Supplementary information for

The Arabidopsis SAFEGUARD1 suppresses singlet oxygen-induced stress responses by protecting grana margins

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SI Materials and Methods

Measurement of the methylation status of the Rubisco complex. Stroma proteins of *flu ex1* and *flu ex1 safe1* mutants were mixed well with an equal volume of gel loading buffer (80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 0.0006% (m/v) bromophenol blue) and fractionated on a 6% Native-PA gel. Then the gel was stained with Coomassie Blue staining solution (0.1% (m/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid) and destained with destaining solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid). The Rubisco complex corresponding to the biggest band in the gel was subjected to MS analysis.

Briefly, the gel piece was washed 3 times with LC-MS grade water and cut into smaller pieces (< 1 mm). Afterwards, each sample was washed for 2×10 min with 100 µl 20 mM ammonium bicarbonate (ABC) and 100 µl acetonitrile (ACN), respectively. To reduce disulfide bridges, 100 µl 10 mM dithiothreitol (in 20 mM ABC) was added to the sample and incubated at 56 °C for 20 min. Then the samples were washed with 100 µl ACN for 10 min and 100 µl 55 mM idoacetamide (IAA, in 20 mM ABC) for 30 min in the dark. Afterwards, the samples were washed for 2×10 min with ABC and ACN separately, and digested overnight with 0.1 µg/µl trypsin at 37 °C. The digested sample was measured on an LTQ Orbitrap (Thermo Fisher Scientific Inc, Waltham, USA) coupled to a Rheos Allegro liquid chromatograph (Flux Instruments GmbH, Basel, Switzerland). For analysis, 3 µl of the digested sample was loaded into the LTQ Orbitrap using a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Data was acquired by Xcalibur 2.0 (Thermo Fisher Scientific) and analyzed by MaxQuant (version 1.6.1.0, Max Planck Institute of Biochemistry, Planegg, Germany).

Transmission electron microscopy (TEM). To visualize the ultrastructure of chloroplasts from *flu ex1* and *flu ex1 safe1-5* mutants before and after the release of $^1O_2$, seeds were first grown under LL for 6 days, incubated in the dark for 4 h, and then grown in the dark, or transferred to light for 1 h and finally transferred to the dark for 3 h to minimize the starch content.

Cotyledons were cut into pieces (1 mm^2) and fixed in fixation buffer (75 mM sodium cacodylate, 2 mM $MgCl_2$, pH7.0) containing 2.5% (v/v) glutaraldehyde for 2 days at 4 °C. The leaf pieces were washed three times at room temperature using fixation buffer and post-fixed with 1% (w/v) osmium tetroxide for 2 h. After washing with fixation buffer and water, the samples were stained *en bloc* with 1% (w/v) uranyl acetate in 20% (v/v) acetone for 30 min. Samples were dehydrated in a series of graded acetone and embedded in Spurr´s resin. Ultrathin sections (thickness: 60 nm) were cut using a diamond knife on a Reichert Ultracut-E ultramicrotome (Reichert-Jung (now: Leica Microsystems), Wetzlar, Germany). Sections were mounted on collodion-coated copper grids, post-stained with lead citrate (80 mM, pH 13.0) and examined with an EM 912 transmission electron microscope (Zeiss, Oberkochen, Germany) equipped with an integrated OMEGA energy filter operated in the zero-loss mode at 80 kV. Images were acquired using a 2k × 2k slow-scan CCD camera (Tröndle Restlichtverstärkersysteme, Moorenweis, Germany).

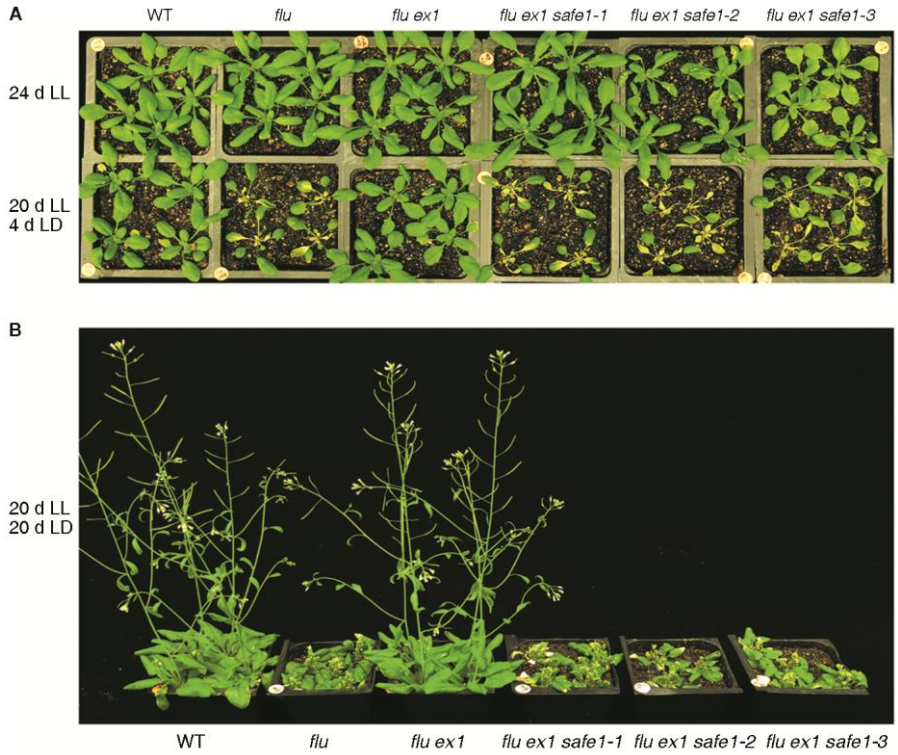
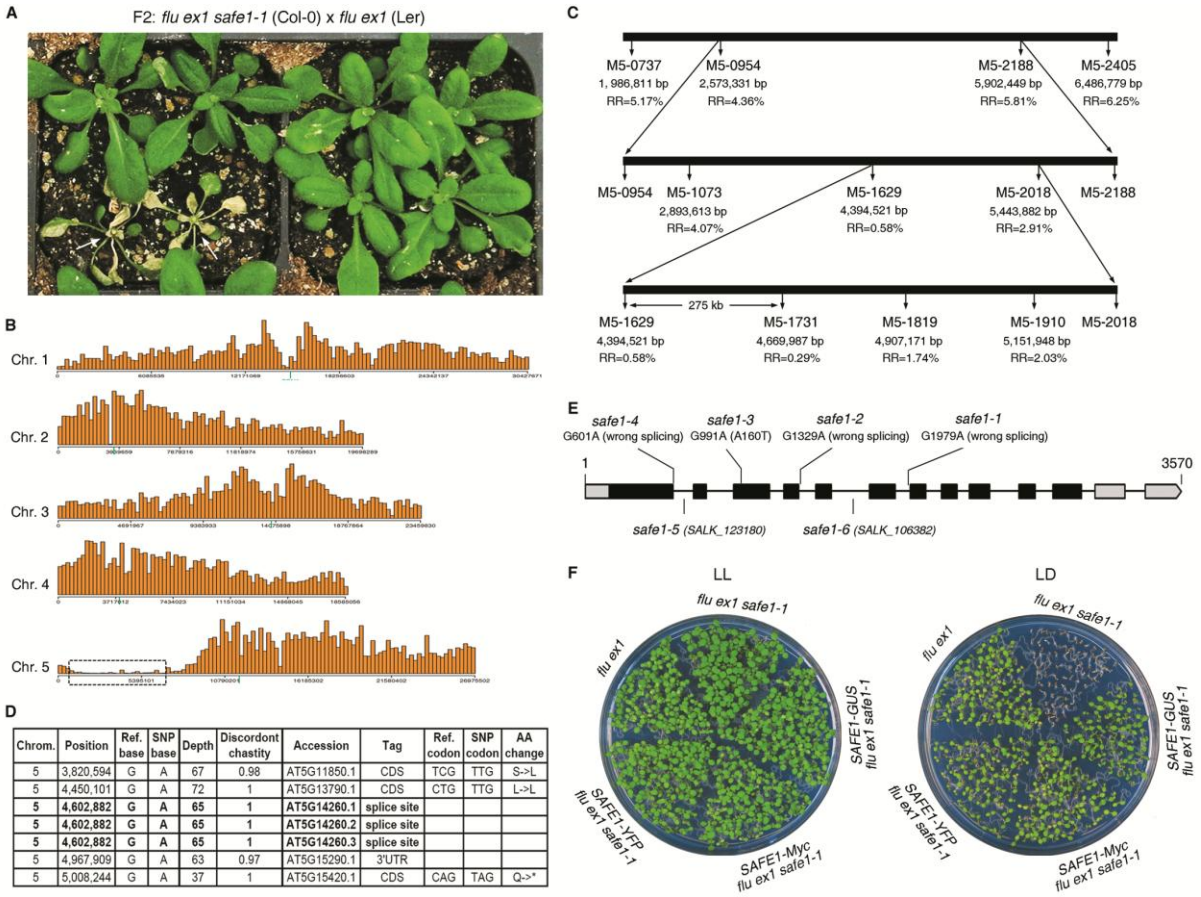


Fig. S1 Singlet oxygen induces cell death and growth inhibition in three allelic lines of mature *flu ex1 safe1* plants. WT, *flu*, *flu ex1* and *flu ex1 safe1* were grown under LL for 20 days and were further kept under LL or transferred to LD and grown for an additional 4 (*A*) or 20 (*B*) days.



Chrom.	Position	Ref. base	SNP base	Depth	Discordont chastity	Accession	Tag	Ref. codon	SNP codon	AA change
5	3,820,594	G	A	67	0.98	AT5G11850.1	CDS	TCG	TTG	S->L
5	4,450,101	G	A	72	1	AT5G13790.1	CDS	CTG	TTG	L->L
5	**4,602,882**	**G**	**A**	**65**	**1**	**AT5G14260.1**	**splice site**			
5	**4,602,882**	**G**	**A**	**65**	**1**	**AT5G14260.2**	**splice site**			
5	**4,602,882**	**G**	**A**	**65**	**1**	**AT5G14260.3**	**splice site**			
5	4,967,909	G	A	63	0.97	AT5G15290.1	3'UTR			
5	5,008,244	G	A	37	1	AT5G15420.1	CDS	CAG	TAG	Q->*

Fig. S2 Identification of mutation sites in the four *safe1* alleles. (*A*) Segregation analysis of an F2 population from a cross between *flu ex1 safe1-1* (Col-0) and *flu ex1* (Ler). About 1/4 of the plants showed typical $^1O_2$-induced cell death in the F2 population after transfer to LD conditions for 4 days, implying that the *safe1* mutation is recessive. (*B*) Identification of the *safe1* mutation by whole genome sequencing. Data represent the distribution of SNP sites in the pooled mutant DNAs on the five chromosomes of *Arabidopsis*. The pooled DNA was extracted from approximately 200 plants that showed the mutant phenotype in the F2 population (*flu ex1 safe1-1* (Col-0) × *flu ex1* (Ler)). A region in the upper part of Chr. 5 with extremely low SNP frequency (labeled by a rectangle) was considered to harbor the *safe1* mutation. (*C*) The process of map-based cloning for definitive identification of the *safe1* mutation. Molecular markers with their chromosomal positions and corresponding recombination rates (RR) are shown. The *safe1* mutation was localized to a region of 275 kb, between the markers M5-1629 and M5-1731. (*D*) Within this region, there are two mutations. One is a silent mutation which causes no amino acid change, and the other is in the splicing site of the gene *At5G14260*. (*E*) A schematic diagram showing the mutation sites of the four *safe1* alleles and two T-DNA insertions in the *SAFE1* (*At5g14260*) gene. The altered nucleotides/T-DNA insertion sites are indicated below/after the allele numbers. 5' UTR: gray box; exons: black boxes; introns: black lines; 3' UTR: gray box/arrow. Nucleotides are numbered from the beginning of the 5' UTR. (*F*) Genetic complementation of *flu ex1 safe1*. Introduction of *SAFE1* (*SAFE1-GUS*, *SAFE1-Myc* or *SAFE1-YFP*) driven by its native promoter into *flu ex1 safe1-1* restores the *flu ex1* phenotype. The growth behavior of *flu ex1*, *flu ex1 safe1-1* and three complemented lines under LL and LD is shown.

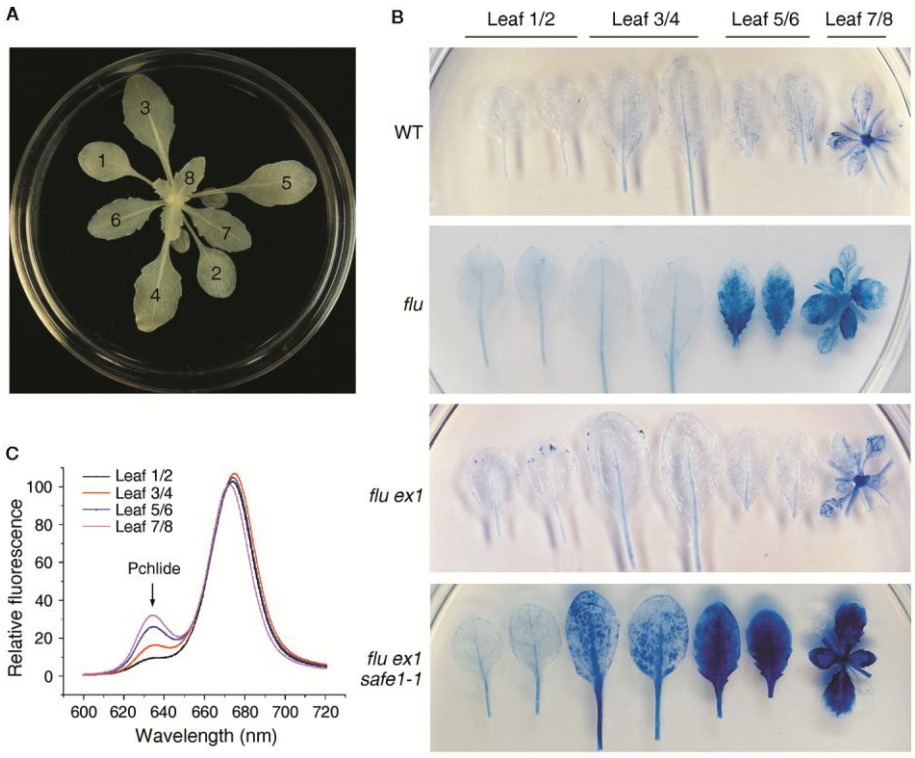


Fig. S3 The extent of $^1O_2$-induced cell death in *flu* and *flu ex1 safe1* is positively correlated with the level of Pchlide accumulation in leaves. (*A*) A bleached mature plant. Leaves are numbered from old to young. (*B*) Singlet oxygen induces much more severe cell death responses in young leaves than old leaves. WT, *flu*, *flu ex1* and *flu ex1 safe1-1* plants (20 days old) were kept in the dark for 8 h and re-exposed to light for 24 h. Dead cells were visualized by trypan blue staining. (*C*) Quantification of Pchlide accumulation in old and young leaves of *flu ex1 safe1-5*. Nonesterified porphyrins were extracted from plants that had been incubated in the dark for 8 h, and their fluorescence emission spectra were recorded. Young leaves accumulated more Pchlide and showed much severe cell death responses than the older leaves.

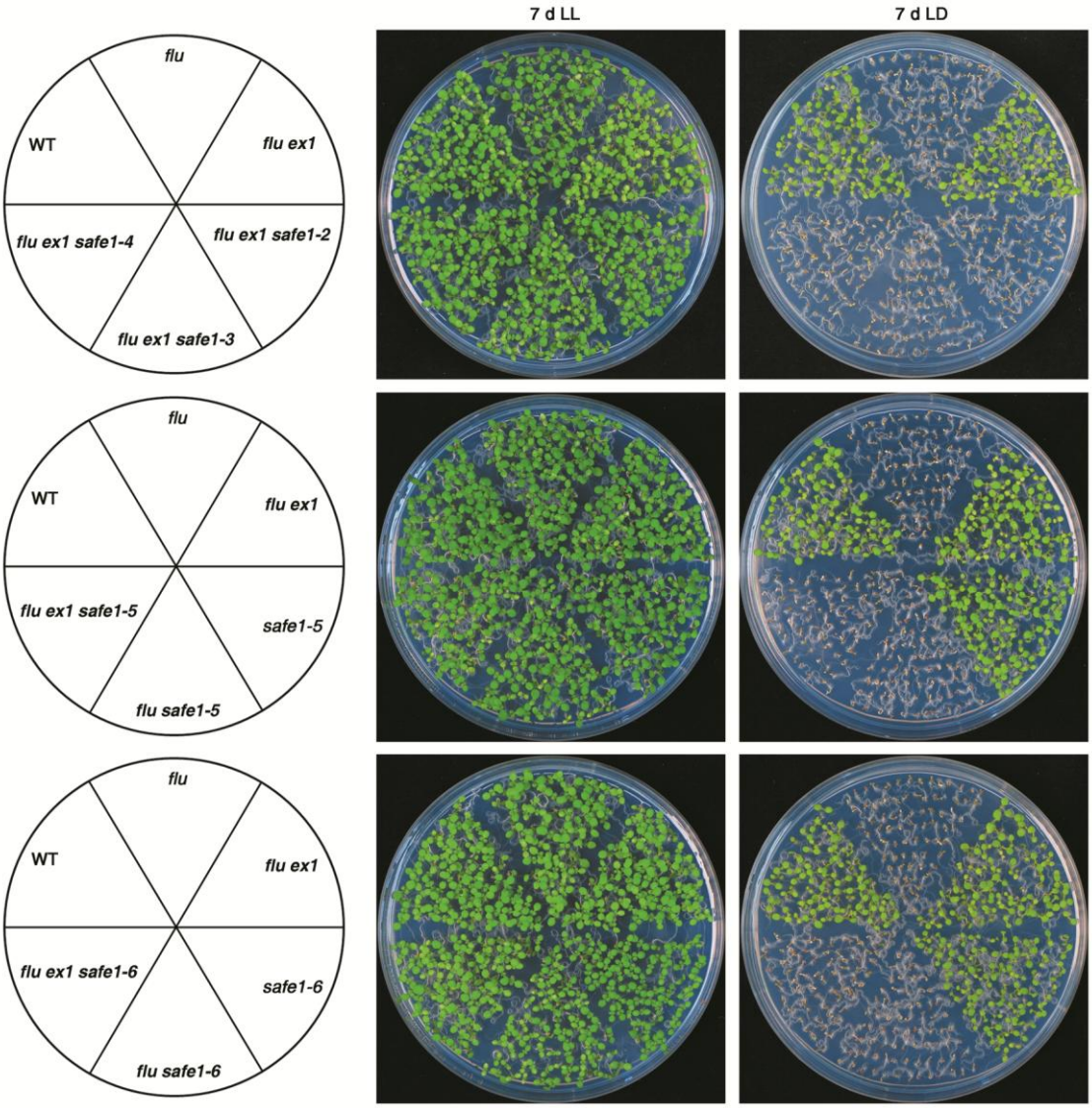


Fig. S4 Seedlings lacking functional SAFE1 are hypersensitive to $^1O_2$ stress. Images of WT, various mutants (left panel) and their growth behaviors under LL (middle panel) and LD (right panel) are shown. Note that the *safe1* single mutants (*safe1-5* and *safe1-6*) grow like WT and *flu ex1*, but that the *flu safe1* double mutants (*flu safe1-5* and *flu safe1-6*) die at early developmental stages when grown under LD, as do *flu* and *flu ex1 safe1*. Generation of $^1O_2$ was achieved by growing seedlings under LD conditions.

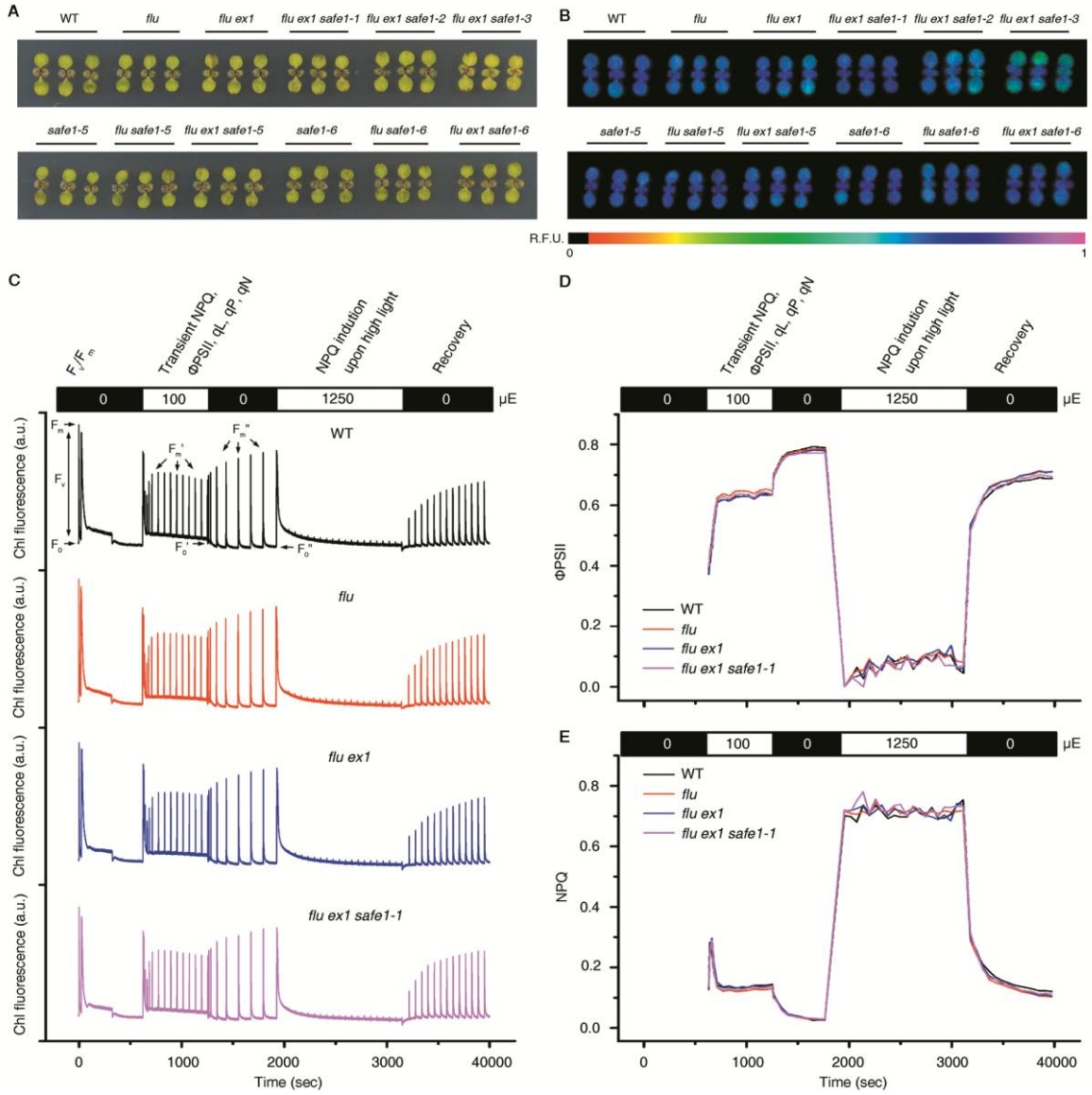


Fig. S5 Functional loss of FLU, EX1 and/or SAFE1 has no obvious effect on photosynthetic performance under moderate or high light. (*A*, *B*) Images and transient chlorophyll fluorescence data for WT, *flu*, *flu ex1*, *safe1*, *flu safe1* and five allelic *flu ex1 safe1* lines following exposure to high light. Seedlings were grown under LL for 6 days and exposed to high light (1000 μmol photons m^{-2} s^{-1}) for 2 days. R.F.U.: relative fluorescence unit. (*C*) Chlorophyll fluorescence (PAM) traces of WT, *flu*, *flu ex1* and *flu ex1 safe1-1* grown under moderate and high light were recorded with an Imaging PAM system. The sequences of light conditions and photosynthetic parameters that were determined during this experimental run are indicated above the PAM traces. (*D*, *E*) Effective quantum yield of PSII (ΦPSII) and non-photochemical quenching (NPQ) are not affected in *flu*, *flu ex1* or *flu ex1 safe1-1*. Six-day-old seedlings grown under LL were dark-adapted for 10 min and then subjected to PAM analysis. F_0: minimal fluorescence in dark-adapted leaves; F_m: maximum fluorescence in dark-adapted leaves; F_v/F_m: maximum quantum yield of PSII photochemistry in dark-adapted leaves; F_0': minimum Chl fluorescence in light-adapted leaves; F_m': maximum Chl fluorescence in light-adapted leaves; qL: fraction of open PSII centers; qP: coefficient of photochemical quenching; qN: coefficient of non-photochemical quenching.

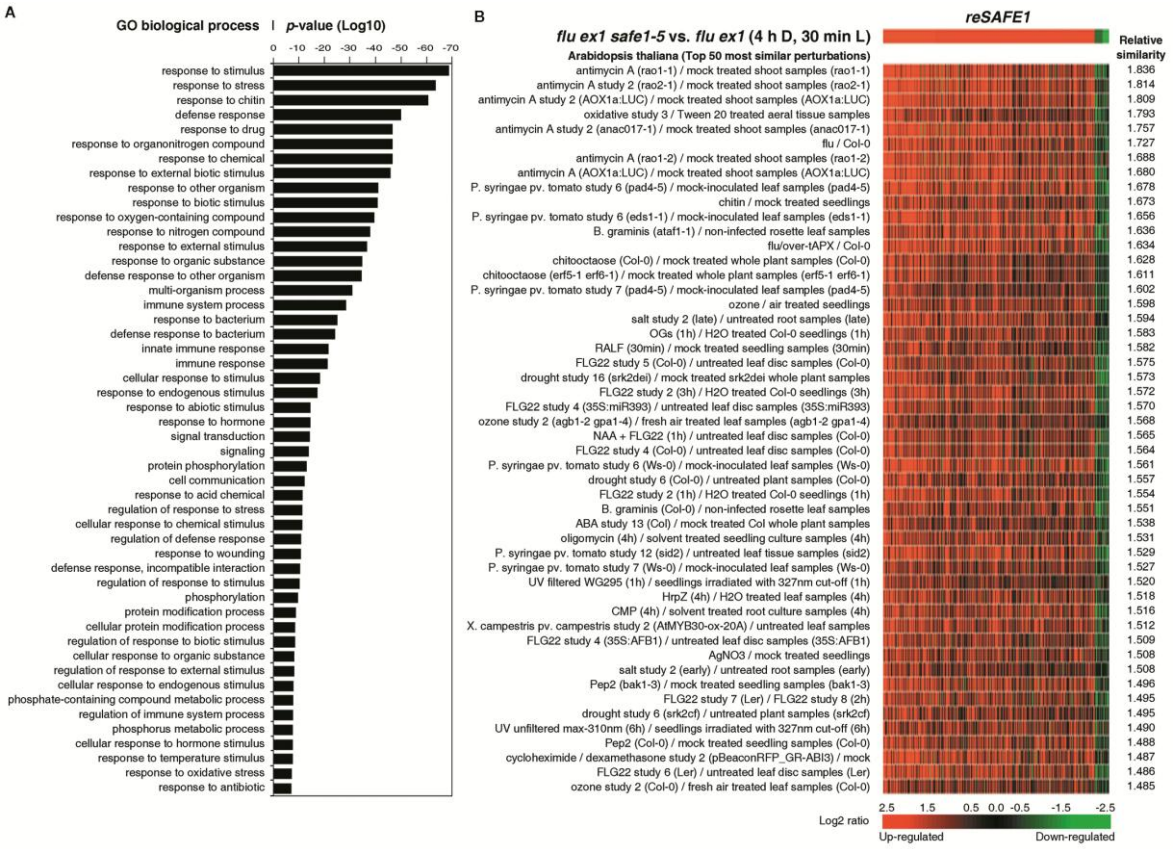


Fig. S6 Gene Ontology (GO) analysis and comparison of *reSAFE1* with genes induced by various stimuli. (*A*) GO analysis of the biological category indicates that stress-, immune- and defense-related genes are highly enriched in *reSAFE1*. (*B*) Mutants/perturbations that evoke gene expression changes most similar to *reSAFE1*.

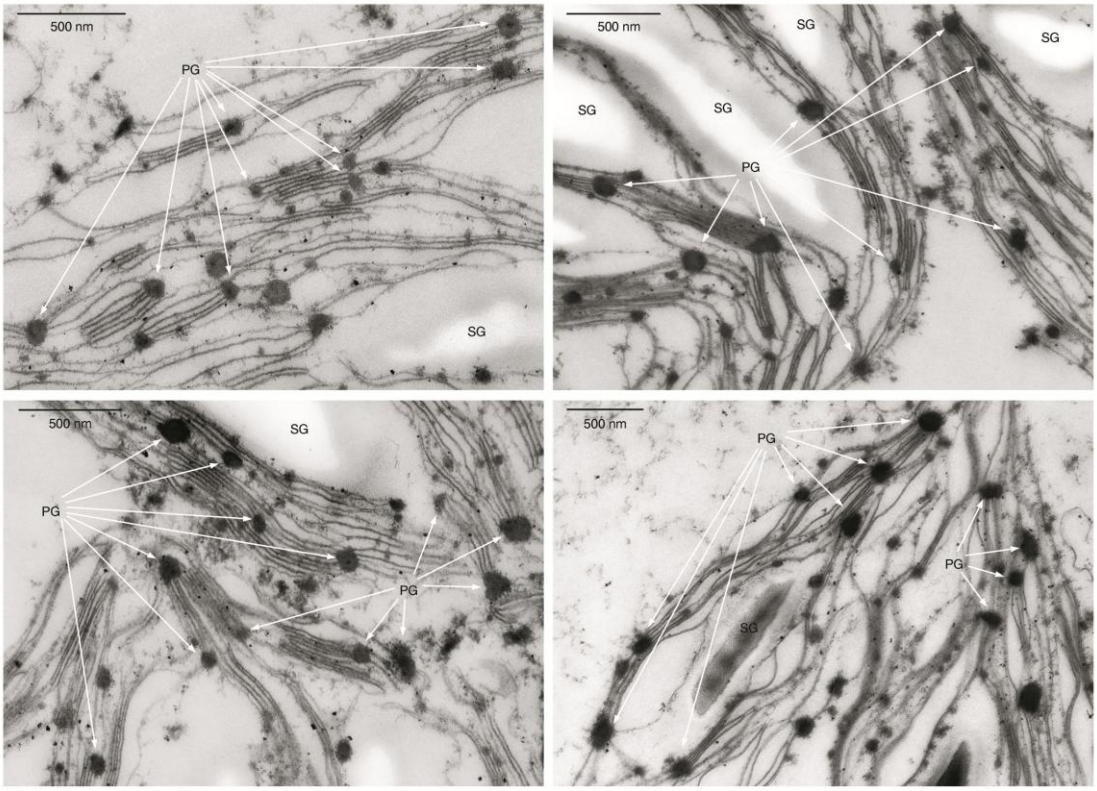


Fig. S7 TEM micrographs showing the specific accumulation of plastoglobules on the grana margins after release of $^1O_2$ in *flu ex1 safe1-5*. PG: plastoglobules; SG: starch granules.

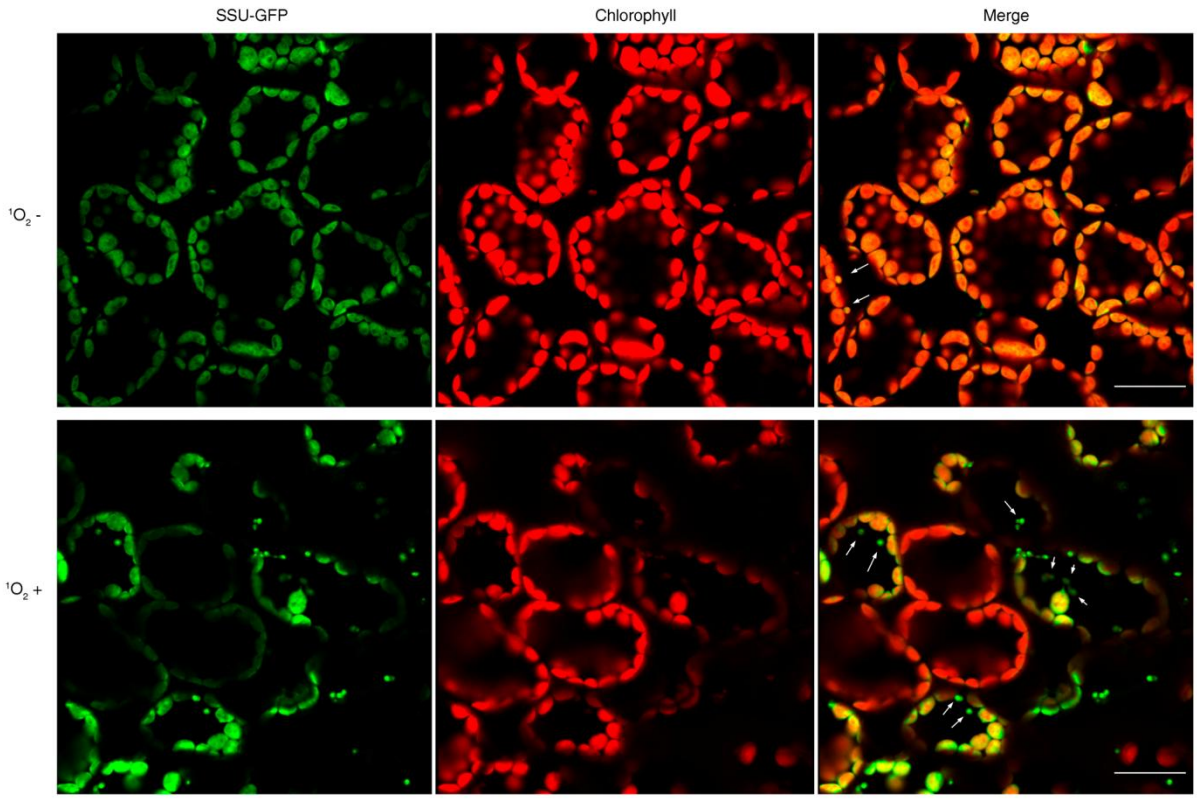


Fig. S8 Rubisco is incorporated in the $^1O_2$-induced chloroplast-originated vesicles. The small subunit of Rubisco (SSU) under control of the cauliflower mosaic virus 35S promoter was labeled with a C-terminal GFP tag and introduced into the *flu ex1* double mutant. Release of $^1O_2$ ($^1O_2$ +) was achieved by incubating 6-d-old seedlings in the dark for 4 hours and exposing them to light for 4 hours. The $^1O_2$-induced chloroplast-originated vesicles are indicated by white arrows. Bar = 20 μm.

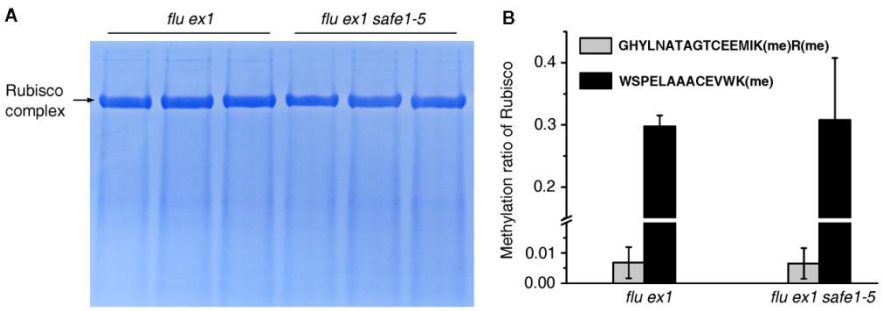


Fig. S9 Lack of functional SAFE1 does not affect the methylation status of the Rubisco complex. (*A*) Stroma proteins of *flu ex1* and *flu ex1 safe1-5* were fractionated on a 7% Native PA gel, and the Rubisco complexes were subjected to mass spectrometric analysis. (*B*) Three methylation sites in two peptides derived from Rubisco complexes were identified and their methylation ratios are shown. Data represent mean values with SD of three independent replicates.

Captions for Datasets S1 to S7:

Dataset S1 List of genes whose expression changed ≥ 2-fold in *flu ex1 safe1-5* compared to *flu ex1* at the end of 4 h dark treatment.

Dataset S2 List of genes whose expression changed ≥ 2-fold in *flu ex1* after 4 h dark/30 min light treatment compared with the end of 4 h dark treatment.

Dataset S3 List of genes whose expression changed ≥ 2-fold in *flu ex1* after 4 h dark/60 min light treatment compared with the end of 4 h dark treatment.

Dataset S4 List of genes whose expression changed ≥ 2-fold in *flu ex1 safe1-5* after 4 h dark/30 min light treatment compared with the end of 4 h dark treatment.

Dataset S5 List of genes whose expression changed ≥ 2-fold in *flu ex1 safe1-5* after 4 h dark/60 min light treatment compared with the end of 4 h dark treatment. Genes marked in bold have been verified by qRT-PCR.

Dataset S6 List of genes that are specifically ≥ 2-fold changed in *flu ex1 safe1-5* (*reSAFE1*).

Dataset S7 Primers used in this study.