Sensing and signaling of oxidative stress in chloroplasts by inactivation of the SAL1 phosphoadenosine phosphatase

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SI Text – Materials and Methods

Stress treatments on plants

Drought stress treatment was performed in a randomized manner as previously described (1, 2). For other oxidative stress treatments, leaf disks were floated on 0.1 % Tween and either exposed to 1000 µmol photons m^{-2} s^{-1} white light (High-light); or to 500 mM H_2O_2, 100 µM methyl viologen (O_2^- generator), or untreated (Control) under standard light intensity for 4 hours.

Yeast growth conditions and stress treatments

All yeast (*Saccharomyces cerevisiae*) strains used were isogenic with the haploid BY4741 background and were cultured at 30 ^oC in yeast synthetic drop-out medium (SD) with the omission of leucine where necessary for plasmid maintenance. The *Δhal2* strain was complemented with WT Sc*HAL2* and Sc*HAL2+3C* cDNA sequences overexpressed under the glyceraldehyde-3-phosphate dehydrogenase promoter in the **pJR1138** vector (3) using a standard lithium acetate transformation protocol. These strains were designated *Δhal2*:ScHAL2 and *Δhal2*:HAL2+3C, respectively. For oxidative stress treatments, cultures were grown till early exponential phase, then H_2O_2 (1 or 5 mM final concentration) was added. Cells were grown for a further 12 h before harvest for PAP quantification.

Cloning, cysteine knock-out and knock-in mutagenesis

The mature full length WT AtSAL1 cDNA was amplified from WT *Arabidopsis* leaf cDNA using proofreading polymerase and cloned into the **pGEM 3zf(+)** vector. Mutagenesis was then carried out using different combinations of primers containing point mutations to change the individual cysteine codons into alanine, with the QuikChange II Site-Directed Mutagenesis kit (Stratagene, USA) as per manufacturer's instructions. All mutations were confirmed by Sanger sequencing. The resulting mutated AtSAL1 sequences were then cloned

into either **pHUE** (4) or **prSETb** (Life Technologies, USA), re-sequenced to confirm presence of mutation(s), and recombinant proteins purified as described below. For introduction of additional cysteines into ScHAL2 to produce ScHAL2+3C, the mutated sequence was synthesized and directly cloned into **prSETb** (Life Technologies, USA).

Recombinant protein purification

The mature full length AtSAL1 cDNA (excluding the transit peptide sequence) was expressed as an AtSAL1-Ubiquitin fusion protein in the **pHUE** expression vector under IPTG induction and purified using Ni-NTA His-Bind Resin (Novagen, USA) according to manufacturer's instructions. AtSAL1-Ubiquitin was then digested with the deubiquitinylating enzyme Usp2 in a digestion buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM KCl, 2 mM β-mercaptoethanol] and re-purified through the Ni-NTA resin to yield the mature AtSAL1 protein of 95% purity as assayed by SDS-PAGE. The mature AtSAL1 protein was further purified into monomeric and dimeric fractions by size-exclusion chromatography (SEC) on a HiLoad 26/60 Superdex-200 SEC column (Life Technologies, USA). The monomeric (eluting between 195-220 mL) and dimeric (eluting between 170-195 mL) fractions obtained were concentrated by filter centrifugation, quantified by Bradford assay and stored in storage buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 15% glycerol] at -80 ^{o}C. Mutagenized variants of AtSAL1, and WT OsSAL1, proteins were purified essentially as described above, but were expressed with an N-terminus TEV protease recognition sequence in **prSETb** (Life Technologies, USA) instead of pHUE and the fusion protein was digested with TEV protease instead of Usp2 to yield the mature protein.

Size exclusion Multiple angle Laser light Scattering (SEC-MALLS)

Purified AtSAL1 protein was dialysed against AtSAL1 assay buffer without reducing agent. The protein was then applied to a WTC-030S5 size-exclusion column (Wyatt Technologies, USA) equilibrated with AtSAL1 assay buffer. Multi angle laser light scattering (Dawn8+: Wyatt Technologies) and differential refractive index (Optilab Rex: Wyatt Technologies) of the eluted protein were monitored and data was analyzed using ASTRA (Wyatt Technologies).

SDS-PAGE

To recombinant proteins incubated with redox agents at RT for 1h, NuPAGE LDS 4× Sample Loading Buffer (Life Technologies, USA) was added to a final concentration of 1×. Samples were heated at 70 ^{o}C for 10 minutes, cooled on ice and then resolved on a 10% Bis-Tris NuPAGE gel with MOPS buffer (Life Technologies, USA) at 150 V for 2.5 hours without addition of reducing agents or antioxidants. The protein bands were visualized by staining with GelCode Blue Safe Stain (Thermo Scientific, USA) according to manufacturer's instructions.

Native Protein Purification from *Arabidopsis*

Approximately 100 mg of either control or stressed *Arabidopsis* leaves were frozen in liquid nitrogen and ground to a fine powder with a 1/8" steel ball with the TissueLyzer II (Qiagen, Germany). Native proteins were resuspended in ice-cold 50 mM Tris-HCl pH 7.5 supplemented with 1 % PVP 360 and 1× Roche Protease Inhibitor Cocktail (Roche, Switzerland). Cellular debris was removed by centrifugation at 4 ^{o}C and the proteins in the supernatant quantified by Bradford assay.

Immunoblotting

5 µg total *Arabidopsis* leaf protein was resolved by SDS-PAGE as described above, in absence of additional redox agents, then electro-transferred to a PVDF membrane and probed

with a 1:1000 dilution of polyclonal antibodies raised against AtSAL1 (Catalog Number AS07 256, Agrisera, Sweden) as described previously (2). After 3 washes with PBST, the blot was incubated with 1:10000 dilution of HRP-conjugated goat anti-rabbit IgG for 10 min. After a further 3 washes with PBST, the membrane was developed with the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Mass spectrometry

Gel bands were manually excised for identification by mass spectrometry. Excised gel bands were destained and digested with trypsin as previously described (5), with the modification of using 55 mM iodoacetamide but omitting DTT to avoid loss of glutathione. Tryptic peptides were extracted in 80% ACN/0.1% formic acid, dried under vacuum and resuspended in 5% ACN, 0.1% formic acid before MS analysis using liquid chromatography connected to an accurate-mass Quadrupole Time-of-Flight MS (Agilent 6550) equipped with a Chip Cube ion source as described previously (6). MS/MS spectra analysis and database searching was performed as described (7) using the following modifications. The results were searched against the TAIR10 database for *A. thaliana* protein sequences (33,621 protein sequences) using the Mascot ver. 2.3 search engine (Matrix Science). The follwing parameters were selected: MS error tolerance of ±100ppm, MS/MS error tolerance of ±0.5Da, "semitryptic" peptides, 1 maximum missed cleavage tolerated, variable modifications oxidation (M), carboxymethyl (C), glutathione (C) and oxidation (C) and finally the instrument was set to ESI-Q-ToF. In addition to MS/MS identification, all data files were searched for the presence of cysteine-containing peptides, as well as glutathionylated peptides. Within the Mass Hunter qualitative software (Agilent), the extracted ion chromatogram was extracted for all peptides of interest.

Amino acid alignment

SAL1 amino acid sequences from the plant kingdom were retrieved from the Phytozome database (www.phytozome.net). Mature amino acid sequences (after manual removal of confirmed and putative transit peptides) of SAL1 orthologs in plants were aligned using Clustal Omega with default parameters, and visualized in JalView.

Quantification of adenosines

Total adenosines were extracted with 0.1 M HCl, derivatized with chloroacetaldehyde and quantified fluorometrically after HPLC fractionation as previously described (1). PAP quantification was performed using calibration curves constructed from authentic standards.

Statistical analyses

For all experiments where three or more sample groups were present, analysis of variance (ANOVA) was used to test for significant differences ($p<0.05$). Where only two sample groups of interest were compared, the two-sample student's t-test was used. All statistical analyses were carried out using GraphPad Instat 3.0 (GraphPad Software, USA) or R3.08 (www.r-project.org).

SI Text - References

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