

Supplementary material for Phil. Trans. R. Soc. B. article

Overexpression of chloroplast-targeted ferrochelatase 1
results in a *genomes uncoupled* chloroplast-to-nucleus
retrograde signalling phenotype

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Table S1. Primers used for molecular cloning of *FC1* and genotyping of transgenic plants.

Reaction	Purpose	Primer	Primer sequences (5' > 3')
1	Amplification of <i>FC1:spGFP</i> , to remove the native FC1 transit peptide and add a BglIII restriction site at the 5' end of the amplicon.	A	<u>AGATCT</u> GCTAAAGCACGTTCTCATG
		B	GCTCTTATTTGTATAGTTCATCCATGC
2	Re-amplification of the amplicon obtained in reaction 1, to add <i>attB</i> Gateway® recombination sites at each end (step 1 of a two-step reaction).	C	<u>AAAAGCAGGCTCA</u> <u>AGATCT</u> GCTAAAGCAC
		D	<u>GAAAGCTGGGCT</u> TTATTTGTATAGTTCATCC
3	Re-amplification of the amplicon obtained in reaction 1, to add <i>attB</i> Gateway® recombination sites at each end (step 2 of a two-step reaction).	E	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCT</u>
		F	<u>GGGGACCACTTTGTACAAGAAAGCTGGGT</u>
4	Amplification of the RecA transit peptide, to add BglIII restriction sites at both ends of the amplicon.	G	<u>AGATCT</u> ATGGATTCACAGCTAGTCTTG
		H	<u>AGATCT</u> TCTGTCATCGAATTCAGAAC
5	Amplification of the CoxIV transit peptide, to add BglIII restriction sites at both ends of the amplicon.	I	<u>AGATCT</u> ATGCTTTCACCTACGCTCAATCT
		J	<u>AGATCT</u> GGGTTTTTGTCTGAAGCAGA
6	Amplification of <i>RecA:spGFP</i> , to add <i>attB</i> Gateway® recombination sites at each end (step 1 of a two-step reaction).	K	<u>AAAAGCAGGCTAC</u> ATGGATTCACAGCTAG
		D	<u>GAAAGCTGGGCT</u> TTATTTGTATAGTTCATCC
7	Amplification of <i>RecA:spGFP</i> from the amplicon obtained in reaction 6, to add <i>attB</i> Gateway® recombination sites at each end (step 2 of a two-step reaction).	E	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCT</u>
		F	<u>GGGGACCACTTTGTACAAGAAAGCTGGGT</u>
8	Amplification of <i>CoxIV:spGFP</i> , to add <i>attB</i> Gateway® recombination sites at each end (step 1 of a two-step reaction).	L	<u>AAAAGCAGGCTAC</u> ATGGTTTCACTACGTC
		D	<u>GAAAGCTGGGCT</u> TTATTTGTATAGTTCATCC
9	Amplification of <i>CoxIV:spGFP</i> from the amplicon obtained in reaction 8, to add <i>attB</i> Gateway® recombination sites at each end (step 2 of a two-step reaction).	E	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCT</u>
		F	<u>GGGGACCACTTTGTACAAGAAAGCTGGGT</u>
10	Amplification of the full-length <i>FC1</i> coding sequence (FL- <i>FC1</i>) fused to <i>GFP</i> , to add <i>attB</i> Gateway® recombination sites at each end (step 1 of a two-step reaction).	M	<u>AAAAGCAGGCTCA</u> ATGCAGGCAACGG
		D	<u>GAAAGCTGGGCT</u> TTATTTGTATAGTTCATCC
11	Amplification of FL- <i>FC1</i> fused to <i>GFP</i> from the amplicon obtained in reaction 10, to add <i>attB</i> Gateway® recombination sites at each end (step 2 of a two-step reaction).	E	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCT</u>
		F	<u>GGGGACCACTTTGTACAAGAAAGCTGGGT</u>
12	Genotyping <i>RecA:FC1:GFP</i> transgenic lines. Product size = 560 bp.	N	GGATTCACAGCTAGTCTTGTCTCTG
		O	CCTCCTCAGTGAACGGATACC
13	Genotyping <i>CoxIV:FC1:GFP</i> transgenic lines. Product size = 416 bp.	P	CAAGCCAGCCACAAGAACCTTG
		O	CCTCCTCAGTGAACGGATACC
14	Genotyping <i>RecA:GFP</i> and <i>CoxIV:GFP</i> transgenic lines. Product sizes = 447 bp and 330 bp respectively.	Q	GGATGACGCACAATCCCACATATC
		R	CAAGAATTGGGACAACCTCCAG
15	Genotyping FL- <i>FC1:GFP</i> transgenic lines. Product size = 783 bp.	Q	GGATGACGCACAATCCCACATATC
		O	CCTCCTCAGTGAACGGATACC

Restriction enzyme recognition sites within primer sequences are given in blue text. Gateway® *att* recombination sequences (including any extra bases to maintain reading frames) within primers are underlined.

Table S2. Information on the plasmids used and created during molecular cloning of *FC1*.

Plasmid name	Insert name	Parent plasmid/ amplicon	<i>Arabidopsis</i> lines	Notes	Reference
pGEM [®] -T Easy				TA cloning plasmid (Amp ^R).	Promega
pDONR [™] 221				Gateway [®] cloning plasmid (Kan ^R).	Invitrogen [™]
pGWB502Ω				Gateway [®] destination plasmid (Spec ^R). The plant selectable marker is hygromycin.	Nakagawa <i>et al</i> (2007)
pGEM [®] -T FC1-GFP	<i>FC1:GFP</i>	pGEM [®] -T Easy & reaction 1		Subcloning of <i>FC1:GFP</i> (no native transit peptide).	
pENTR FC1-GFP	<i>FC1:GFP</i>	pDONR [™] 221 & reaction 3		Cloning <i>FC1:GFP</i> (no native transit peptide) into a Gateway [®] plasmid.	
pGEM [®] -T RecA	<i>RecA</i>	pGEM [®] -T Easy & reaction 4		Subcloning of <i>RecA</i> transit peptide.	
pGEM [®] -T CoxIV	<i>CoxIV</i>	pGEM [®] -T Easy & reaction 5		Subcloning of <i>CoxIV</i> transit peptide.	
pENTR RecA-FC1-GFP	<i>RecA:FC1:GFP</i>	pENTR FC1-GFP & pGEM [®] -T RecA (BglII digest)		Ligation of <i>RecA</i> transit peptide upstream of <i>FC1:GFP</i> .	
pENTR CoxIV-FC1-GFP	<i>CoxIV:FC1:GFP</i>	pENTR FC1-GFP & pGEM [®] -T CoxIV (BglII digest)		Ligation of <i>CoxIV</i> transit peptide upstream of <i>FC1:GFP</i> .	
pGWB502Ω RecA-FC1-GFP	<i>RecA:FC1:GFP</i>	pENTR RecA-FC1-GFP & pGWB502Ω	pFC1	Recombination of <i>RecA:FC1:GFP</i> into a destination plasmid.	
pGWB502Ω CoxIV-FC1-GFP	<i>CoxIV:FC1:GFP</i>	pENTR CoxIV-FC1-GFP & pGWB502Ω	mFC1	Recombination of <i>CoxIV:FC1:GFP</i> into a destination plasmid.	
pENTR RecA-GFP	<i>RecA:GFP</i>	pDONR [™] 221 & reaction 7		Cloning <i>RecA:GFP</i> into a Gateway [®] plasmid.	
pGWB502Ω RecA-GFP	<i>RecA:GFP</i>	pENTR RecA-GFP & pGWB502Ω	pGFP	Recombination of <i>RecA:GFP</i> into a destination plasmid.	
pENTR CoxIV-GFP	<i>CoxIV:GFP</i>	pDONR [™] 221 & reaction 9		Cloning <i>CoxIV:GFP</i> into a Gateway [®] plasmid.	
pGWB502Ω CoxIV-GFP	<i>CoxIV:GFP</i>	pENTR CoxIV-GFP & pGWB502Ω	mGFP	Recombination of <i>CoxIV:GFP</i> into a destination plasmid.	
pENTR FL-FC1-GFP	<i>FL-FC1:GFP</i>	pDONR221 [™] & reaction 11		Cloning <i>FL-FC1:GFP</i> into a Gateway [®] plasmid.	
pGWB502Ω FL-FC1-GFP	<i>FL-FC1:GFP</i>	pENTR FL-FC1-GFP & pGWB502Ω	FLFC1	Recombination of <i>FL-FC1:GFP</i> into a destination plasmid.	

The reactions referred to in column three relate to the amplicons created in the reactions described in Supplementary Table S1.

Table S3. Information on the primers used for qRT-PCR analysis of gene expression.

Gene name	Accession No. (source)	Forward primer sequence (5' > 3')	Reverse primer sequence (5' > 3')	Amplicon length (bp)
<i>FC1</i>	At5g26030 (TAIR)	CCTGAACTCTTAACGATGTTTC	CCACCAATAGCAGCATAACC	164
<i>GFP</i>	U70496.1 (GenBank)	GAGGACCATCTCTTTCAAGGAC	GTTGTGGGAGTTGTAGTTGTATTC	163
<i>FC2</i>	At2g30390 (TAIR)	GCAGAGATGGAAGAATGTGTTG	CAGTAATGGCTTCTTCAGTGATG	139
<i>ADF2</i>	At3g46000 (TAIR)	CGATTTGACTTTGTCCTGTC	TCATCTTGTCTCTCACTTTGGC	95
<i>YLS8</i>	At5g08290 (TAIR)	GCTGAAATATCCCGTGAACG	AATGGAGAACAACCGAAACAG	93
<i>GUN4</i>	At3g59400 (TAIR)	CAATCTCACTTCGGACCAAC	TTGAAACGGCAGATACGG	121
<i>CA1</i>	At3g01500 (TAIR)	GCTTCTTTCTCACTTCACTTTCTC	CAATGATAGGAGCAGGAGCG	189
<i>HEMA1</i>	At1g58290 (TAIR)	GCTTCTTCTGATTCTGCGTC	GCTGTGTGAATACTAAGTCCAATC	128
<i>LHCB2*1</i>	At2g05100 (TAIR)	CTCCGCAAGGTTGGTGTATC	CGGTTAGGTAGGACGGTGTAT	142
<i>CHLH</i>	At5g13630 (TAIR)	CATTGCTGACACTACAACGTC	CTTCTCTATCTCACGAACTCCTTC	145
<i>RecA:FC1</i>	Created in this study	CTTCACTCCTCTTTCTCCTCTCT	CAACAACATGAGAACGTGCTTTA	191
<i>CoxIV:FC1</i>	Created in this study	CAAGCCAGCCACAAGAAGCTT	CATCGTTAAGAGTTTCAGGACCA	146

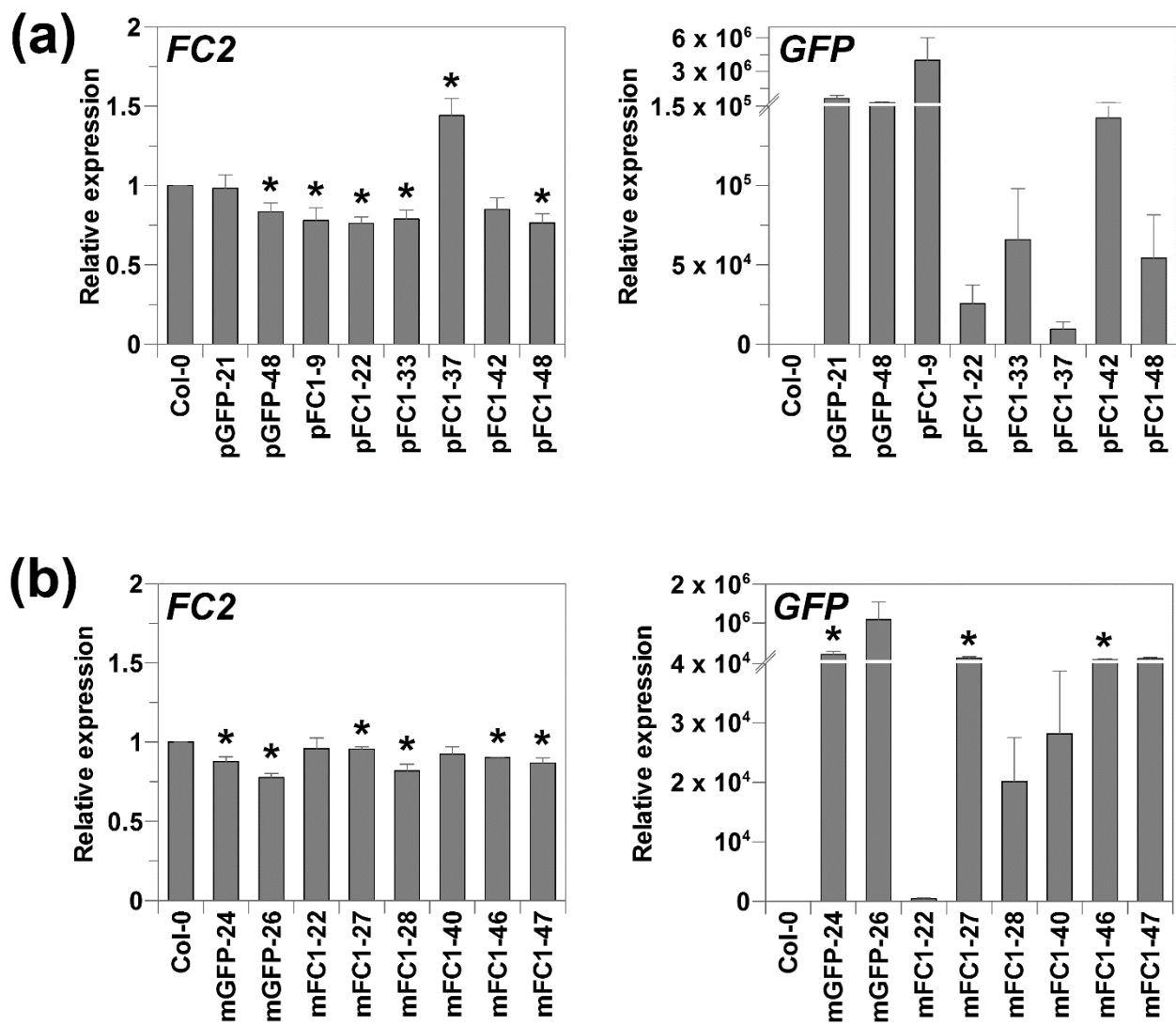


Figure S1. Expression of *FC2* and *GFP* in *FC1* overexpressing lines. (a,b) Expression of *FC2* and *GFP* was determined in the same pFC1 (a) and mFC1 (b) seedlings used to generate Figure 1 and is shown relative to Col-0. Lines expressing only *GFP* in plastids (pGFP) or mitochondria (mGFP) were included as controls. Data represents the mean + SEM of three independent biological replicates and asterisks indicate a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

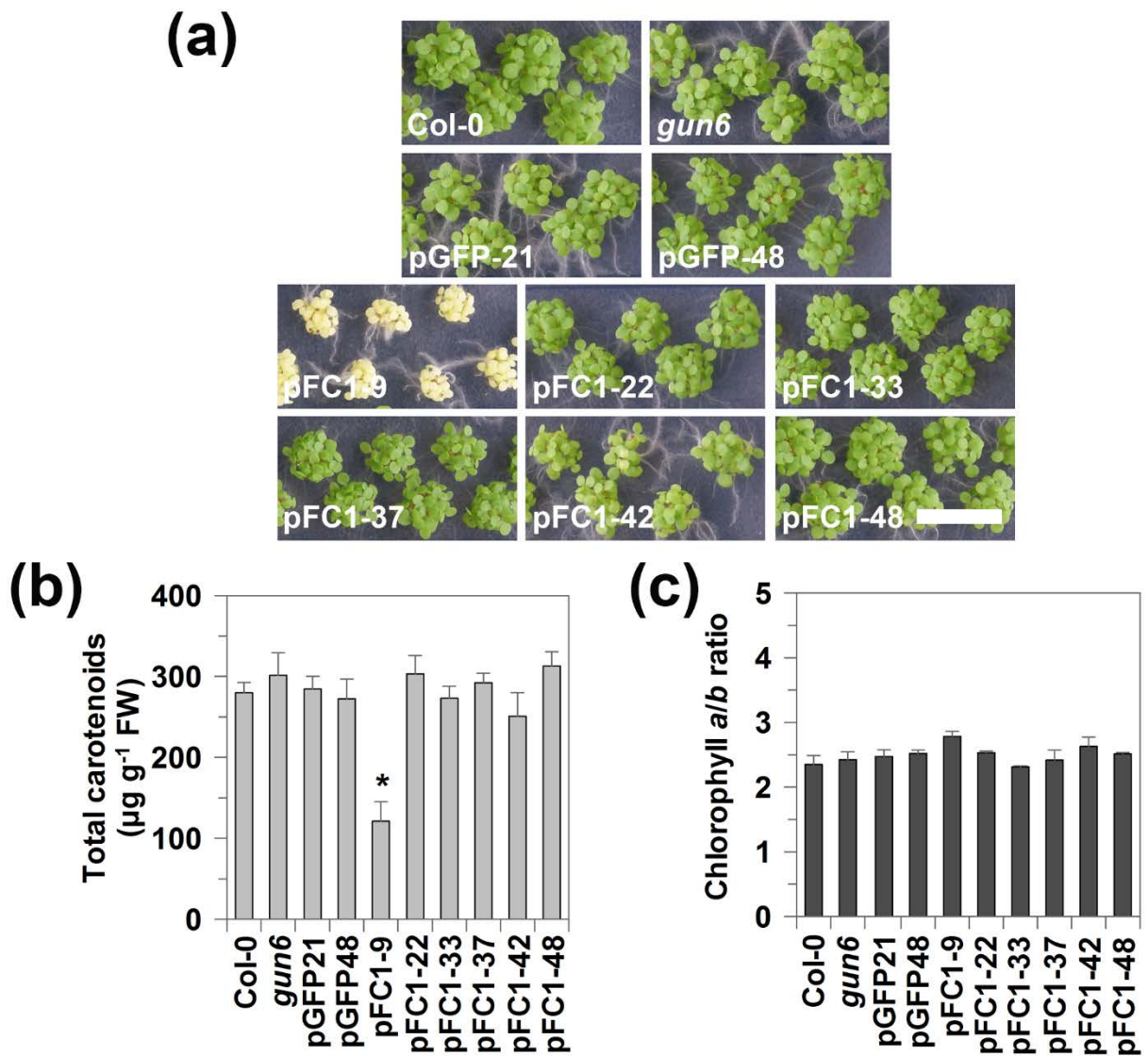


Figure S2. Characterisation of 5 day-old WLC-grown seedlings overexpressing plastid-targeted *FC1*. (a) Representative seedling phenotype of pFC1 and pGFP lines, bar = 10 mm. (b) Total carotenoid and (c) chlorophyll *a/b* ratio of the same transgenic lines. For (b, c), data shown is the mean + SEM of three independent biological replicates and the asterisk denotes a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

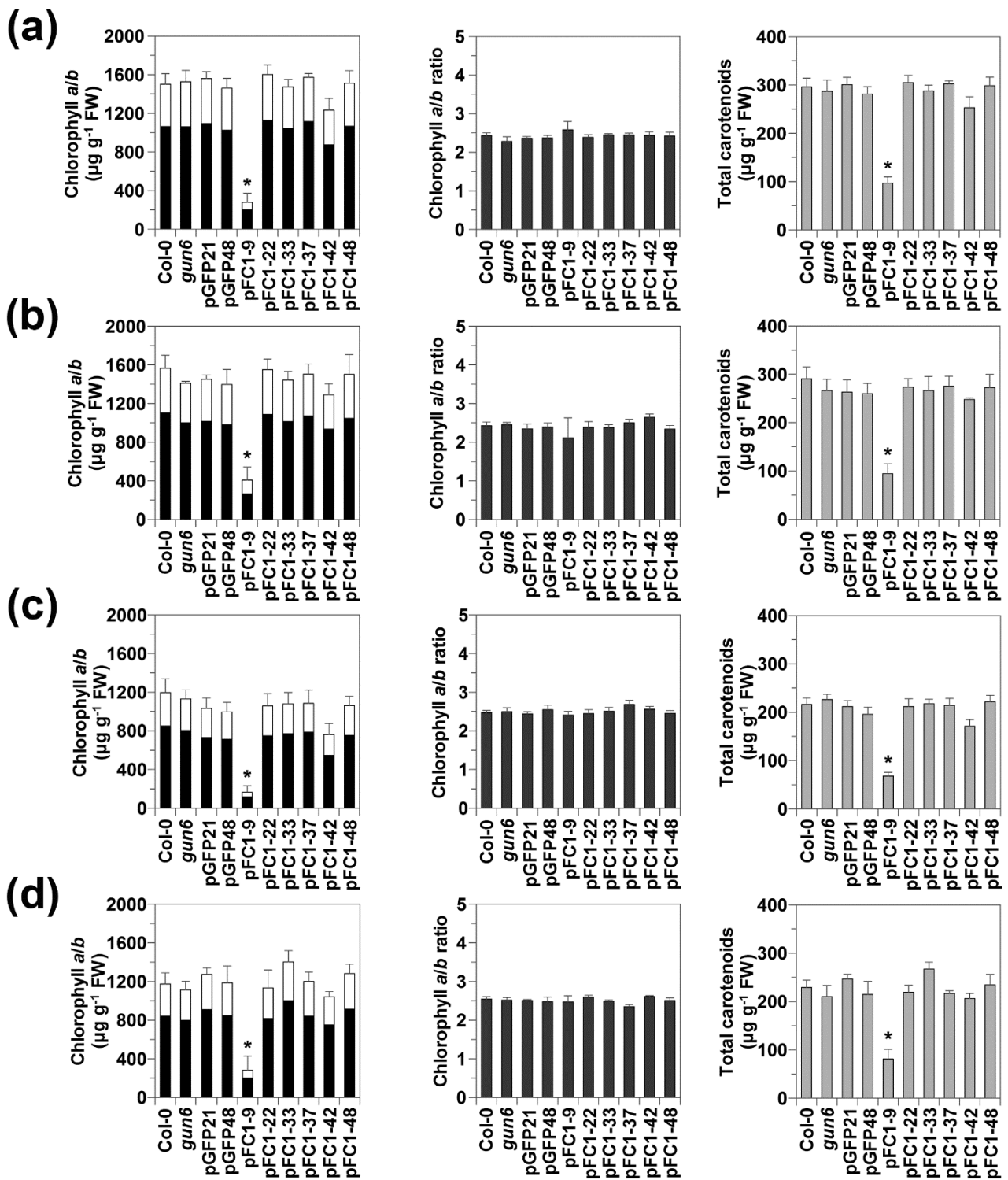


Figure S3. Analysis of chlorophyll and carotenoid levels in pFC1 seedlings grown in different light conditions. (a-d) Total chlorophyll, chlorophyll *a/b* ratio and total carotenoids were measured in pFC1, pGFP (control) and *gun6* 5 d-old seedlings under a range of conditions. (a) LWLc (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$), (b) HWLc (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$), (c) SD (8 h light, 16 h dark, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), (d) LD (16 h light, 8 h dark, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). For graphs of chlorophyll content, black bars represent chlorophyll *a* and white bars represent chlorophyll *b*. Data shown are the mean + SEM of three independent biological replicates and asterisks indicate a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

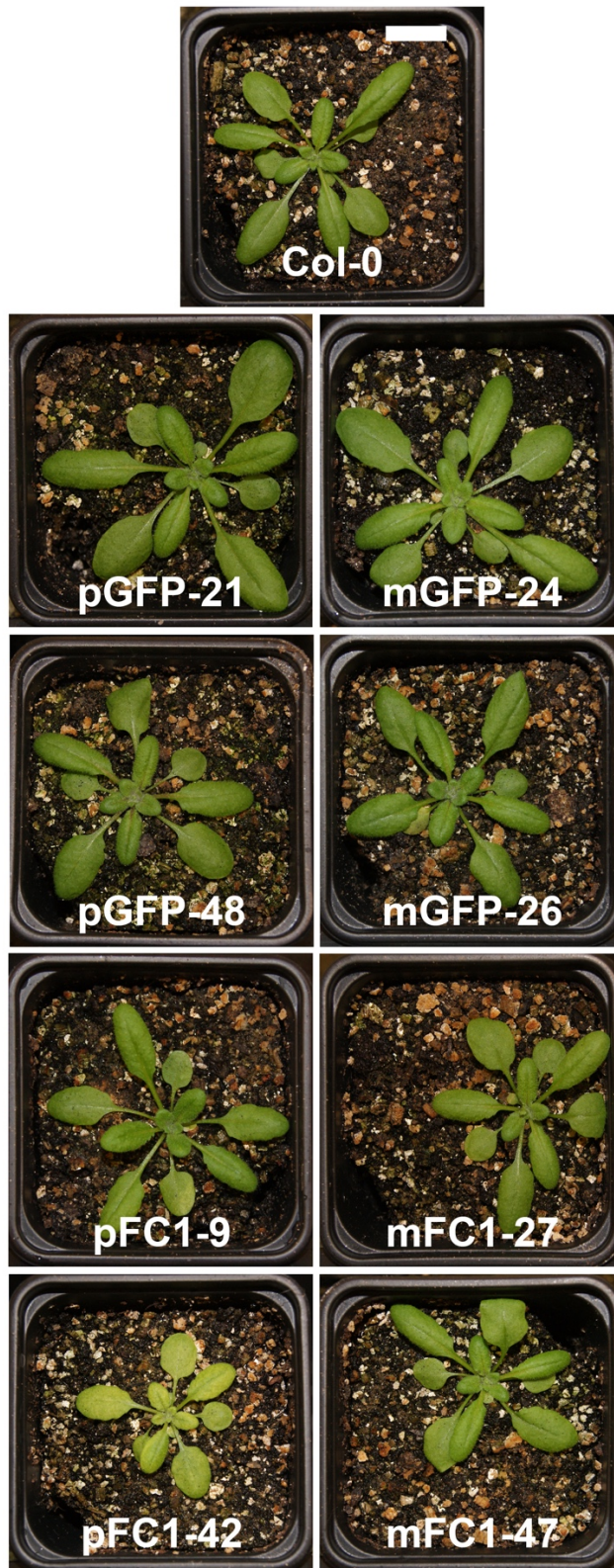


Figure S4. Phenotype of *FC1* overexpressing lines at the rosette stage. Representative photographs of pGFP, pFC1, mGFP and mFC1 lines. All photographs were taken 23 days after sowing (DAS), except pFC1-9 (34 DAS). Plants were grown on soil in LD conditions (16 h light, 8 h dark, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$), scale bar = 10 mm.

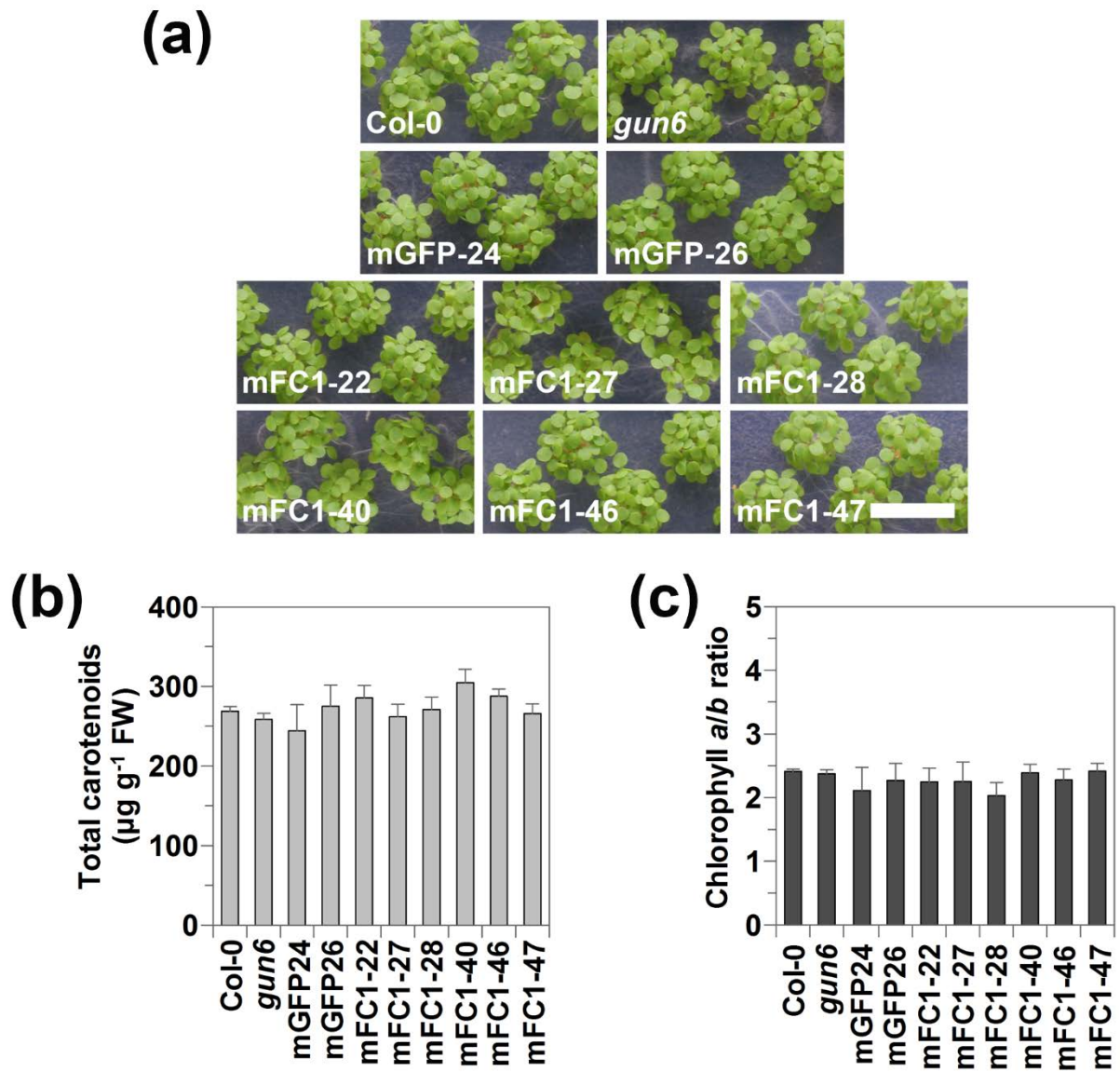


Figure S5. Characterisation of 5 d-old WLC-grown seedlings overexpressing mitochondria-targeted FC1. (a) Representative seedling phenotype of mFC1 and mGFP lines, bar = 10 mm. (b) Total carotenoid and (c) chlorophyll *a/b* ratio of the same transgenic lines. For (b) and (c), data shown is the mean + SEM of three independent biological replicates.

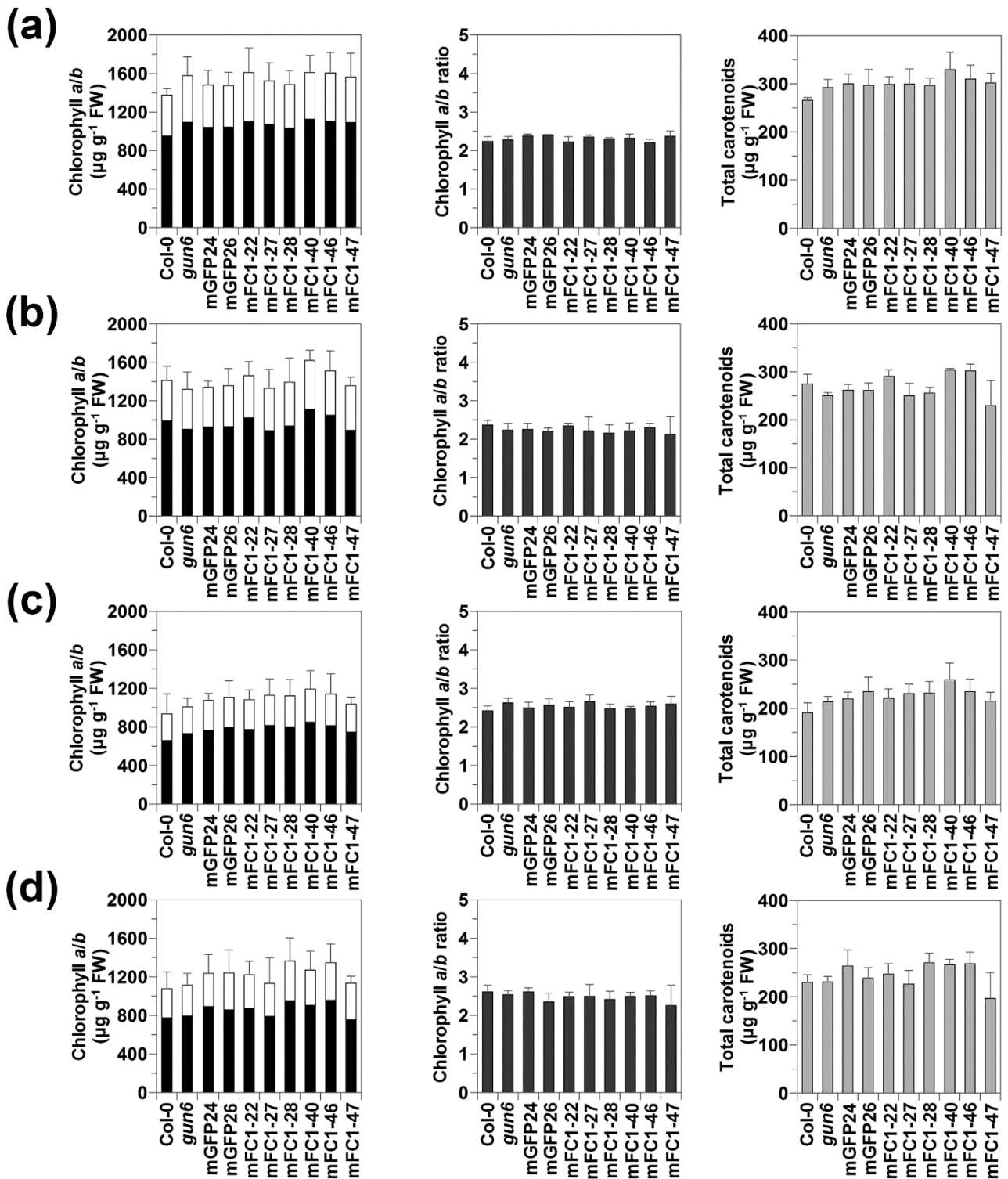


Figure S6. Analysis of chlorophyll and carotenoid levels in mFC1 seedlings grown in different light conditions. (a-d) Total chlorophyll, chlorophyll *a/b* ratio and total carotenoids were measured in mFC1, mGFP (control) and *gun6* 5 d-old seedlings under a range of conditions. (a) LWLc ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$), (b) HWLc ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$), (c) SD (8 h light, 16 h dark, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$), (d) LD (16 h light, 8 h dark, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$). For graphs of chlorophyll content, black bars represent chlorophyll *a* and white bars represent chlorophyll *b*. Data shown are the mean + SEM of three independent biological replicates.

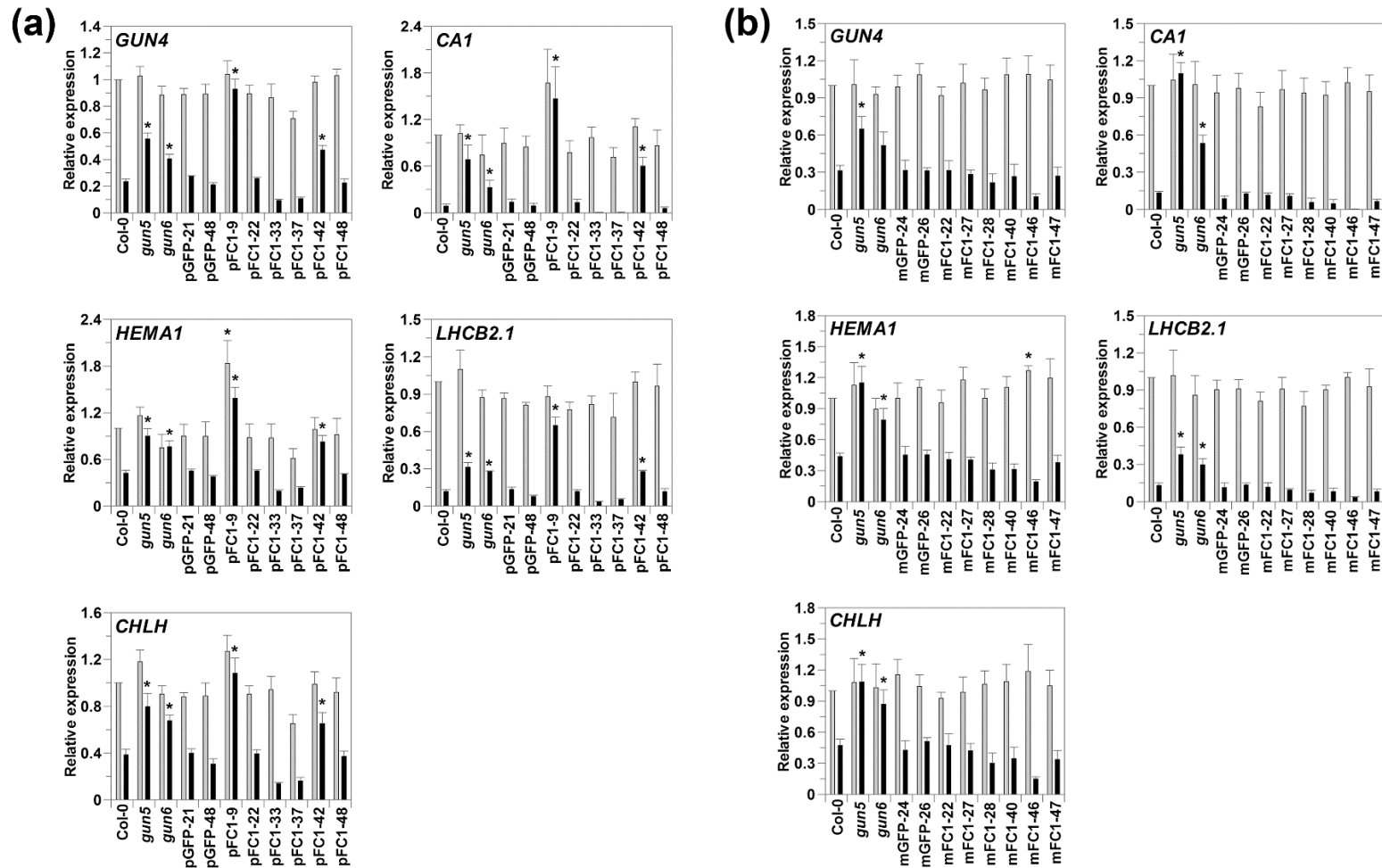


Figure S8. Expression of photosynthesis-associated genes on NF is rescued in plastid *FC1* overexpressors, but not mitochondrial *FC1* overexpressors. (a,b) The expression of *GUN4*, *CA1*, *HEMA1*, *LHCB2.1* and *CHLH* was determined by qRT-PCR in pFC1 (a) and mFC1 (b) seedlings grown for 7 d in LWLc on plates in the absence (grey bars) or presence (black bars) of NF. The control lines pGFP (a) and mGFP (b), as well as *gun5* and *gun6*, were included. Data shown are the mean fold changes vs. Col-0 on NF + SEM of three independent biological replicates and asterisks indicate a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test). The data in this figure was used to produce the graphs in Figure 3.

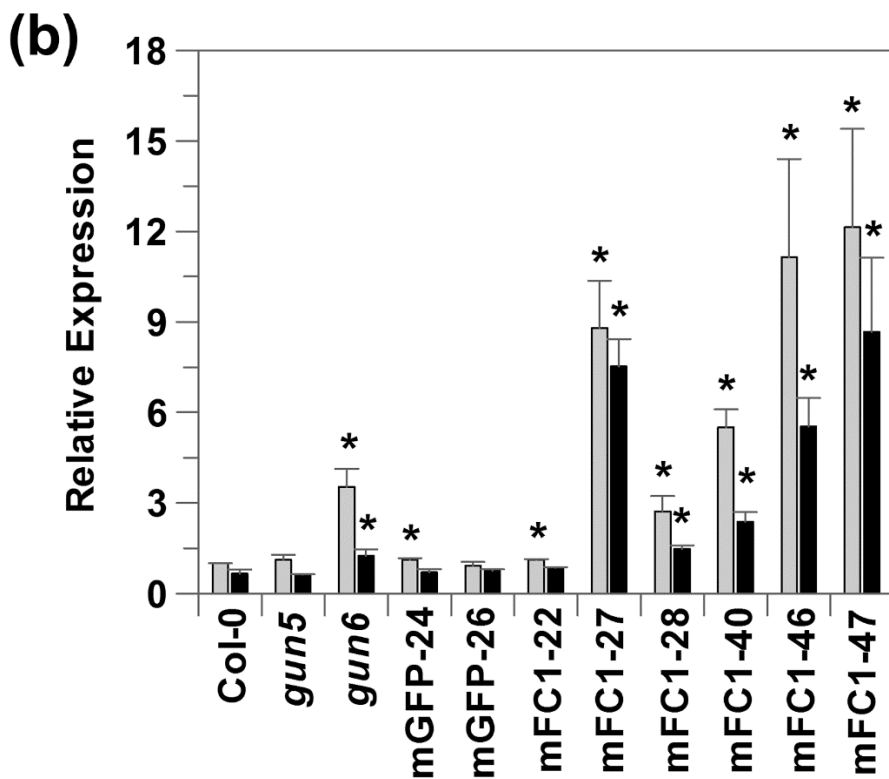
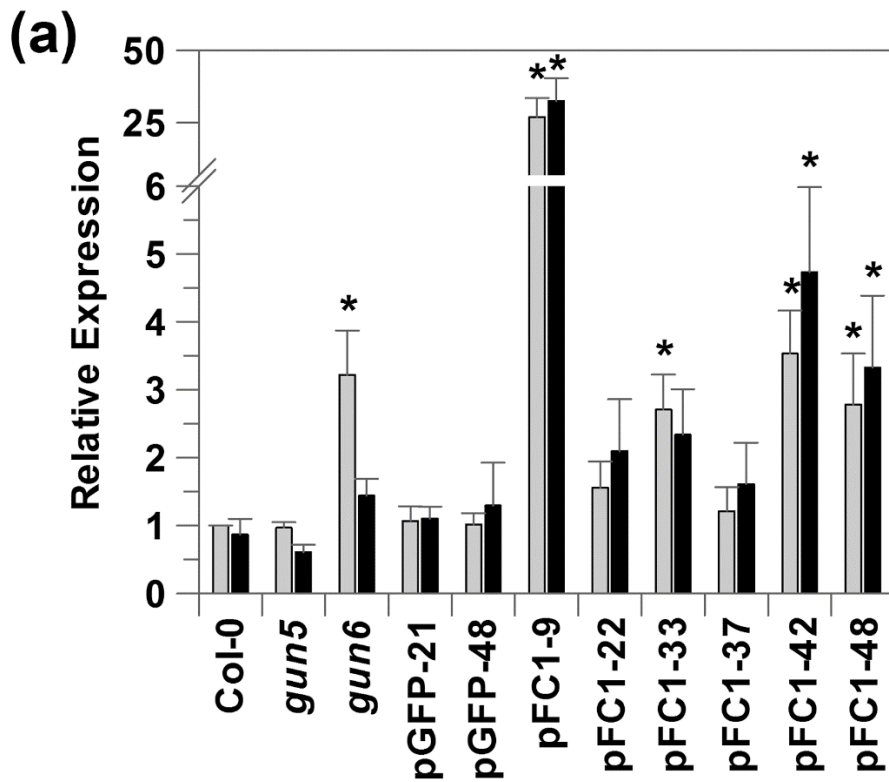


Figure S9. FC1 expression in pFC1 and mFC1 lines in the NF screen. (a,b) FC1 expression was determined by qRT-PCR in pFC1 (a) and mFC1 (b) seedlings in the absence (grey bars) or presence (black bars) of NF. Data represents the mean + SEM of three independent biological replicates and asterisks indicate a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

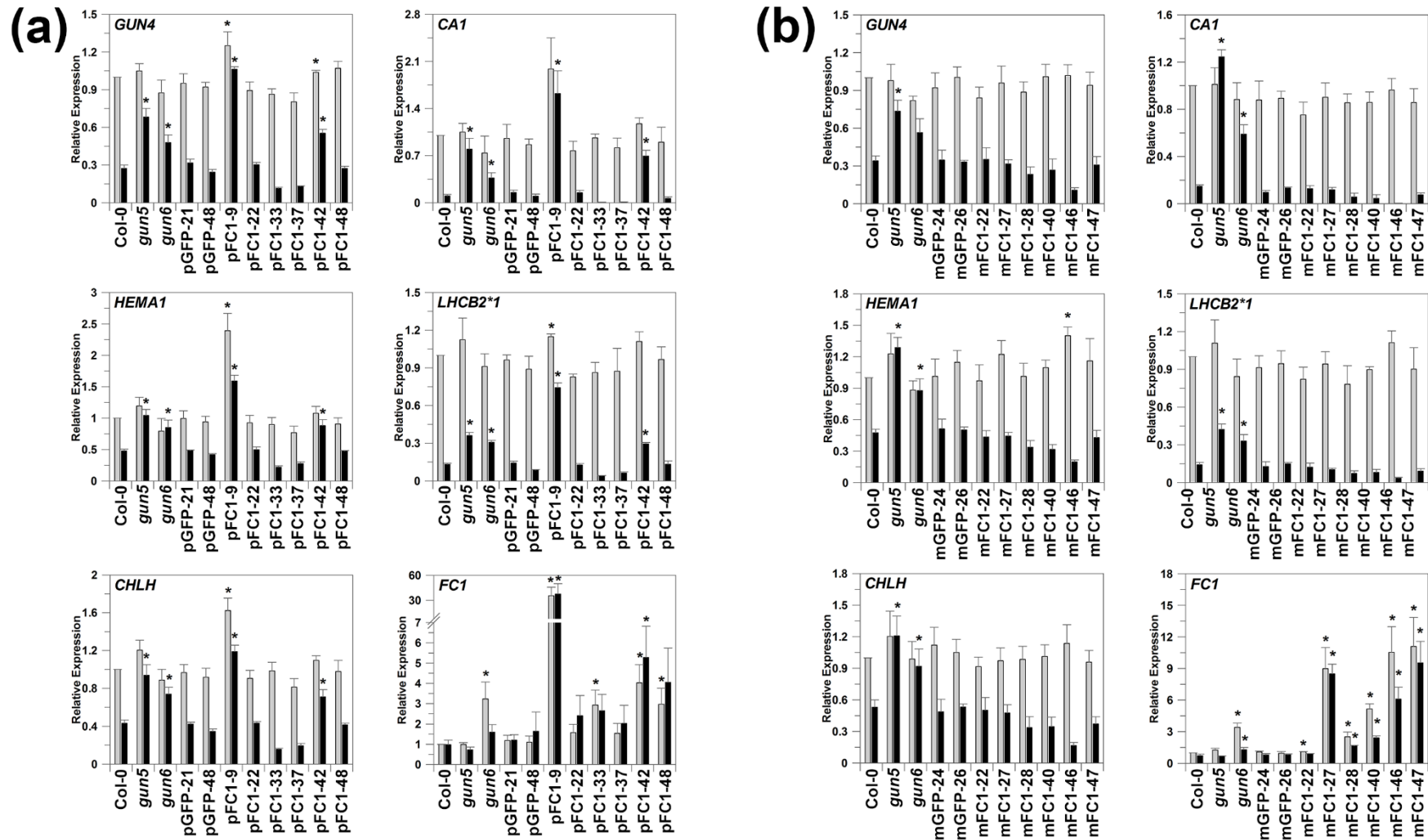


Figure S10. Gene expression changes on NF in pFC1 seedlings are not dependent on the qRT-PCR reference gene. (a,b) qRT-PCR data shown in electronic supplementary material figures S8 and S9 were normalised to a different reference gene, *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290). The expression of *GUN4*, *CA1*, *HEMA1*, *LHCB2.1*, *CHLH* and *FC1* was determined by qRT-PCR in pFC1 (a) and mFC1(b) seedlings grown for 7 d in LWLc on plates in the absence (grey bars) or presence (black bars) of NF. The control lines pGFP (a) and mGFP (b), as well as *gun5* and *gun6*, were included. Data shown are the mean fold changes vs. Col-0 on NF + SEM of three independent biological replicates and asterisks indicate a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

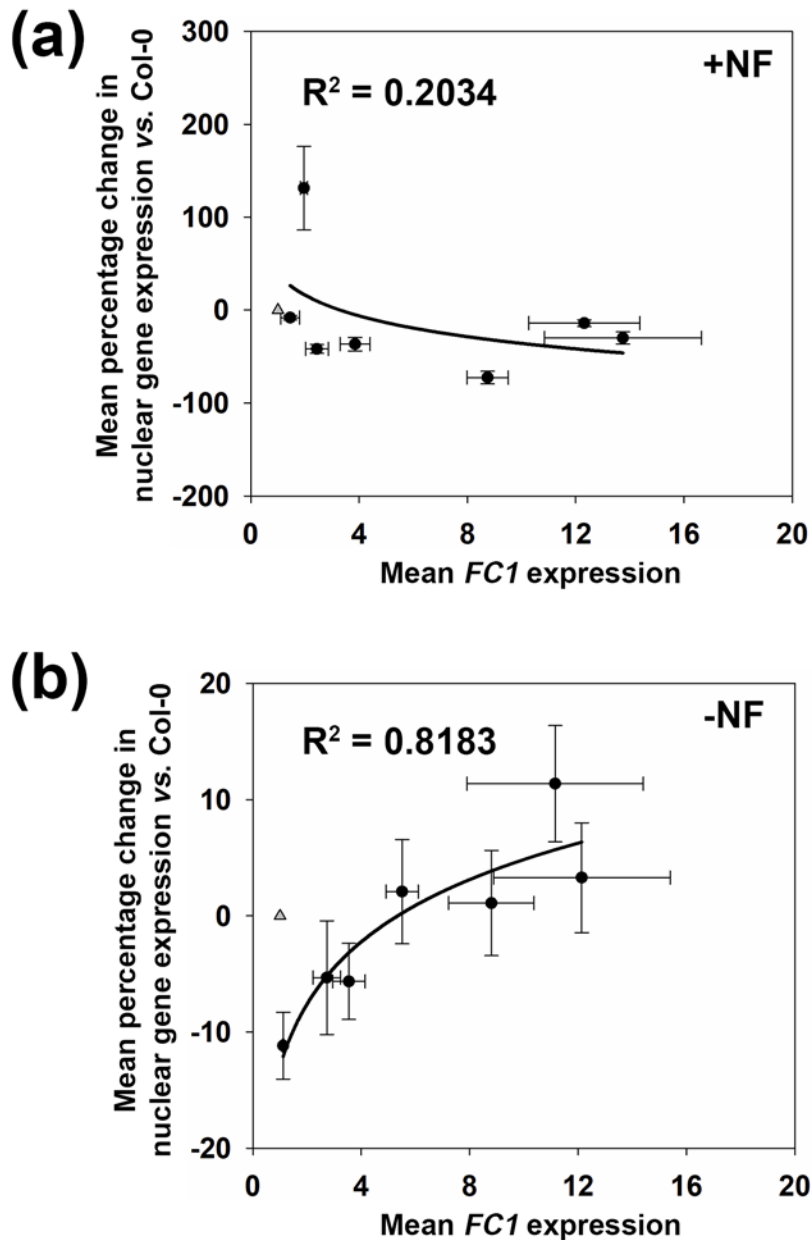


Figure S11. Mitochondria-targeted *FC1* expression does not correlate with enhanced nuclear gene expression on NF. Correlation plots of the combined mean percentage change in expression of *GUN4*, *CA1*, *HEMA1*, *LHCB2.1*, and *CHLH*, vs. *FC1* expression for m*FC1* seedlings in the presence (a) or absence (b) of NF. Data is relative to Col-0 +NF (a) or -NF (b). For both graphs, data points include *gun6* and the six transgenic m*FC1* overexpressing lines. The triangle indicates WT response. SigmaPlot 13.0 was used to fit logarithmic best-fit lines and derive coefficients of determination. Data shown is the mean \pm SEM of three independent biological replicates.

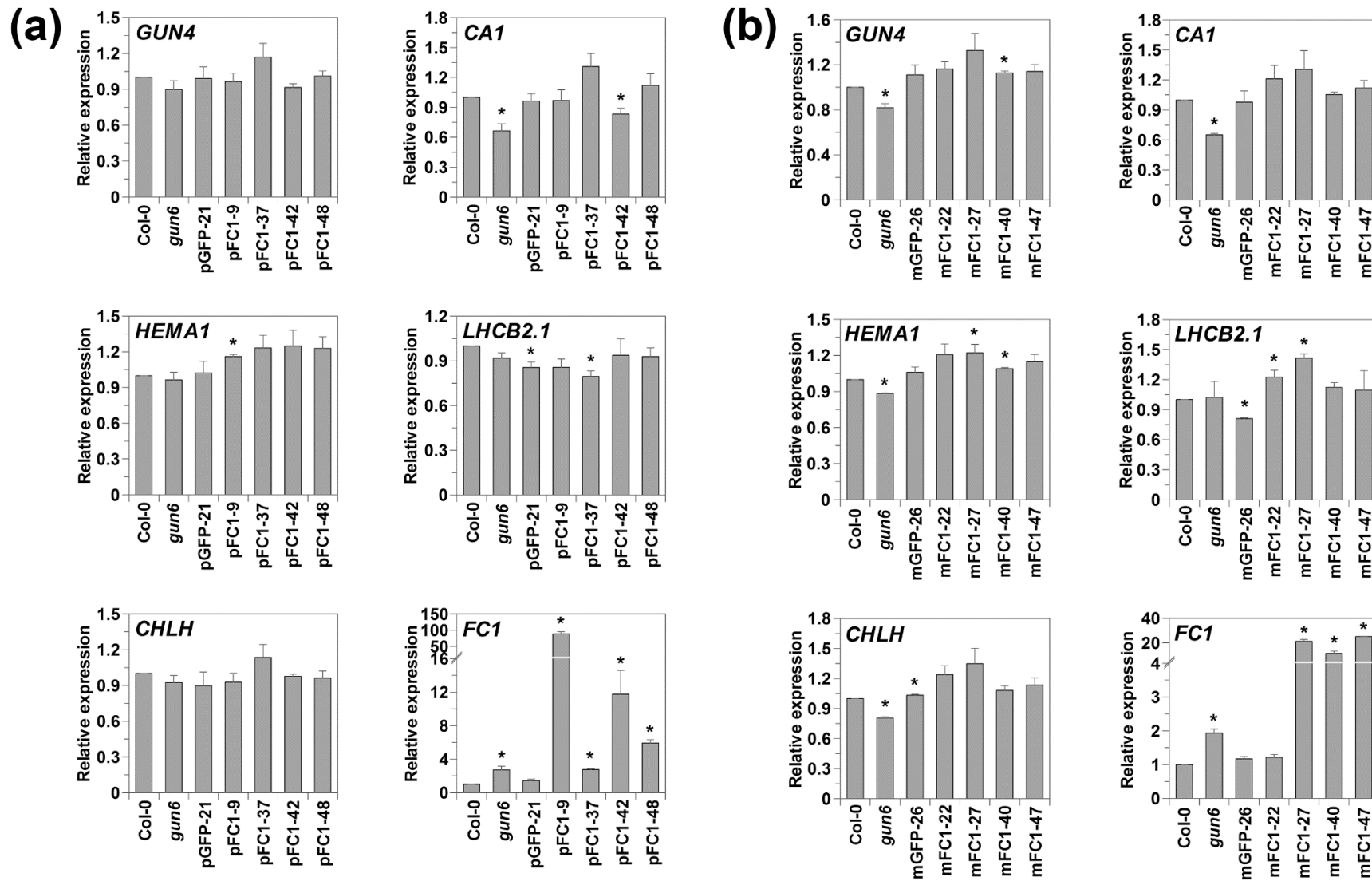


Figure S12. Increased *FC1* expression does not confer elevated nuclear gene expression in dark-grown seedlings. (a,b) The expression of *GUN4*, *CA1*, *HEMA1*, *LHCB2.1*, *CHLH* and *FC1* was determined by qRT-PCR in pFC1 (a) and mFC1 (b) seedlings grown for 4 d in the dark. Data shown is the mean + SEM of three independent biological replicates and asterisks denote a significant difference vs. Col-0 ($p < 0.05$, Student's *t*-test).

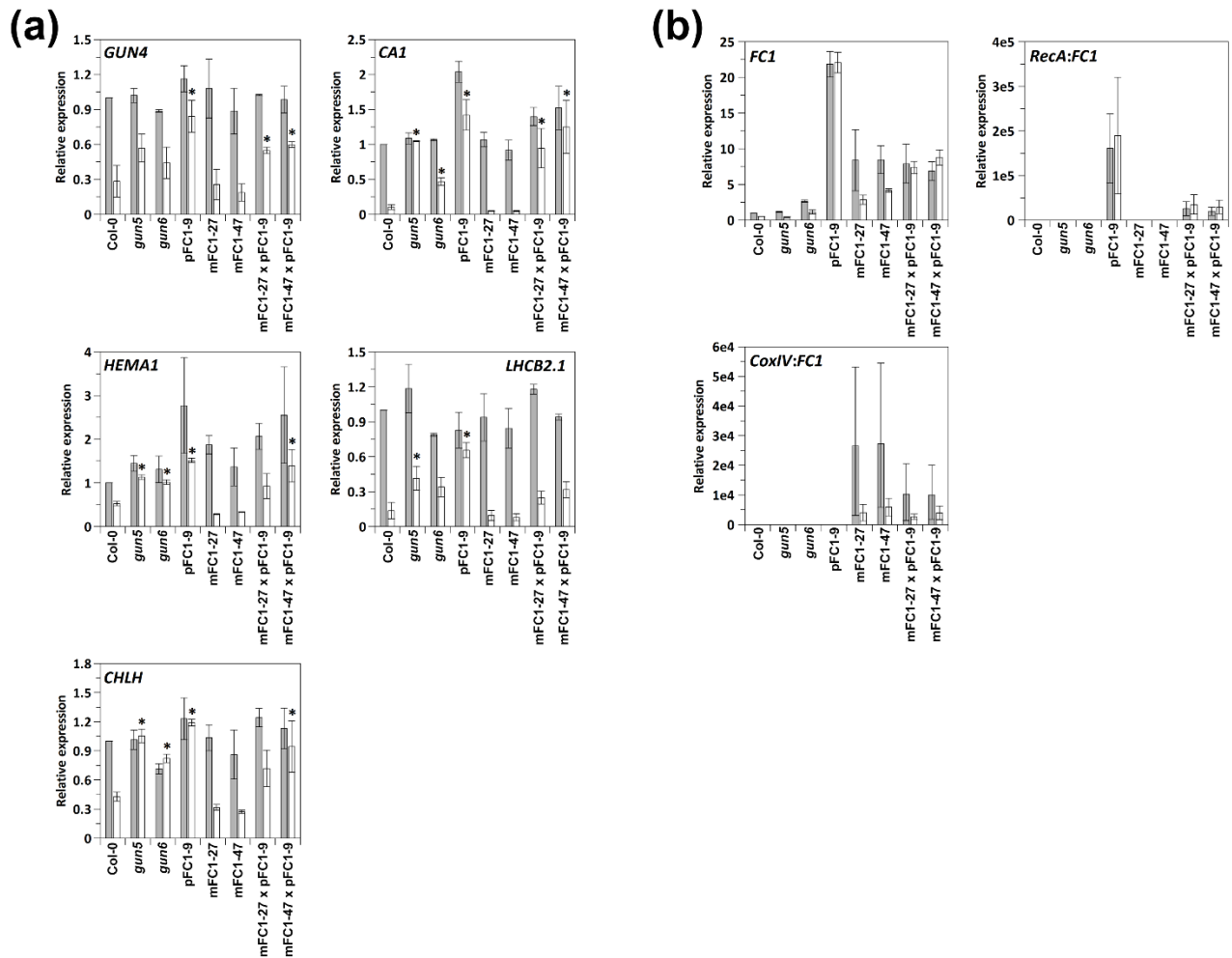


Figure S13. *FC1* overexpression in crosses of pFC1 and mFC1 transgenic lines. (a,b) Analysis of gene expression by qRT-PCR in F₁ seedlings derived from a cross between pFC1-9 and mFC1-27, or pFC1-9 and mFC1-47 was assessed in the absence (grey bars) or presence (white bars) of NF by qRT-PCR. The parent lines pFC1-9, mFC1-27 and mFC1-47, as-well-as *gun5* and *gun6*, were included as controls. Expression of *GUN4*, *CA1*, *HEMA1*, *LHC2.1* and *CHLH* (a) and total, plastid-targeted (*RecA:FC1*) and mitochondria-targeted (*CoxIV:FC1*) *FC1* (b) is shown relative to Col-0. Data shown is the mean \pm range of two independent biological replicates and asterisks denote a significant enhancement of nuclear gene expression vs. Col-0 +NF (determined as no overlap of the 95% confidence limits).

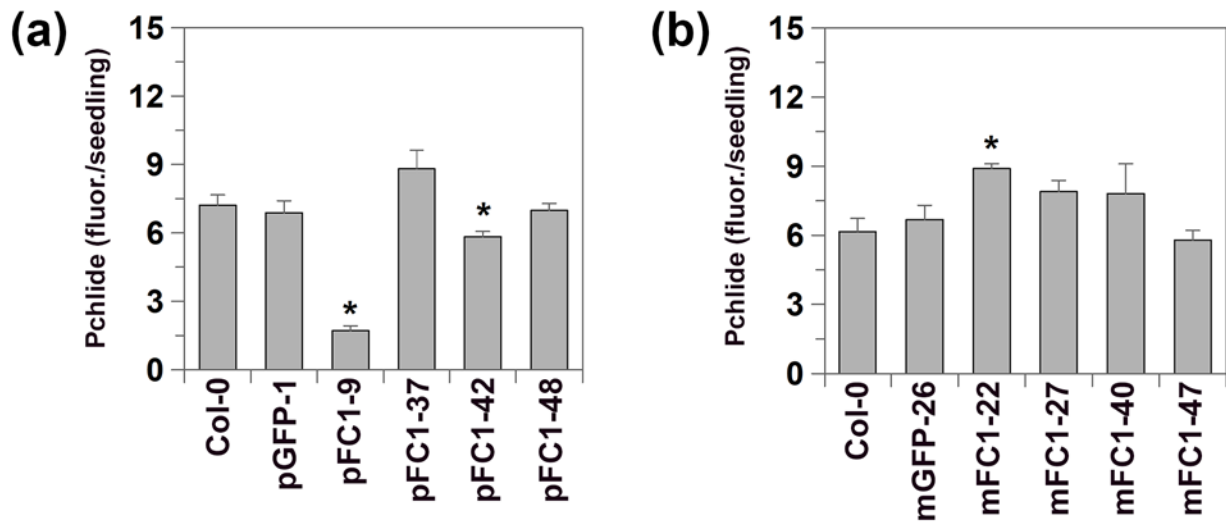


Figure S14. Protochlorophyllide is reduced in pFC1 lines. (a,b) Protochlorophyllide (Pchlide) content of pFC1 (a) and mFC1 (b) seedlings grown for 4 d in the dark. Data shown is the mean + SEM of three independent biological replicates and asterisks indicate a significant difference in percentage change vs. Col-0 for the same treatment (ANOVA, followed by Tukey's test).

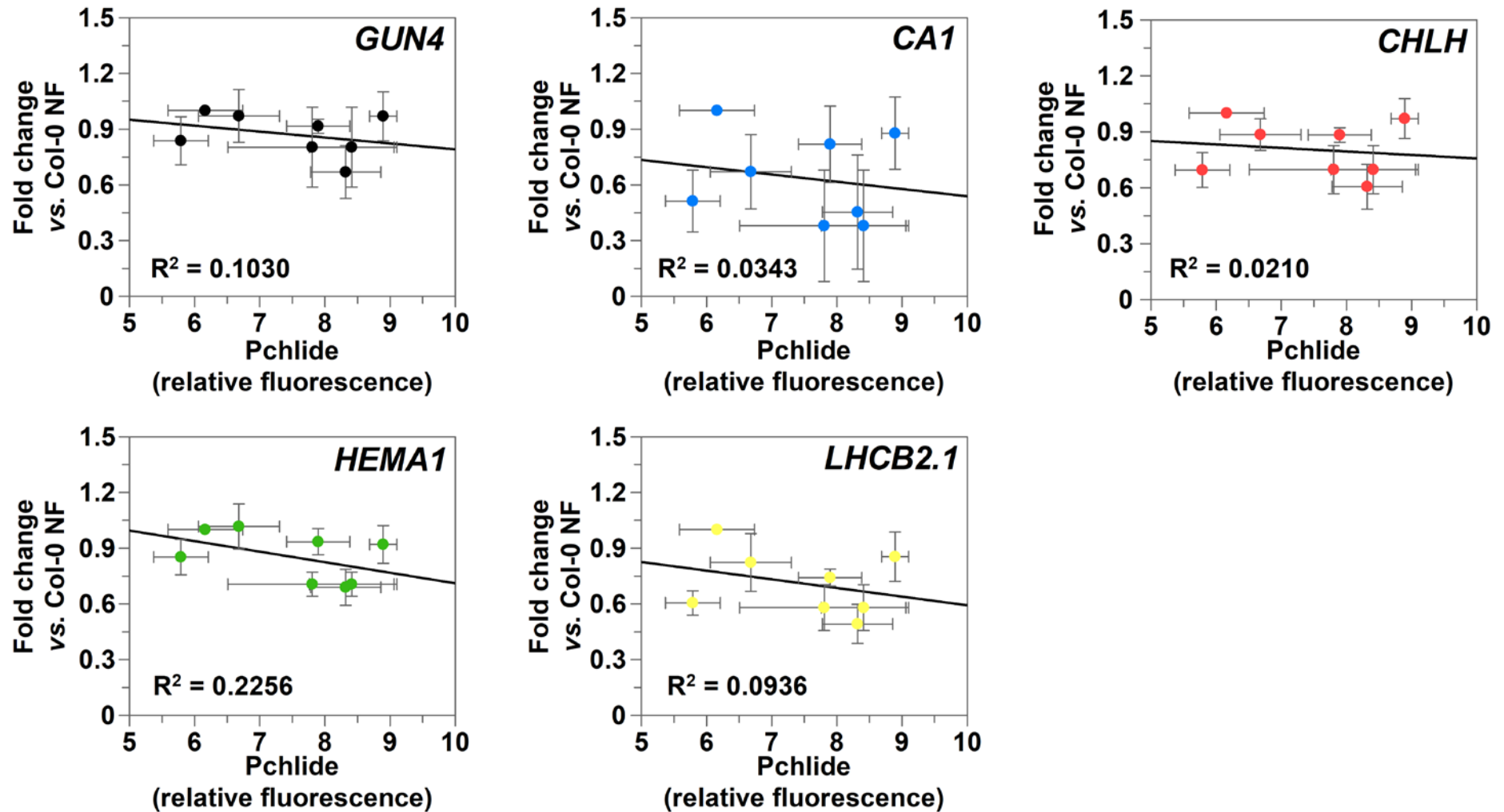


Figure S15. Enhancement of nuclear gene expression on NF does not correlate with protochlorophyllide levels in dark-grown mFC1 seedlings. Correlation plots of protochlorophyllide (Pchlide) in 4 d-old dark-grown mFC1 seedlings and against fold change in expression of *GUN4*, *CA1*, *HEMA1*, *LHCB2.1*, and *CHLH* vs. Col-0 on NF. Data represent the mean \pm SEM of three independent biological replicates.