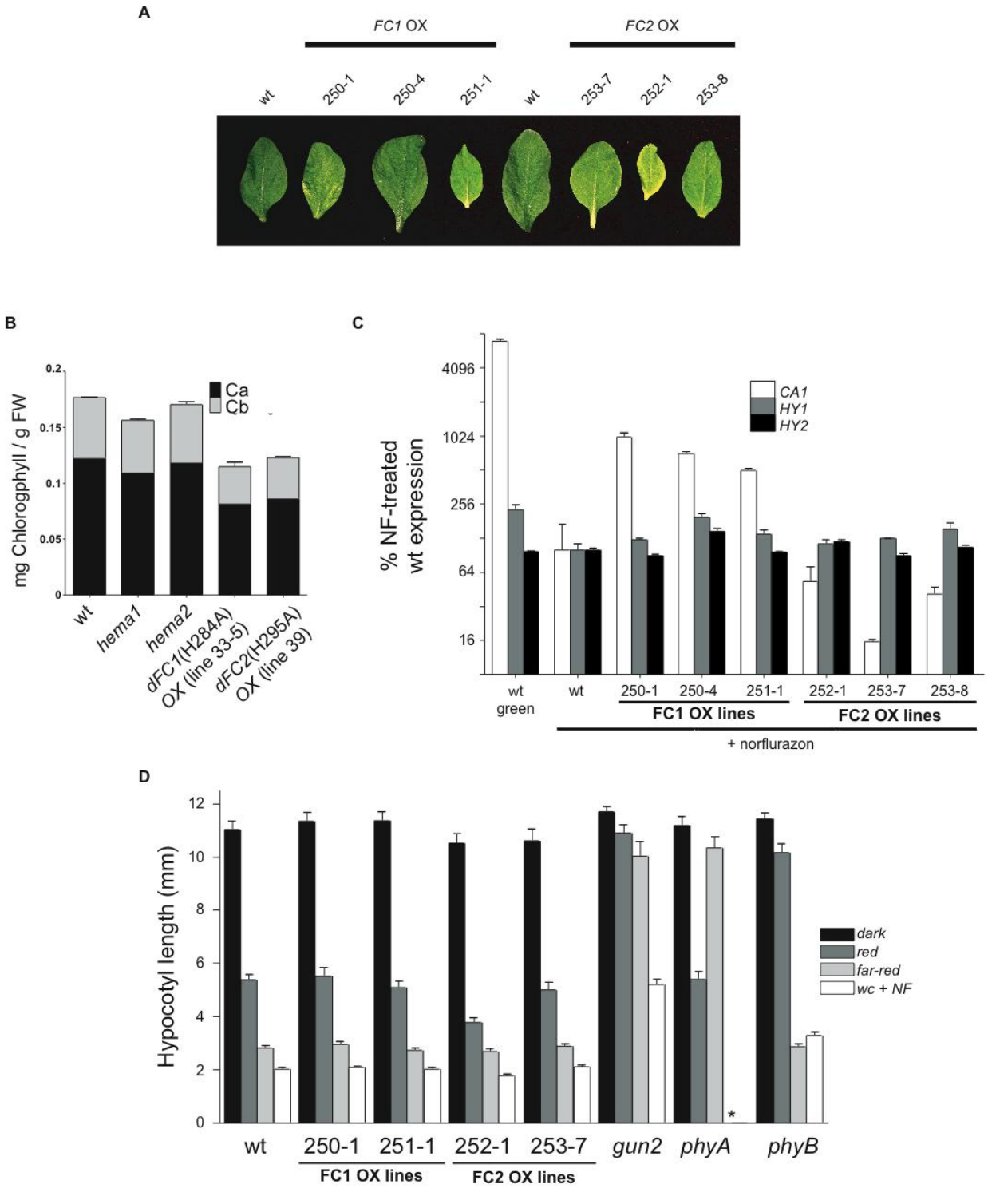
A
FC1 OX
FC2 OX
wt
250-1
250-4
251-1
wt
253-7
252-1
253-8
B
mg Chlorogphyll / g FW
Ca
Cb
wt
hema1
hema2
dFC1(H284A) OX (line 33-5)
dFC2(H295A) OX (line 39)
C
% NF-treated wt expression
CA1
HY1
HY2
wt green
wt
250-1
250-4
251-1
252-1
253-7
253-8
FC1 OX lines
FC2 OX lines
+ norflurazon
D
Hypocotyl length (mm)
dark
red
far-red
wc + NF
wt
250-1
251-1
252-1
253-7
gun2
phyA
phyB
FC1 OX lines
FC2 OX lines

Figure S2.

Figure S2. Effects of *FC1* and *FC2* Overexpression on the Tetrapyrrole Pathway (Related to Figure 2)

(A) Shown are the first cauline leaf of four-week-old *FC1* and *FC2* overexpression lines (indicated above the image).

(B) Chlorophyll levels of 3-day-old seedlings grown under cycling conditions (16 hours light/8 hours dark) of 65 μmol/m^2/s without sucrose supplementation. Data shown are the mean +/- SEM of biological duplicates.

(C) RT-qPCR analysis of steady-state *HY1* and *HY2* mRNA transcript levels of 7-day old NF-treated seedlings. Data shown are the mean +/- SEM of triplicate reactions of a representative experiment.

(D) Effect of light on hypocotyl elongation. Shown are the lengths of hypocotyls (mm) of mutants and *FC OX* lines after 4 days in continuous dark, red (1 μmol/m^2/s), far-red (1 μmol/m^2/s), or white light (wc) (7.5 μmol/m^2/s) at 22°C. Seedlings grown in white light were also treated with 5 μM NF and failed to green. $n \geq 13$ seedlings. Data shown are the mean +/- SEM of biological duplicates. *Not determined.

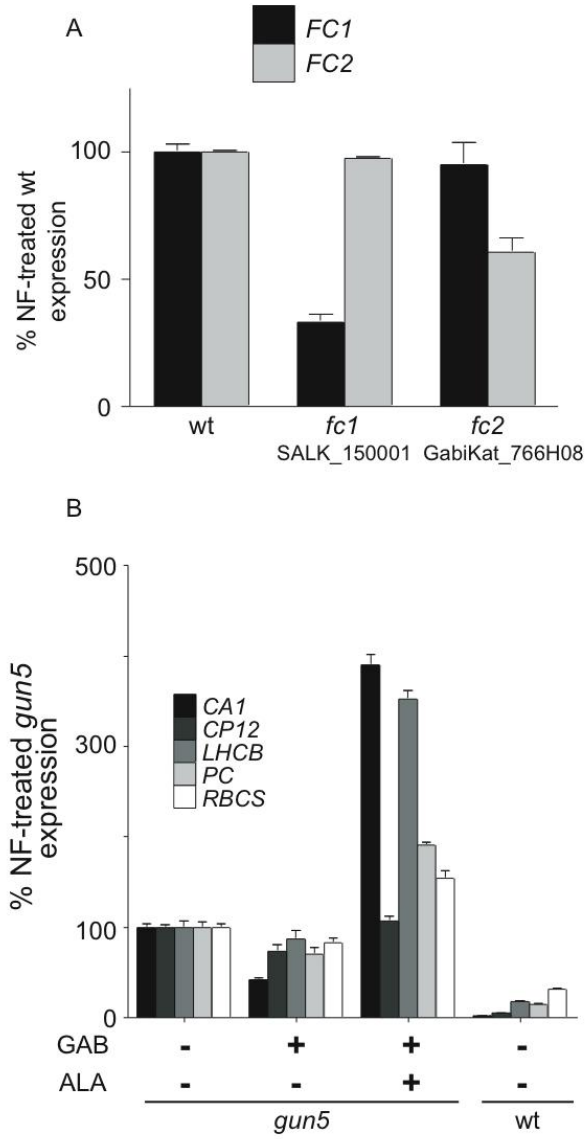

Figure S3. Analysis of Transcript Levels in NF-Treated Seedlings (Related to Figure 3)

RT-qPCR analysis of NF-treated seedlings.

(A) Steady-state *FC1* and *FC2* mRNA transcript levels in T-DNA knockdown lines.

(B) Six-day-old *gun5* seedlings were treated with 0.5 mM GAB and 1 mM ALA for 24 hr as indicated. Data shown are the mean +/- SEM of triplicate reactions of a representative experiment.

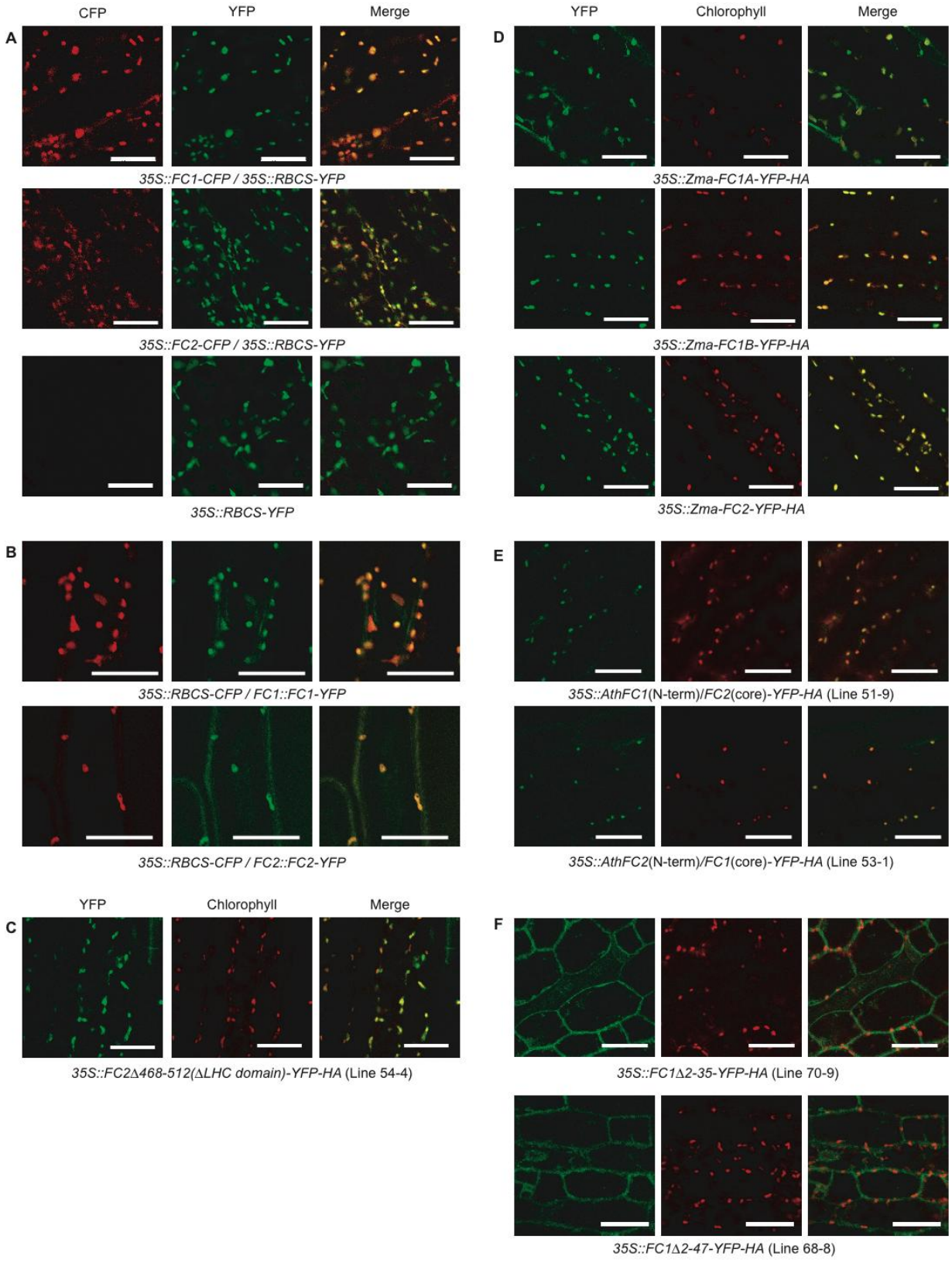
A
CFP
YFP
Merge
35S::FC1-CFP / 35S::RBCS-YFP
35S::FC2-CFP / 35S::RBCS-YFP
35S::RBCS-YFP
B
35S::RBCS-CFP / FC1::FC1-YFP
35S::RBCS-CFP / FC2::FC2-YFP
C
YFP
Chlorophyll
Merge
35S::FC2Δ468-512(ΔLHC domain)-YFP-HA (Line 54-4)
D
YFP
Chlorophyll
Merge
35S::Zma-FC1A-YFP-HA
35S::Zma-FC1B-YFP-HA
35S::Zma-FC2-YFP-HA
E
35S::AthFC1(N-term)/FC2(core)-YFP-HA (Line 51-9)
35S::AthFC2(N-term)/FC1(core)-YFP-HA (Line 53-1)
F
35S::FC1Δ2-35-YFP-HA (Line 70-9)
35S::FC1Δ2-47-YFP-HA (Line 68-8)

Figure S4.

Figure S4. Plastid Localization of Overexpressed and Natively Expressed FC1 and FC2 (Related to Figure 4)

(A) Overexpressed FC1- and FC2-CFP colocalizes with YFP fused to the tobacco RBCS N-terminal sorting signal [2].

(B) FC1- and FC2-YFP expressed by their respective native promoters colocalize with CFP fused to the tobacco RBCS N-terminal sorting signal.

(C-E) Overexpressed FC2-YFP-HA lacking the LHC domain (Δ468-512) (C), overexpressed *Z. mays* FC1A-YFP-HA, FC1B-YFP-HA, FC2-YFP-HA (D), and overexpressed hybrid *Arabidopsis* FC1-YFP-HA and FC2-YFP-HA proteins with swapped N-terminal sorting signals (E) are localized to plastids.

(F) Overexpressed FC1-YFP-HA lacking the N-terminal plastid localization signal peptide (Δ2-35/Δ2-47) is not localized to a single compartment. Plastid localization was confirmed by overlay of YFP signal with chlorophyll auto fluorescence using confocal microscopy. The yellow color in the digitally merged images indicates colocalization. Scale bars represent 30 μm.

Supplemental Experimental Procedures

Growth of Bacterial Strains

For cloning purposes, *E. coli* and *Agrobacterium tumefaciens* strains were grown in liquid Miller nutrient broth or solid medium containing 1.5% agar (w/v). Cells were grown at 37°C (*E. coli*) or 28°C (*A. tumefaciens*) with the appropriate antibiotics. Liquid medium was shaken at 225 rpm.

GUS Staining

Seven-day-old NF-treated seedlings were soaked in cold 90% acetone for 20 min and then vacuum infiltrated with 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 0.2 % (w/v) Triton X-100, in 500 mM sodium phosphate, pH 7.2. Tissue was incubated at 37°C for 24 hr before images were taken.

gun Activation-Tagging Mutant Screen

Flowering Col-0/107 plants were transformed with the pSKI015 plasmid containing multimerized Cauliflower Mosaic Virus (CaMV) 35 enhancer elements and a Basta herbicide selection marker [39]. Transformation was accomplished by dipping *Arabidopsis* flowers in a stationary culture of *A. tumefaciens* that had been washed in MS medium containing 5% sucrose and 0.03% Silwet L-77 (v:v) (Osi Specialities Inc.). Plants were then kept covered in the dark for 1 day before being returned to greenhouse conditions. Seeds were then harvested and sown on medium containing Basta to select for T1 generation transformants. T1 plants were then grown normally and seeds were collected from individual plants. About 30 T2 generation seeds from 20,000 individual T1 lines were germinated on medium containing 2% sucrose, 5 μM norflurazon, and 20 μg/ml of hygromycin. Seedlings were grown for seven days in 25 μ mol/m^2/s continuous light, and hygromycin-resistant seedlings were transferred to medium without drug for recovery. Once seedlings had greened, they were transferred to soil and seeds were collected for analysis. One candidate, called *gun6-1D* in this study, was isolated by this method and used for further study. 100% of T3 and F1 seedlings (backcrossed to wt) were hygromycin resistant during NF-treatment indicating a dominant gain-of-function mutation.

The *Arabidopsis* genome sequence flanking T-DNA's in the *gun6-1D* mutant were obtained according to [39]. Briefly, genomic DNA from *gun6-1D* mutant seedlings was extracted using the C-TAB method [40]. Total DNA was digested with either HinDIII, EcoRI, or BamHI restriction enzymes, ligated, and transformed into *E. coli*. Kanamycin resistant colonies were isolated and their plasmids were sequenced using T-DNA specific primers described in [39]. The location of T-DNA's was confirmed by PCR and sequencing to be inserted into gene At5g26015 replacing six bases upstream of its start codon and the first 68 bases of its coding region (Figure S1A).

Construction of Overexpression Plasmids

Plasmids and plant lines used in this study are listed in Supplemental Experimental Procedures. DNA fragments were amplified by using Phusion enzyme (Finnzymes Espoo, Finland) using either wt *Arabidopsis* genomic DNA or cDNA or wt *Z. mays* cDNA as template. The Primers used are listed in Supplemental Experimental Procedures. DNA fragments were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the Gateway compatible vector pENTR-D/TOPO (Invitrogen) according to the manufacturer's instructions.

Directed mutagenesis of *FC1* and *FC2* genes was achieved by designing anti-parallel primers with the desired nucleotide change near the center. Using plasmids pJDW5 (*FC1*) and pJDW7 (*FC2*) as templates, mutagenized plasmid was amplified using *pfu* turbo (Stratagene). Reactions were treated with *Dpn*I and plasmid was transformed into *E. coli*. Single colonies were selected and plasmids were sequenced to confirm the mutation was present.

To generate hybrid *FC1* and *FC2* genes, sequence encoding the FC1 N-terminus (amino acids 1-88), FC1 core (amino acids 89-466), FC2 N-terminus (amino acids 1-99), and FC2 core (amino acids 100-512) were amplified separately from cDNA with introduced in-frame BamHI sites at the 3' end of the N-terminal encoding fragments and the 5' end of the core protein encoding fragments. DNA fragments were digested with BamHI and ligated in the desired combinations. Flanking primer pairs were used to amplify the hybrid fragment that was gel-purified and cloned as described above.

The cloned DNA fragments were then transferred to the 35S overexpression vectors (pEARLEYGATE100-2) [41] or promoter-less vectors pGBGWC/Y [42] using LR clonase (Invitrogen) according to manufacturer's instructions. Plasmids were then transformed into the *A. tumefaciens* strain GV301 and these cells were used to transform *Arabidopsis*.

TargetP 1.1 program (www.cbs.dtu.dk/services/TargetP/) was used to predict that amino acid residue 35 is the cleavage site of the FC1 N-terminal plastid sorting signal. The two FC1 N-terminal deletion constructs used in this study begin at residues 36 and 48 in order to exclude the entire plastid sorting signal.

Heme Measurements

Total non-covalently-bound heme (normalized to fresh weight) was extracted from 7-day old seedling shoots by homogenizing liquid N_2-frozen tissue in acetone with 2% HCL (v:v) and cell debris was removed by centrifugation at 10,000 x g for 20 min at 4°C. Samples were diluted 1:100 in 100 mM Tris-Cl pH 8.4. Heme content was measured by the chemiluminescence-based method described by [38] that measures reconstituted activity from apo-horseradish peroxidase (Biozyme, Blaenovan, Wales). A Promega Glomax 96 microplate luminometer was used to measure luminescence.

Hypocotyl Measurements

After a five day vernalization period at 4°C, seeds were germinated by exposing to 75 $\mu mol/m^2/s$ white light at 22°C for 2 hr and then placed under the appropriate light conditions. After 4 days, at least 13 seedlings of each sample group was placed on a transparency sheet and scanned to a digital image file. Hypocotyl lengths were calculated using ImageJ 1.42q software (http://rsbweb.nih.gov/ij/).

Ferrochelatase Assay

Total protein extracts and crude plastid fractions were made from 5 day old NF-treated seedlings as described [43] except that plastids were pelleted at 4,000 x g to exclude other organelles. Ferrochelatase activity was monitored using the codeuteroporphyrin IX assay [43] that measures the aerobic synthesis of codeuteroporphyrin IX from cobalt and deuteroporphyrin IX (Frontier Scientific, Logan, UT). 1 mg of total protein was added to each reaction and deuteroporphyrin consumption was monitored over time by stopping the reaction with 30-fold dilution in 80% acetone (v:v) and measuring fluorescence in a Tecan Safire^2 fluorimeter. Excitation and

fluorescence wavelengths of 400 nm and 620 were used, respectively. Known concentrations of deuteroporphyrin IX dissolved in 80% acetone were used to calibrate the fluorimeter.

Protein Extraction and Western Blot Analysis

Seedlings were frozen in liquid N_2 and homogenized in extraction buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40 (v:v), and protease inhibitors (Roche). Cell debris was removed by centrifugation at 10,000 x g for 20 min at 4°C. Total protein concentration was measured using the Bradford assay (Bio-Rad) and 20 μg of each sample was loaded into a 4-20% SDS-PAGE gel and proteins were fractionated by electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad). Filters were incubated with specific primary and secondary antibodies. Detection was performed using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific Rockford, IL). Monoclonal HA and Actin antibodies were purchased from Roche (Basel, Switzerland) and MP Biomedicals (Solon, OH), respectively. Polyclonal plastocyanin (PC), ATPC, and Rubisco large subunit (RBCL) were purchased from Agrisera (Vännäs, Sweden). Polyclonal GFP antibodies were produced in our laboratory.

Plastid-enriched extracts were prepared from 200 mg of 5-day old NF-treated that were frozen in liquid N_2, homogenized, re-suspended in isolation buffer (10 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$, 400mM Sucrose, 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche)), and incubated for 10 min on ice. Cell debris was removed by centrifugation at 500 x g for 5 min at 4 °C and supernatant (total protein) was re-centrifuged (4,000g, 10 min). The resulting pellet, enriched in plastids, was washed with isolation buffer three times and re-suspended in lysis buffer (62.5 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$) to disrupt the chloroplasts. To ensure the rupture of thylakoids, samples were subjected to freeze-thaw cycles and centrifuged 75,000 x g for 45 min to separate supernatant (soluble fraction) and pellet (envelope and thylakoid membranes). To remove any possible residual soluble proteins from the membranes, the pellet was re-suspended in lysis buffer and an additional spin of 20 min at 75,000 x g was performed. The resulting pellet (plastid membrane fraction), was re-suspended in Lysis buffer containing 0.5% w/v Nonidet P-40. Protein from Total (15ug) and Plastid Membrane (6ug) fractions were separated by 4-12% SDS-PAGE, transferred to Nitrocellulose membrane (Bio-Rad) and immunoblotted with the specified antibodies.

***Arabidopsis* Mutants Used in This Study**

Mutant	Gene / protein	Mutation	notes	Ref
gun2-1	At2g26670 (*HY1*) / Heme oxygenase	EMS		[1]
gun4-1	At3g59400 / GUN4	EMS, L88F		[1]
gun5-1	At5g13630 (*CHLH*) / Mg-chelatase subunit H	EMS, A990V		[1, 3]
gun6-1D	increases expression of At5g26030 / Ferrochelatase 1	Activation tag T-DNA	dominant *gun* mutant	This study
lin2	At1g03475 (*HEMF1*) / Coproporphyrinogen III oxidase	T-DNA, 5'-UTR		[4]
fc1 (SALK_150001)	At5g26030 / Ferrochelatase 1	T-DNA, 5'-UTR		[5]
fc1 (GabiKat_110D02) null mutant	At5g26030 / Ferrochelatase 1	T-DNA, exon 3/8	Unable to obtain homozygotes	This study
fc2 (GabiKat_766H08)	At2g30390 / Ferrochelatase 2	T-DNA, 5'-UTR		This study
hema1 (SALK_056220)	At1g58290 / Glutamate tRNA reductase 1	T-DNA, 5'-UTR		This study
hema1 (SALK_053036) null mutant	At1g58290 / Glutamate tRNA reductase 1	T-DNA, exon 3/3	homozygous is seedling lethal	This study
hema2 (SALK_052000)	At1g09940 / Glutamate tRNA reductase 2	T-DNA, exon 3/3		This study

Primers Used in This Study

Gene	Primer orientation/name	Sequence
RT-qPCR primer pairs		
18S rRNA At3g41768	For. / JP190	TATAGGACTCCGCTGGCACC
	Rev. / JP191	CCCGGAACCCAAAAACTTTG
Actin2 At3g18780	For. / JP199	GCACTTGCACCAAGCAGCAT
	Rev. / JP200	CCTTTCAGGTGGTGCAACGAC
LHCB 1.2, CAB3 At1g29910	For. / JP197	GGACTTGCTTTACCCCGGTG
	Rev. / JP198	TCGGTAGCAAGACCCAATGG
CA1 AT3g01500	For. / JP209	TGTGTCCATCACACGTTCTGG
	Rev. / JP210	GGACCACGAAGGCATCTCCT
RBCS At1g67090	For. / JP205	GCAACGGCGGAAGAGTTAAC
	Rev. / JP206	TCTTTCCAATCGGAGGCCA
Plastocyanin At1g76100	For. / JP203	TGGTGTTCGACGAAGACGAG
	Rev. / JP204	AGATCTTGCTTGCGTCCACA
CP12 At3g62410	For. / JP271	CGGACCCTTTGGAGGAATACTG
	Rev. / JP272	GGCACTCGTTGGTCTCAGGA
Hema1 At1g58290	For. / JP254	GCTTCCGCAGTCTTCAAACG
	Rev. / JP255	CCAGCGCCAATTACACACATC
Hema2 At1g04490	For. / JP211	AGCTCCTGCACGGTCCAAT
	Rev. / JP212	TGCTATCGTTCCCATCGCAT
FC1 At5g26030	For. / JP110	ATACCAGAGTCGTGTTGGCCC
	Rev. / JP111	TCATCGGTGTATGGCTTCAGC
FC2 At2g30390	For. / JP239	TGGTGCTATGGCTGTCTCAAAC
	Rev. / JP240	AGCGGAACTAACGACTGTCGA
HY1 At2g26670	For. / JP409	TGCTGGTGGACGAATGATTG
	Rev. / JP410	TTATCGAGTATCCGCTCTGCC
HY2 At3g09150	For. / JP411	AAGCAAAGGCAAAGGAGCTG
	Rev. / JP412	CATCCACCCCATTGAACAGG
Genotyping primers		
SALK T-DNA left border	LB1.3	ATTTTGCCGATTTCGGAAC
GABI-KAT Left border	Gabi-KAT 08409	ATATTGACCATCATACTCATTGC
fc1 (SALK_150001)	LP JP138	TTTTGGATTGAGGAACTTTTACAAC
	RP JP139	AAACTCAATCCACGATTCGTG
fc1 (GabiKat_110D02)	LP JP281	CACTCTTCCATCTGCTTCTGG
	RP JP282	GCTCGTTCTGTGACTGGAGAC
fc2 (GabiKat_766H08)	LP JP283	GAGCAACGCCAAACATAGAAG
	RP JP284	TCAAAGGCAATGAATGTTTCC
hema1 (SALK_056220)	LP JP235	TTCCACAAATCTATCCATCGG
	RP JP236	AAGCAGAGAGCTCACAACGAG
hema1 (SALK_053036)	LP JP237	CTGAATGGCCAAGAGCTATTG
	RP JP240	AACCTCTGGAGAAGCTTGAGG

hema2 (SALK_052000)	LP 142	ATCTCAAATGCTGCATTGGAC
	RP JP143	TGTTTTGGGCATTTCTCATTC
gun5-1	For (Cac8I digest)	ACCAACCGGTAAAAACATGCATG
	Rev	CTCACACCAATCATCCAAAGAAC
Cloning primers		
FC1 coding region for Gateway	For JP115	CACCATCGGTTCTGAAATTTGTAGCTATG
	Rev JP118	TAGGTTCCGGAACGCATGGA
FC1 + native promoter (1700 bp upstream ATG)	For JP219	CACCTTCAATTACGATAATTCATGGTGTG
FC1Δ2-35	For JP297	CACCATGTGCGATATAAAAGAGAGATCTT
FC1Δ2-47	For JP295	CACCATGACTATCACGAATCGTGGATT
FC2 coding region for Gateway	For JP119	CACCTCAAGTTTCAATCTTTTTGCAATG
	Rev JP122	TAATGAAGGCAAGATGCCCCAC
FC2 + native promoter (1061 bp upstream ATG)	For JP220	CACCGCTTTCGAAATCATGCAACA
FC2Δ468-512 (ΔLHC)	Rev JP264	CATTGTCACCGGTGCTGGTAAC
HEMA2 coding region for Gateway	For JP216	CACCAAACTCAAAATCTATGGCGGTTT
	Rev JP218	CTTTTTTTCCACCTTTGCTCTAA
Z. Mays FC1A cDNA for Gateway	For JP321	CACCAAGCAGCCCATGTCTTCGT
	Rev JP322	AAGCATGGTGCTCCGGAAG
Z. Mays FC1B cDNA for Gateway	For JP325	CACCCTGCAGGAAGGATCATGGA
	Rev JP326	AAGCATGGTGTTCCGGAAAG
Z. Mays FC2 coding region for Gateway	For JP323	CACCGGAGGAGAGGGAGAGGATGT
	Rev JP324	GCGGAACAGAGGCAGGATTC
FC1 mutagenesis (H284A)	For JP221	CTTCAGTGCTGCTGGTGTTCCGGTC
	Rev JP222	GACCGGAACACCAGCAGCACTGAAG
FC2 mutagenesis (H295A)	For JP245	TTCAGTGCAGCTGGCGTGCCTCTTG
	Rev JP246	CAAGAGGCACGCCAGCTGCACTGAA
FC1 N-term sorting signal (Residues 1-88)	Rev JP260	ATAATAGGATCCATCTTCTGCAACAACATG AGAAC
FC1 core (residues 89-466)	For JP261	ATAATAGGATCCGCTAAGATTGGTGTCTTG CTTTTGAATT
FC2 N-terminal sorting signal (residues 1-99)	Rev JP262	ATAATAGGATCCGGCATCATCAGTGATAA CAGAG
FC2 core (residues 100-512)	For JP263	ATAATAGGATCCAAAATTGGTGTCTTGTTA TTAAACCT

Plasmids Used in This Study

Plasmid	Insert	Parent	*Arabidopsis* Lines	notes	ref
pENTR-D/TOPO				Gateway cloning vector	Invitrogen
PEARLEYGATE 101				35S promoter, C-term YFP-HA fusion	[6]
PEARLEYGATE 102				35S promoter, C-term CFP-HA fusion	[6]
pGBGWC				No promoter, C-term CFP	[7]
pGBGWY				No promoter, C-term YFP	[7]
pJDW5	Genomic *FC1* coding region w/o stop codon	pENTR-D/TOPO			
pJDW7	Genomic *FC2* coding region w/o stop codon	pENTR-D/TOPO			
pJDW10	*35S::FC1-YFP-HA*	pEARLEYGATE101 + pJDW5	250		
pJDW11	*35S::FC1-CFP-HA*	pEARLEYGATE102 + pJDW5	251		
pJDW12	*35S::FC2-YFP-HA*	pEARLEYGATE101 + pJDW7	252		
pJDW13	*35S::FC2-CFP-HA*	pEARLEYGATE102 + pJDW7	253		
pJDW21	Genomic *FC1* coding region w/o stop codon	pJDW5		FC1 H284A mutation	
pJDW28	Genomic *FC2* coding region w/o stop codon	pJDW7		FC2 H295A mutation	
pJDW33	*35S::dFC1-YFP-HA*	pEARLEYGATE101 + pJDW21	33	FC1 H284A mutation	
pJDW39	*35S::dFC1-YFP-HA*	pEARLEYGATE101 + pJDW28	39	FC2 H295A mutation	
pJDW25	Genomic *HEMA2* coding region w/o stop codon	pENTR-D/TOPO			
pJDW37	35S::*HEMA2-YFP-HA*	pEARLEYGATE101 + pJDW25	37		
pJDW87	*MzaFC1A* cDNA w/o stop codon	pENTR-D/TOPO			
pJDW88	*MzaFC2* cDNA w/o stop codon	pENTR-D/TOPO			
pJDW89	*MzaFC1B* cDNA w/o stop codon	pENTR-D/TOPO			

pJDW90	*35S::MzaFC1A-YFP-HA*	pEARLEYGATE101 + pJDW87	90		
pJDW91	*35S::MzaFC2-YFP-HA*	pEARLEYGATE101 + pJDW88	91		
pJDW92	*35S::MzaFC1B-YFP-HA*	pEARLEYGATE101 + pJDW89	92		
pJDW26	Genomic *FC1* w/o stop codon + native promoter (1700 bp upstream ATG)	pENTR-D/TOPO			
pJDW27	Genomic *FC2* w/o stop codon + native promoter (1061 bp upstream ATG)	pENTR-D/TOPO			
pJDW30	*FC1::FC1-YFP*	PGBGWY + pJDW26	30		
pJDW31	*FC2::FC2-CFP*	PGBGWC + pJDW27	31		
pJDW32	*FC2::FC2-YFP*	PGBGWY + pJDW27	32		
pJDW43	Fusion of FC1 N-terminal sorting signal and core FC2 (FC1/FC2) w/o stop codon	pENTR-D/TOPO			
pJDW45	Fusion of FC2 N-terminal sorting signal and core FC1 (FC2/FC1) w/o stop codon	pENTR-D/TOPO			
pJDW46	*FC2* cDNA (Δ468-512)	pENTR-D/TOPO			
pJDW51	*35S::FC1*(N-term)/ *FC2*(core)-*YFP-HA*	pENTR-D/TOPO	51		
pJDW53	*35S::FC2*(N-term)/ *FC1*(core)-*YFP-HA*	pEARLEYGATE101 + pJDW45	53		
pJDW54	*35S::FC2*(Δ468-512)-*YFP-HA*	pEARLEYGATE101 + pJDW46	54		
PJDW61	*FC1* cDNA (Δ2-47)	pENTR-D/TOPO			
PJDW63	*FC1* cDNA (Δ2-35)	pENTR-D/TOPO			
pJDW68	*35S::FC1*(Δ2-47)-*YFP-HA*	pEARLEYGATE101 + pJDW61	68		
pJDW70	*35S::FC1*(Δ2-35)-*YFP-HA*	pEARLEYGATE101 + pJDW63	70		

Transgenic Lines Used in This Study

Line	Construct	plasmid	Parent	
Col-0/107			Col-0	[1]
250	*35S::FC1-YFP-HA*	pJDW10	Col-0/107	
251	*35S::FC1-CFP-HA*	pJDW11	Col-0/107	
252	*35S::FC2-YFP-HA*	pJDW12	Col-0/107	
253	*35S::FC2-CFP-HA*	pJDW13	Col-0/107	
33	*35S::dFC1-YFP-HA*	pJDW33	Col-0/107	
39	*35S::dFC2-YFP-HA*	pJDW39	Col-0/107	
37	*35S::HEMA2-YFP-HA*	pJDW37	Col-0/107	
30	*FC1::FC1-YFP*	pJDW30	Col-0	
31	*FC2::FC2-CFP*	pJDW31	Col-0	
32	*FC2::FC2-YFP*	pJDW32	Col-0	
51	*35S::FC1*(N-term)/*FC2*(core*)-YFP-HA*	pJDW51	Col-0/107	
53	*35S::FC2*(N-term)/*FC1*(core*)-YFP-HA*	pJDW53	Col-0/107	
54	*35S::FC2*(Δ468-512)*-YFP-HA*	pJDW54	Col-0/107	
68	*35S::FC1*(Δ1-47)*-YFP-HA*	PJDW68	Col-0/107	
70	*35S::FC1*(Δ1-35)*-YFP-HA*	pJDW70	Col-0/107	
90 (ZmaFC1A)	*35S::ZmaFC1A-YFP-HA*	pJDW90	Col-0/107	
91 (ZmaFC2)	*35S::ZmaFC2-YFP-HA*	pJDW91	Col-0/107	
92 (ZmaFC1B)	*35S::ZmaFC1B-YFP-HA*	pJDW92	Col-0/107	
Plastid-CFP marker	*35S::TobaccoRBCS(1-79)-CFP*	pt-cb CD3-994	Col-0	[2]
Plastid-YFP marker	*35S::TobaccoRBCS(1-79)-YFP*	pt-yb CD3-998	Col-0	[2]

Supplemental References

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