Supplementary Information

Oxidative post-translational modification of EXECUTER1 is required for singlet oxygen sensing in plastids

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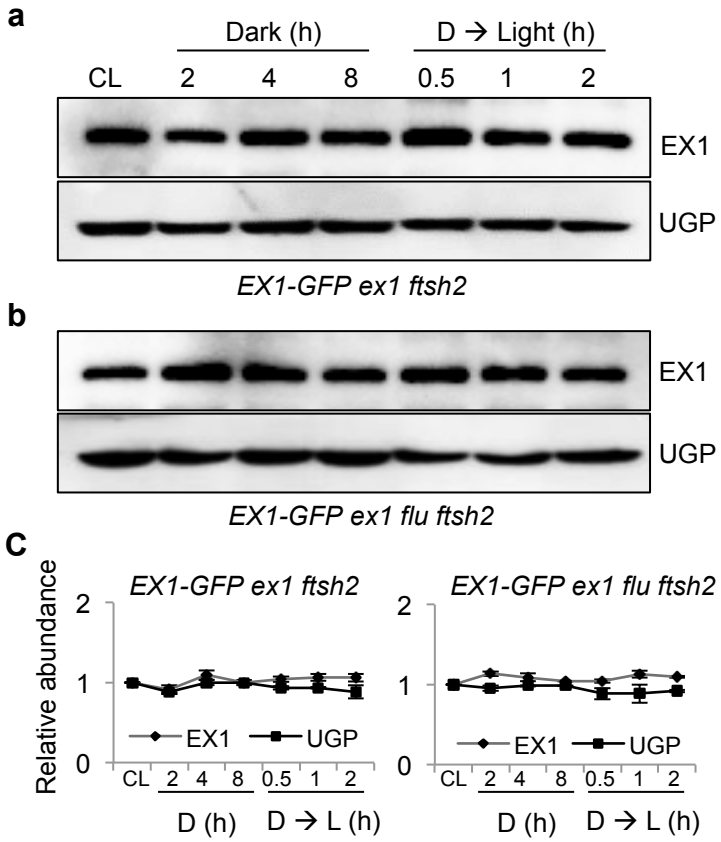
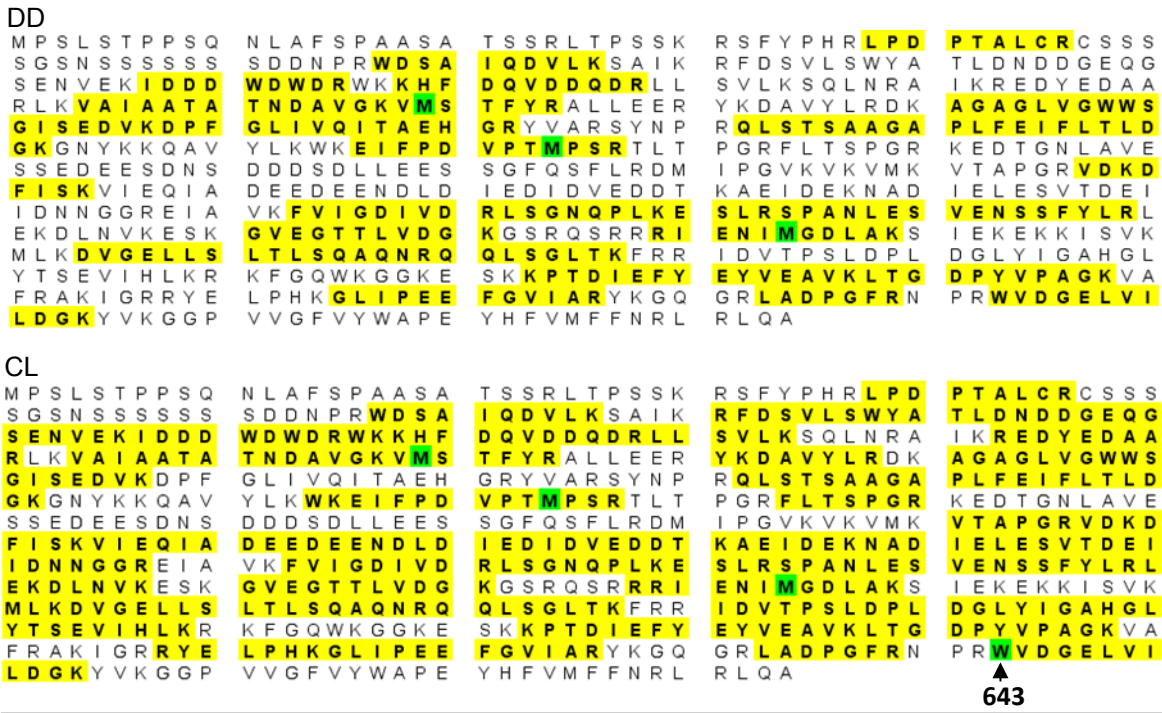


Supplementary Fig. 1 Light- and $^1O_2$-dependent EX1 degradation is FtsH protease dependent. CL-grown 5-d-old transgenic seedlings of **(a)** *ftsh2 ex1* and **(b)** *ftsh2 ex1 flu* expressing *EX1-GFP* under the control of the 35S promoter were transferred to the dark (for 2, 4, 8 h) and then re-exposed to light (for 0.5, 1, 2 h) at the light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Total protein was extracted and analyzed by western blot. Cytosolic UDP-glucose pyrophosphorylase (UGP) was used to show equal loading. EX1-GFP and UGPase were detected using antibodies against GFP, and UGP, respectively. (**c**) The levels of EX1-GFP in the dark or after re-exposing to light were compared to its abundances under CL conditions. Average intensity values of the protein bands were calculated using AzureSpot software v14.0 (AZURE). Data represents mean of three biological repeats. Error bars show standard error of the mean.

a



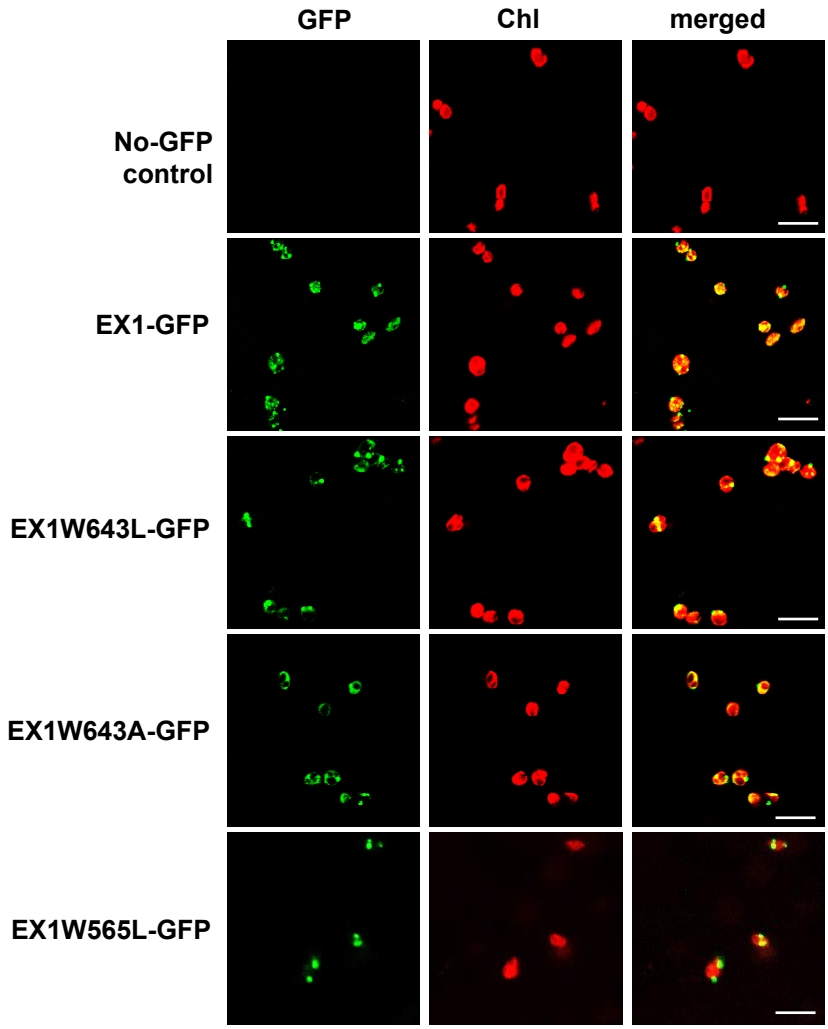
Sample	Protein identification Probability (%)	Protein in total spectra (%)	Exclusive unique peptide count	Exclusive unique spectrum count	Total spectrum count	Coverage (%)
DD	100	0.22	26	37	53	40
CL	100	0.32	34	59	98	**53**

b

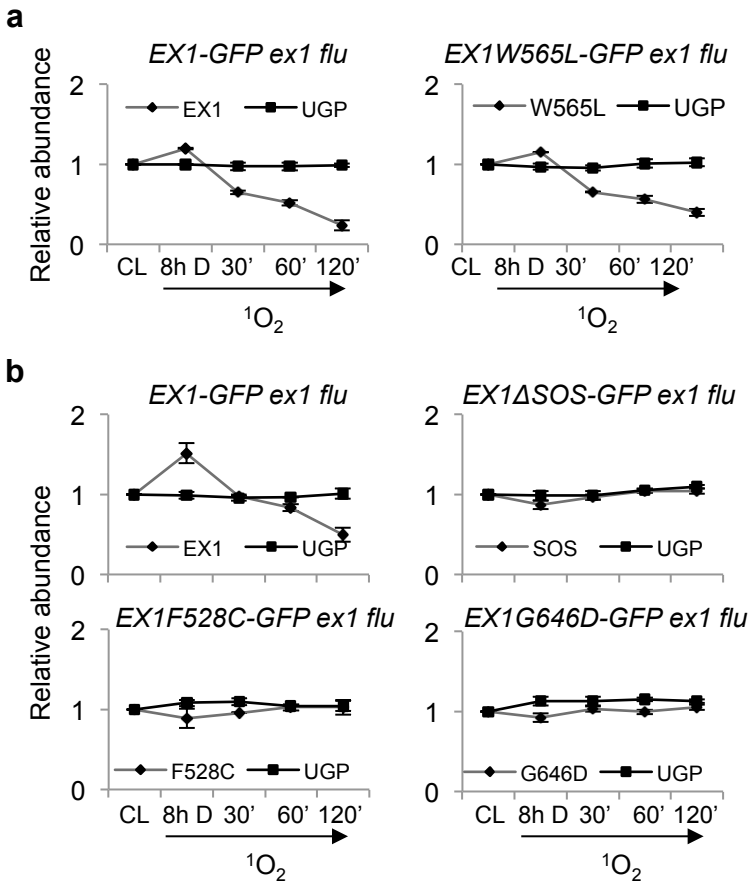
MPSLSTPPSQNLAFSPAASATSSRLTPSSKRSFYPHRLPDPTALCRCSSSSGSNSSSSSSSDDNPR**W**DSA 70
IQDVLKSAIKRFDSVLS**W**YATLDNDDGEQGSENVEKIDDD**W**D**W**DR**W**KKHFDQVDDQDRLLSVLKSQLNRA 140
IKREDYEDAARLKVAIAATATNDAVGKVMSTFYRALLEERYKDAVYLRDKAGAGLVG**WW**SGISEDVKDPF 210
GLIVQITAEHGRYVARSYNPRQLSTSAAGAPLFEIFLTLDGKGNYKKQAVYLK**W**KEIFPDVPTMPSRTLT 280
PGRFLTSPGRKEDTGNLAVESSEDEESDNSDDDSDLLEESSGFQSFLRDMIPGVKVKVMKVTAPGRVDKD 350
FISKVIEQIADEEDEENDLDIEDIDVEDDTKAEIDEKNADIELESVTDEIIDNNGGREIAVKFVIGDIVD 420
RLSGNQPLKESLRSPANLESVENSSFYLRLEKDLNVKESKGVEGTTLVDGKGSRQSRRRIENIMGDLAKS 490
IEKEKKISVKMLKDVGELLSLTLSQAQNRQQLSGLTKFRRIDVTPSLDPLDGLYIGAHGLYTSEVIHLKR 560
KFGQ**W**KGGKESKKPTDIEFYEYVEAVKLTGDPYVPAGKVAFRAKIGRRYELPHKGLIPEEFGVIARYKGQ 630
GRLADPGFRNPR**W**VDGELVILDGKYVKGGPVVGFVY**W**APEYHFVMFFNRLRLQA 684

643

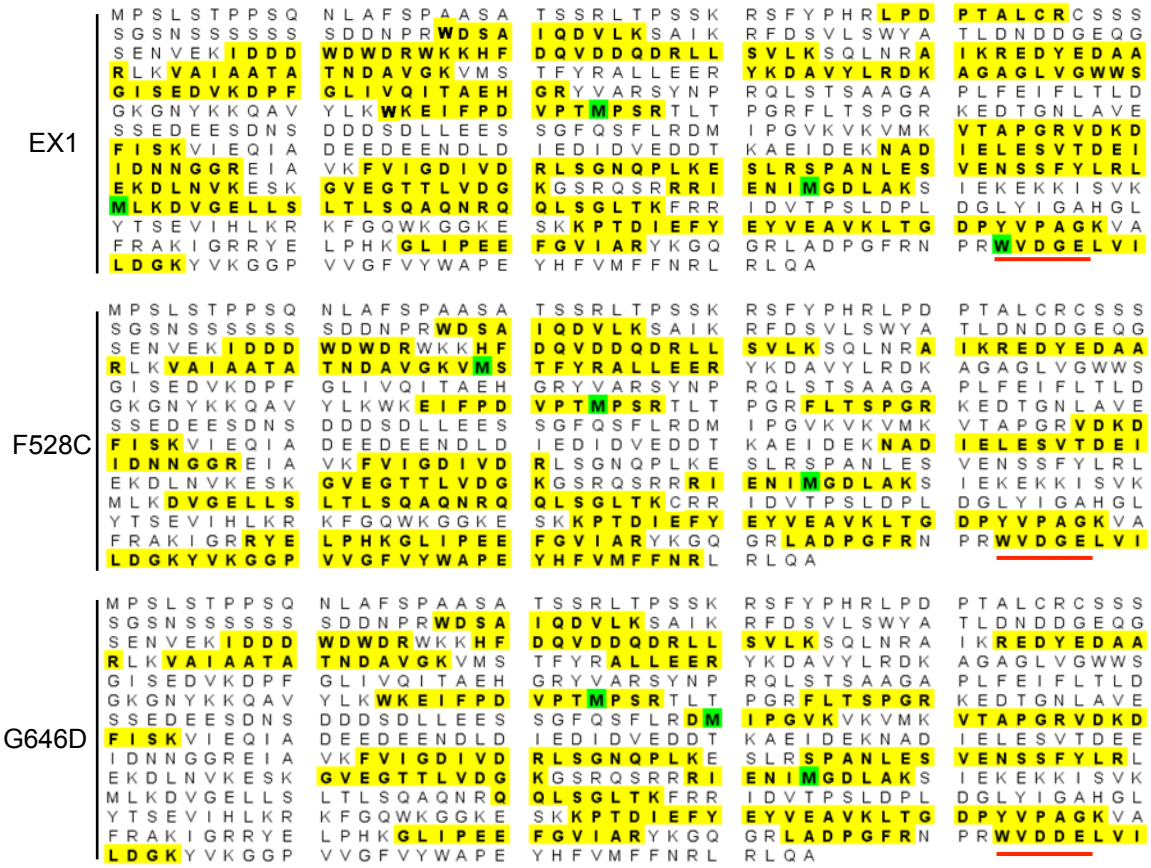
Supplementary Fig. 2 MS analysis showed a significant peptide coverage for EX1. **a** Peptide coverage of EX1 protein from dark-(DD) and light-(CL) grown seedlings as visualized in Scaffold viewer (Scaffold 4.0.7, Proteome Software Inc.). Peptides identified by tandem-MS analysis are shown in yellow. Oxidized Met (M) and Trp (W) residues are highlighted in green. Protein identification parameters are enlisted at the bottom. **b** EX1 protein contains 11 Trp residues (highlighted in bold), and all were covered in MS analysis (shown in grey background). Trp643 undergoing $^1O_2$-induced oxidation is highlighted in red. UVR B/C and DUF3506 (SOS) domains are underlined.



Supplementary Fig. 3 EX1 proteins carrying Trp643 substitutions are targeted to chloroplasts. Confocal images showing the localization of EX1-GFP, EX1W643A-GFP, EX1W643L-GFP, and EX1W565L-GFP in the chloroplasts of *N. benthamiana* leaves. Healthy leaves of 3-weeks-old *N. benthamiana* were infiltrated with Agrobacterium carrying 35S::*EX1-GFP*, 35S::*EX1W643A-GFP*, 35S::*EX1W643L-GFP,* and 35S::*EX1W565L-GFP*. The GFP signal was observed under confocal laser scanning microscope. Leaves without infiltration were used as negative controls.

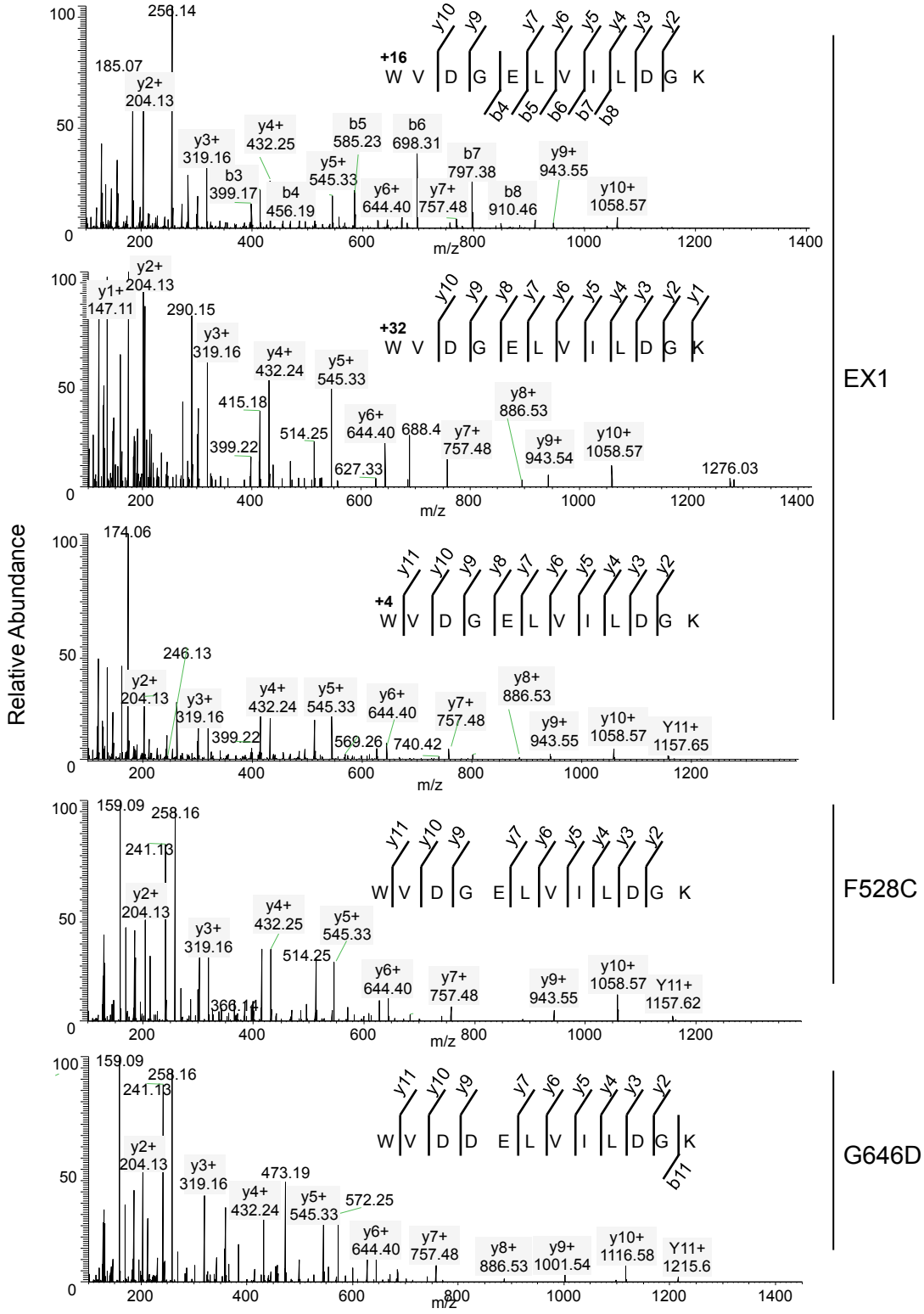


Supplementary Fig. 4 Relative abundances of intact and modified EX1 proteins upon $^1O_2$ burst. The steady state levels of intact and modified EX1-GFP proteins upon dark- or after re-exposing to light were compared to their abundances under CL conditions as shown in (**a**) figures 4g and (**b**) 5e. The abundance of UGP was shown as equal loading control. Average intensity values of the protein bands were calculated using AzureSpot software v14.0 (AZURE). Data represents mean of three biological repeats. Errors bars show standard error of the mean.

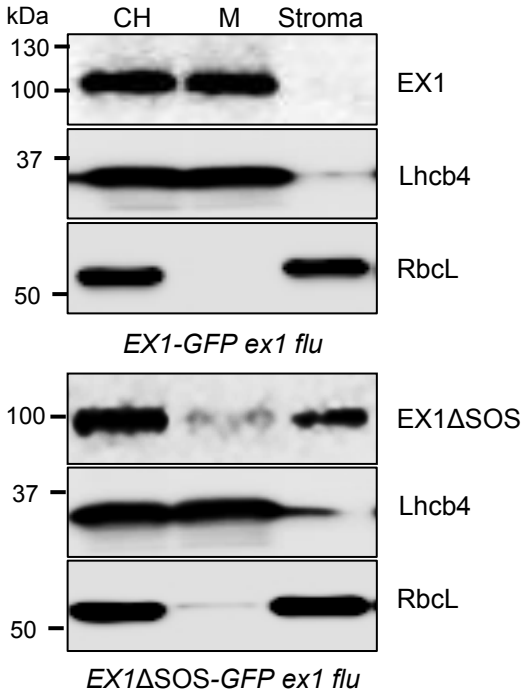


Protein	Protein identification Probability (%)	Total spectrum count	Coverage (%)
EX1	100	53	40
F528C	100	30	39
G646D	100	29	37

Supplementary Fig. 5 Oxi-PTM analysis revealed Trp643 undergoes oxidation in EX1-GFP but not in EX1F528C-GFP and EX1G646D-GFP proteins. For oxidation analysis, 5-d-old transgenic seedlings of *EX1-GFP ex1 flu, EX1F528C-GFP ex1 flu*, and *EX1G646D-GFP ex1 flu* initially grown under CL condition were transferred in the dark, kept for 2 h, and re-exposed to light for 10 min. Total protein was extracted and the immune-reactive EX1-GFP proteins were enriched by using magnetic agarose beads conjugated with GFP antibody. Each protein sample was then subjected to PTM analysis. Peptide coverage of EX1 protein from EX1-GFP, EX1F528C-GFP and EX1G646D-GFP samples was visualized in Scaffold viewer. Peptides identified by tandem-MS analysis are shown in yellow. Oxidized Met (M) and Trp (W) residues are highlighted in green.



Supplementary Fig. 6 MS spectra of peptides carrying Trp643 in native and modified EX1 proteins. All three oxidized variants of Trp643 in peptide ^{643}WVDGELVILDGK^{654} were detected in EX1-GFP. However, Trp643 remains unoxidized in peptides ^{643}WVDGELVILDGK^{654} in EX1F528C-GFP and ^{643}WVDDELVILDGK^{654} in EX1G646D-GFP.



Supplementary Fig. 7 SOS domain-deleted EX1 is unable to attach to the thylakoid membrane which is indispensible for perceiving $^1O_2$. EX1-GFP proteins reside in the thylakoid membrane whereas the majority of the EX1ΔSOS-GFP were found in the stroma. Chloroplast (CH) isolated from 3-weeks-old plants of *EX1-GFP ex1 flu* and *EX1ΔSOS-GFP ex1 flu* were lysed and separated by centrifugation into membrane (M), and stroma fractions. Proteins were solubilized, separated by SDS-PAGE, and analyzed on western blots using specific antibodies against GFP, Lhcb4, and Rubisco large subunit (RbcL). Lhcb4 and RbcL serve as membrane and stroma controls, respectively.

Supplementary Table 1: MS-based PTM analysis identified various Trp oxidation products at Trp643 in peptide ^{643}WVDGELVILDGK^{654}.

Oxidation status	Mass shift	Observed mass (m/z)	Charge state	Actual mass (Da)	Error (ppm)	Probability (%)	Mascot ion score
No	0	672.37	2+	1342.72	0.0026	100	62.2
Mono (OIA)	+16	680.36	2+	1358.71	1.4	100	43.1
Di (NFK)	+32	688.36	2+	1374.71	4.7	100	63.8
Di* (KYN)	+4	674.36	2+	1346.71	-0.0011	100	64.7

OIA: oxindolylalanine, NFK: *N*-formylkynurenine, KYN: Kynurenine

Supplementary Table 2: MS-based PTM analysis identified $^1O_2$-induced oxidation at Trp643 side chains in EX1 but not in EX1 proteins carrying F528C and G646D substitutions.

Protein	Sequence	Residue modified	Mass shift	Observed mass (m/z)	Charge state	Actual mass (Da)	Error (ppm)	Probability (%)
EX1	$^{643}W^{ox}$VDGELVILDGK^{654}	Trp643	+16	680.36	2+	1358.71	3.0	98
EX1	$^{643}W^{ox}$VDGELVILDGK^{654}	Trp643	+32	688.36	2+	1374.70	-0.68	100
EX1	$^{643}W^{ox}$VDGELVILDGK^{654}	Trp643	+4	674.36	2+	1346.71	1.2	100
EX1F528C	^{643}WVDGELVILDGK^{654}	No	0	672.36	2+	1342.71	-0.91	100
EX1G646D	^{643}WVDDELVILDGK^{654}	No	0	701.37	2+	1400.37	-0.59	100

Supplementary Table 3: List of primers used in this study.

Gene ID	Gene name	Primer sequence (5' to 3')	Primer length	Product size (bps)	Used for
AT3G18780	*ACT2*	F: TATGTATGTCGCCATCCAA	19	76	qRT-PCR
		R: ACCAGAATCCAGCACAATA	19		
AT2G38470	*WRKY33*	F: GAAACAAATGGTGGGAATGG	20	217	
		R: TGTCGTGTGATGCTCTCTCC	20		
AT3G56710	*SIB1*	F: CGACTTTTCTCACCACGACA	20	235	
		R: TCGGAGGAAGGGGAATAGAT	20		
AT2G41180	*WRKY40*	F: GTCAAGACGCCGCTGATCTA	20	166	
		R: GCCGACATCTGAGGAGCATT	20		
AT3G18780	*ACT2*	F: CTGGGATTTGTAAAGCAGCTG	21	265	RT-PCR
		R: TCAAGTTCCATGGAGCAAAAG	21		
AT4G33630	*EX1-GFP*	F: ATGCCTTCCTTATCCACCCCG	20	2159	
		R: CCATTAACATCACCATCTAAT	21		
AT4G33630	*EX1*	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCTTCCTTATCCACCCCG	20	EX1	Cloning and AA substitution and domain deletion [1st PCR: F & R1, F1 and R; 2nd PCR: F + R using mixture of purified primary products]
		R: GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCTTGAAGCCTCAGGCGGTT	21		
		F1: CGTAATCCGAGAGCGGTAGATGGCGAG	27	643A	
		R1: CTCGCCATCTACCGCTCTCGGATTACG	27		
		F1: CGTAATCCGAGATTGGTAGATGGCGAG	27	643L	
		R1: CTCGCCATCTACCAATCTCGGATTACG	27		
		F1: CAAATTTGGCCAGTTGAAAGGTGG	24	565L	
		R1: CCACCTTTCAACTGGCCAAATTTG	24		
		F1: CTCAAAACCGTCAACAGGGCAAGTATGTAAAAGG	34	ΔSOS	
		R1: CCTTTTACATACTTGCCCTGTTGACGGTTTTGAG	34		