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

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3	Sensing and signaling of oxidative stress in chloroplasts by inactivation of the SAL1
4	phosphoadenosine phosphatase
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6	
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#### 1 Abstract

Intracellular signaling during oxidative stress is complex, with organelle-to-nucleus retrograde 2 3 communication pathways ill-defined or incomplete. Here we identify the 3'-phosphoadenosine 5'-phosphate (PAP) phosphatase SAL1 as a novel and conserved oxidative stress sensor in plant 4 chloroplasts. Arabidopsis thaliana SAL1 (AtSAL1) senses changes in photosynthetic redox 5 poise, hydrogen peroxide, and superoxide concentrations in chloroplasts *via* redox regulatory 6 mechanisms. AtSAL1 phosphatase activity is suppressed by dimerization, intramolecular 7 disulfide formation and glutathionylation, allowing accumulation of its substrate, PAP; a 8 chloroplast stress retrograde signal that regulates expression of Plastid Redox Associated 9 Nuclear Genes (PRANGs). This redox regulation of SAL1 for activation of chloroplast signaling 10 11 is conserved in the plant kingdom, and the plant protein has evolved enhanced redox sensitivity compared to its yeast ortholog. Our results indicate that, in addition to sulfur metabolism, SAL1 12 orthologs have evolved secondary functions in oxidative stress sensing in the plant kingdom. 13

#### 14 Significance Statement

Management of oxidative stress in plant chloroplasts involves signaling pathways to the nucleus that trigger stress response mechanisms. Yet, how oxidative stress is initially sensed in the chloroplast to activate accumulation of a stress signal remains enigmatic. We show that inactivation of a phosphatase, SAL1, by oxidative stress in chloroplasts controls accumulation of its substrate, as a plant stress signal. This regulatory mechanism is highly conserved across the plant kingdom and confers a second function to this metabolic enzyme as an oxidative stress sensor.

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#### 1 Introduction

Alleviating oxidative stress is a common challenge across evolution, occurring at the cellular, 2 3 organellar and systemic levels. In plant chloroplasts, drought and high light (HL) stress induce production of reactive oxygen species (ROS) such as singlet oxygen  $({}^{1}O_{2})$  at Photosystem II 4 (PSII), and hydrogen peroxide ( $H_2O_2$ ) as well as superoxide ( $O_2^-$ ) at Photosystem I (PSI) (1). 5 6 There is also a shift from reducing to more oxidizing states in the redox poise of plastoquinone (PQ) and other stromal redox couples such as glutathione (GSH/GSSG). All of these changes are 7 associated with adjustment of photosystem stoichiometry and chloroplastic metabolic enzymes 8 by chloroplast-resident kinases (2) and redox-sensitive thioredoxins (3) respectively; as well as 9 activation of signaling pathways for the induction of common and unique sets of nuclear genes 10 11 (4, 5).

12 The nuclear transcriptional response to stress in chloroplasts is mediated by chemical signals 13 emanating from the chloroplasts to the nucleus in a process called retrograde signaling (6). There 14 are at least seven distinct retrograde signaling pathways responding to changes in chloroplastic ROS and redox state (7); including beta-cyclocitral for PSII-<sup>1</sup>O<sub>2</sub> responses (8) and PAP-XRN 15 pathway which alters expression of 25% of the HL-associated transcriptome, many of which are 16 17 ROS and redox associated (9). The unique gene sets which expression are induced by PSI ROS and changes in chloroplast redox poise are collectively referred to herein as PRANGs (Plastid 18 Redox Associated Nuclear Genes) (7); they include key and common stress marker genes such as 19 ASCORBATE PEROXIDASE 2 (APX2) (10, 11) and ZAT10 (12) critical for acclimation. The 20 nuclear regulators of PRANGs and the subsequent chloroplast-targeted stress responses, 21 22 including induction of chloroplast antioxidant and redox regulation enzymes such as redoxin proteins, have been extensively elucidated for the different retrograde pathways (7, 12). Despite 23

these advances, however, in all of the PRANG retrograde signaling pathways no chloroplastic
sensor(s) of ROS and redox state has been conclusively identified (7). For instance, a previously
hypothesized sensor kinase for the PQ redox state (2) has recently been re-ascribed to facilitate
H<sub>2</sub>O<sub>2</sub> production rather than redox sensing *per se* (13).

5 A substantial proportion of PRANGs are regulated by the phosphonucleotide, 3'-

phosphoadenosine 5'-phosphate (PAP), which acts as a mobile chloroplast-to-nucleus stress 6 retrograde signal (9). PAP accumulation is induced by drought and high-light stress, and the 7 metabolite signal moves between the chloroplast, cytosol and nucleus (9). PAP is produced by 8 sulfotransferase-catalyzed sulfation reactions in secondary sulfur metabolism, which transfer 9 activated sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to various key acceptor 10 molecules including peptides and hormones (14). This sulfate transfer generates PAP as a by-11 product that inhibits sulfotransferase activity and feedback-regulates overall sulfur flux (14, 15). 12 During unstressed conditions, PAP is enzymatically degraded by the Arabidopsis thaliana SAL1 13 14 (AtSAL1) phosphatase in the chloroplast (9). AtSAL1 loss-of-function leads to constitutive PAP accumulation, up-regulation of PRANGs, increased stress tolerance and altered sulfur 15 metabolism (9, 15). Hence, SAL1 and PAP perform dual functions in sulfur metabolism (15) and 16 17 stress signaling (9). The role of SAL1-PAP in chloroplast stress signaling is likely conserved beyond Arabidopsis to other members of the plant kingdom, since transient silencing of SAL1 18 19 also enhanced stress tolerance in wheat (Triticum aestivum) (16). PAP controlling PRANG expression during drought and HL has known degradation and 20

21 production site(s) for the signal, a mechanism for signal movement, and a protein target for the

signal (7, 9). Yet, similar to all other PRANG-regulating retrograde pathways, the mechanism by

23 which chloroplast oxidative stress and redox state are initially sensed and transduced is

unknown. Here we demonstrate that contrary to expectation for a metabolic enzyme, SAL1 can
in and of itself act as a molecular sensor for oxidative stress. The switches for accumulation of
the PAP chloroplast retrograde signal reside within the SAL1 protein, thereby providing a
common site of perception of PSI ROS and redox couples for regulating PRANGs.

5 **Results** 

### Accumulation of the stress signal PAP occurs *via* oxidative down-regulation of AtSAL1 activity

8 Given the multiple redox couples altered in the chloroplast in response to HL and drought (1, 14), we hypothesized that PAP accumulation during these stress conditions (9) can be regulated 9 by AtSAL1 if the enzyme acted as a sensor of, and its activity regulated by, oxidative stress. 10 Indeed, PSI-sourced ROS that invoke PRANG regulation such as H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> significantly 11 lowered in vivo AtSAL1 activity (Fig. 1A). This was replicated in plants exposed to the abiotic 12 stresses drought and HL that induce  $H_2O_2$  and  $O_2^-$  formation (Fig. 1A). These treatments did not 13 significantly alter AtSAL1 protein abundance however (Fig. 1A), suggesting that the regulation 14 of AtSAL1 activity in the chloroplast is therefore most likely via a post-translational 15 16 mechanism(s).

It is well established that in mutants deficient in regeneration of oxidized proteins, redox buffer homeostasis (NADPH/NADP<sup>+</sup>, GSH/GSSG), or the water-water cycle that degrades PSI  $O_2^-$  and H<sub>2</sub>O<sub>2</sub> *via* ascorbate (1); there is increased ROS accumulation, shifts in redox balance towards more oxidizing states, and /or deregulated PRANG expression (see **Table S1**). In six of such mutants, *in vivo* AtSAL1 activity in response to HL-induced oxidative stress was further reduced by up to 50% relative to wild type (WT) (**Fig. 1B**).

### AtSAL1 is redox-regulated by dual mechanisms of intramolecular disulfide formation and dimerization

3 The suppression of *in vivo* AtSAL1 activity under oxidative stress conditions (Fig. 1A), coregulation of PRANG expression by photosynthetic ROS and PAP (4, 5, 9), and redox regulation 4 of some sulfur metabolism enzymes in chloroplasts (14), led us to hypothesize that down-5 regulation of in vivo AtSAL1 activity is directly mediated by redox potential. Indeed, in vitro 6 AtSAL1 activity is decreased under oxidizing conditions (Fig. 2A). The amino acid sequence of 7 AtSAL1 includes four cysteine residues as potential targets for redox regulation (Cys21, Cys119, 8 Cys167, and Cys190). When these cysteine residues were mutated to redox-insensitive alanine 9 residues, total activity decreased 50% but, more importantly, oxidative down-regulation of 10 AtSAL1 activity was lost (Fig. 2A). The down-regulation of AtSAL1 activity coincided with the 11 appearance of multiple protein bands on SDS-PAGE (Fig. 2A, bottom panels). Significantly, the 12 multiple band patterns of oxidized AtSAL1 on SDS-PAGE was reversible upon addition of 13 14 reducing agent (Fig. 2B); the altered migration of proteins with disulfide bond(s) due to 15 intramolecular loop formation is well established (17, 18).

Combinatorial analysis of Cys to Ala mutations facilitated identification of the Cys pairs involved in the dominant disulfide bonded bands (**Fig. 2C**). This revealed two important features of the oxidation/inactivation process: first, full oxidation of recombinant AtSAL1 was not possible and only a fraction of the protein was converted to anomalously migrating oxidized bands; second, a specific, dominant, band that was observed when the WT protein was oxidized was lost when either Cys167 or Cys190 was mutated to Ala, suggesting that they form a disulfide bond. Kinetic analysis of Cys to Ala mutations showed that when Cys119 or Cys190

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were mutated to Ala no loss of activity was observed in oxidizing conditions, suggesting that Cys167-Cys190 and Cys119 may be required for inactivation of AtSAL1 (**Fig. 2A**).

3 To investigate this redox sensing mechanism in more detail, we subsequently crystallized and solved the molecular structure of AtSAL1 to 3.05 Å resolution in its apo form (PDB 5ESY, Fig. 4 5 **3**, **Table S2**). AtSAL1 is an  $\alpha/\beta$  protein belonging to the carbohydrate phosphatase fold and superfamily (19) showing the closest structural homology to the yeast (Saccharomyces 6 *cerevisiae*) PAP phosphatase ortholog, ScHAL2 [PDB 1KA1(20), r.m.s.d for C $\alpha$  atoms = 2.4 Å, 7 8 with amino acid sequence identity of 37% as calculated by the DALI server (21)]. Interestingly, 9 we found that AtSAL1 crystallized as a dimer; a crystallographic 2-fold interface was clearly visible and detected with the Protein Interfaces, Surfaces and Assemblies (PISA) server (22). 10 The dimer interface is centered on a symmetrical pair of Cys119 side chains from each monomer 11 in the dimer (Fig. 3A), suggesting a role for an intermolecular disulfide in dimerization. Each 12 13 monomer also contains a potential intramolecular Cys167-Cys190 disulfide pair located across 14 adjacent beta strands (Fig. 3A); such a cross-strand disulfide is often a metastable switch used to control protein activity (23). Cys21 was not located near any potential disulfide bonding 15 partners. Notably, none of the cysteine residues are located in the vicinity of the active site, 16 suggesting that the regulation must be remote, or allosteric. 17

Although previously reported to be a monomer (24), we found that AtSAL1 exists in monomerdimer equilibrium in solution through size exclusion chromatography (SEC) and SEC-multi angle laser light scattering (MALLS) (**Fig. 4A, Fig. S1**). Native PAGE analysis of recombinant AtSAL1 reveals that the dimeric fraction is stable under oxidizing conditions, owing to the presence of an intermolecular disulfide, but under sufficiently reducing conditions the protein returns to equilibrium between monomer and dimer (**Fig. 4B**). Interestingly, the monomeric and

1 dimeric species displayed strikingly different redox sensitivity: whereas the monomer displayed high catalytic activity and is resistant to oxidation and inactivation, the dimer is less active under 2 reducing conditions, but is extremely sensitive to oxidation and is rapidly inactivated, with 3 formation of disulfide bonds, including the intramolecular Cys167-Cys190 bond, in the presence 4 of oxidized DTT (Fig. 4C, Table 1). Thus, it appears that dimerization is a precursor to oxidative 5 inactivation, and that the oligomeric equilibrium can be altered under oxidizing conditions 6 through the formation of an intermolecular disulfide bridge. This result is consistent with the 7 data presented in Fig. 2 showing that the mixed oligomeric species of AtSAL1 do not undergo 8 9 full oxidation owing to the resistance of monomer to oxidation, and that the Cys119 and Cys190 residues are involved in separate disulfide bonds that are both important for inactivation. 10

11 Given that dimerization appears to be essential for the intramolecular Cys167-Cys190 disulfide bond formation, and that oxidation can inactivate AtSAL1, we sought to investigate the 12 mechanisms that underlie these processes. Allosteric regulation of protein activity is 13 14 commonplace (25), and frequently involves alteration of protein dynamics and trapping of 15 proteins in inactive conformations (26-29). Thus, we performed molecular dynamics (MD) 16 energy minimization to generate monomeric (reduced) and dimeric (oxidized) AtSAL1 models 17 to investigate the impact of dimerization and the disulfide bonds on the protein structure and dynamics. Normal mode analysis of elastic network models (NMA-ENM) can effectively 18 19 predict and analyze large-scale collective motions in proteins (30, 31), and was used here to 20 investigate the impact of the Cys119-Cys119 and Cys167-Cys190 disulfide bridges and dimerization on the flexibility of AtSAL1. 21

As shown in **Fig. 3B**, loop 1 overhangs the active site in an open conformation; the equivalent loop to loop 1 in the AtSAL1 ortholog, ScHAL2, has been shown to close over the active site to stabilize substrate (20). In AtSAL1, NMA-ENM indicates that loop 1 fluctuates between open
and closed conformations in a manner common to active site loops in almost all phosphatases
(Fig. 3B) (32, 33), suggesting that this movement is likely to be important for catalytic activity.
Loops 3 and 4 are located at the dimer interface and adjacent to loop 1, while loop 8 is also
adjacent to loop 1.

The mobility of these loops in the oxidized dimer and reduced monomer are strikingly different, 6 with loops 1, 3, 4, and 8 all being substantially rigidified through dimerization and disulfide bond 7 formation (Fig. 3C). The dynamic coupling of these loops is also significantly different between 8 the monomer and dimer, and these loops become significantly more hindered in the dimer (Fig. 9 S2). These data also allow a plausible explanation for the resistance of the monomer to 10 oxidation, since the formation of the Cys167- Cys190 disulfide bond will require the two 11 residues to be located in close proximity and to be relatively stable, which would be the case in 12 the dimer but not the monomer. Thus, the decreased activity of AtSAL1 in the 13 14 dimerized/oxidized state likely results from the rigidification of the active site loops, 15 allosterically inhibiting the enzyme by preventing it from adopting conformations that are 16 essential for activity and substrate binding. Indeed, both  $k_{cat}$  and  $K_{M}$  are affected by dimerization 17 and oxidation (Table 1). This result is consistent with the current view of allosteric inhibitory regulation of proteins (28, 29), and provides a rapid means of reversible enzyme inactivation. 18

#### 19 Formation of the intramolecular disulfide bond controlling AtSAL1 activity can be

#### 20 mediated by the chloroplast redox buffer GSH/GSSG

21 We then investigated whether *in vitro* AtSAL1 inactivation by the redox regulatory mechanisms

shown in **Fig. 3** can be induced by *in vivo* redox couples present in the chloroplast, such as

1	glutathione (GSH). During oxidative stress GSH can be oxidized to oxidized glutathione
2	(GSSG). GSSG is known to glutathionylate cysteine residues of chloroplast proteins to regulate
3	their activity (3), and it promotes formation of an intramolecular disulfide bond between
4	proximal cysteine residues via thiol-disulfide exchange (34) (Fig. S3). Therefore, we tested
5	whether glutathionylation may also induce formation of the Cys167-Cys190 bond in AtSAL1.
6	The GSSG-treated AtSAL1 was able to form the Cys167-Cys190 disulfide in all recombinant
7	proteins containing both residues (Fig. 5A). Additionally, decreased activity in GSSG-treated
8	AtSAL1 correlated with glutathionylation of Cys119 and Cys190 as detected by mass-
9	spectrometry (Fig. 5B). Critically, glutathionylation down-regulated activity in both monomeric
10	and dimeric AtSAL1 samples (Fig. 5C). The redox titration of both monomeric and dimeric
11	AtSAL1 with GSH/GSSG yielded redox midpoint potentials ( $E_m$ ) close to the physiological
12	glutathione redox potential $(-317 \pm 8 \text{ mV})$ in <i>Arabidopsis</i> chloroplasts (35) (Fig. 5C). Therefore,
13	two redox processes can decrease AtSAL1 activity: the first involving dimerization and
14	intermolecular disulfide bonding, and the second a dimerization-independent process involving
15	glutathionylation by the chloroplast redox couple GSH/GSSG. Both mechanisms result in the
16	formation of the Cys167-Cys190 intramolecular disulfide and down-regulation of AtSAL1
17	activity.

#### 18 In vitro redox regulatory mechanisms of AtSAL1 are recapitulated in vivo

19 If the SAL1-PAP pathway is a primary regulator of PRANGs, and PAP concentrations are 20 controlled by redox regulation of SAL1 as hypothesized, then the multiple redox mechanisms 21 regulating AtSAL1 *in vitro* should be recapitulated *in vivo* in response to chloroplast redox cues 22 that initiate PRANG regulation. We tested this hypothesis by analyzing AtSAL1 activity, and intramolecular disulfide formation and dimerization, when photosynthetic ROS and the
 chloroplast redox state were manipulated in various ways.

3 First, we observed in vivo formation of the characteristic Cys167-Cys190 disulfide band in AtSAL1 during drought stress. *In vivo* abundance of the Cys167-C190 disulfide bonded form of 4 AtSAL1 progressively increased in correlation with decreasing AtSAL1 activity and increasing 5 6 PAP accumulation in leaves of drought-stressed Arabidopsis (Fig. 6A). Second, across the multiple abiotic stress treatments that increase abundance of PSI-sourced ROS and lead to 7 reduction of *in vivo* AtSAL1 activity shown in Fig. 1A, the proportion of dimeric AtSAL1 in 8 oxidatively-stressed *Arabidopsis* leaves increased relative to control (Fig. 6B). This is in 9 agreement with the observed Cys119 intermolecular disulfide-mediated dimerization under 10 11 oxidative conditions (Fig. 4B).

To complement the *in vivo* results shown in Fig. 6, either one of two approaches can demonstrate 12 13 that redox regulation of AtSAL1 enables PAP accumulation and PRANG expression. First, 14 AtSAL1 Cys-Ala mutants that are redox-insensitive in vitro can be expressed in Arabidopsis, however abundance of the Cys-Ala proteins were lower than WT when expressed in E. coli (Fig. 15 S4). It is well established in plants that point mutations, let alone four mutations in a single gene, 16 can affect a variety of protein characteristics, including in vivo stability, activity, and/or protein-17 protein interactions (36). Indeed, two other SAL1 point mutations, *alx8* and *hos2*, affect protein 18 19 stability and activity in a temperature-dependent manner, respectively (37, 38).

A second approach involves analyzing the same gene across many species to identify strong conservation of the same characteristic, which would indicate strong evolutionary selection to maintain this function (39, 40). The SAL1-PAP pathway for PRANG regulation and stress

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1	tolerance is functional across dicotyledonous (9, 37) and monocotyledonous plants (16).
2	Therefore, if AtSAL1 is genuinely redox-regulated in vivo, then the cysteine residues conferring
3	in vitro redox sensitivity in AtSAL1 should be strongly conserved in evolution beyond
4	Arabidopsis to other plant species; and distantly-related orthologs should also show redox-
5	sensitive biochemical activity.
6	We found that the redox-responsive cysteine residues in AtSAL1 are indeed highly conserved
7	(Fig. S5). The Cys167-C190 intramolecular disulfide pair is strongly conserved across the
8	representative bryophyte, chlorophyte, early angiosperm, eudicot and monocot species
9	examined. The Cys119 residue that mediates the intermolecular dimerization is less conserved,
10	but is still present in 33% of eudicot SAL1 orthologs and in two monocot proteins. In the
11	distantly related Poaceae SAL1 orthologs including the Oryza sativa SAL1 (OsSAL1) protein,
12	the position of the conserved AtSAL1 Cys190 is C-terminally shifted by 7 amino acids.
13	Interestingly, these proteins possess an additional conserved cysteine residue (Fig. S5).
14	We investigated OsSAL1 in detail, given that it has been shown to have activity against PAP
15	(41), and monocots are estimated to have diverged from dicots $140 - 150$ million years ago (42)
16	Conserved similarities in redox sensitivity between dicot AtSAL1 and monocot OsSAL1 would
17	therefore be functionally and evolutionarily significant. As was observed for AtSAL1, OsSAL1
18	activity is inhibited by oxidation (Table 2) and redox titration of the protein shows a
19	physiologically-relevant $E_m$ (Fig. 7A). OsSAL1 conformation can also be modified by
20	glutathionylation (Fig. 7B). Homology modelling of OsSAL1 reveals that both the strongly
21	conserved cysteine residues are surface-exposed (Fig. 7C), which may potentiate redox
22	regulation.

1 Finally, a corollary to the results described above is that if a SAL1 ortholog lacks the redoxresponsive cysteine residues, then introduction of these residues should enhance redox sensitivity 2 in the new protein. The activity of the yeast (Saccharomyces cerevisiae) ortholog, ScHAL2, 3 shows some redox-sensitivity to glutathionylation (Table S3), possibly due to presence of a 4 surface-exposed cysteine (Fig. S6A). However, ScHAL2 lacks the three cysteines at positions 5 structurally equivalent to those of AtSAL1, including the Cys119 side chain that promotes 6 dimerization (Fig. S5) and the Cys167-Cys190 intramolecular disulfide (Fig. 8A). This suggests 7 that the redox-sensing mechanisms regulating AtSAL1 activity in plants (Figs. 1-6) are absent in 8 9 yeast, and may have evolved in plants for chloroplast redox sensing and PRANG regulation. We therefore introduced three additional cysteine residues (Thr21Cys, Phe127Cys, Tyr196Cys) into 10 ScHAL2 (ScHAL2+3C). 11

Phe127Cys is the equivalent of AtSAL1 Cys119 that promotes dimerization, while Tyr196Cys 12 introduces a potential disulfide pair in the same position as the critical intramolecular disulfide 13 14 that regulates activity of the dimeric AtSAL1 (Fig. 8A). Indeed, ScHAL2+3C showed 15 significantly greater redox sensitivity compared to WT ScHAL2 *in vitro* (Fig. 8B, Table S3). 16 When expressed *in vivo*, the engineered ScHAL2+3C was as efficient as WT ScHAL2 in 17 degrading PAP under unstressed conditions (Fig. S6B). Critically, introduction of the intramolecular disulfide also increases redox sensitivity in vivo compared to the WT form: yeast 18 19 *Ahal2* overexpressing ScHAL2+3C significantly accumulated PAP when challenged with mild 20 H<sub>2</sub>O<sub>2</sub> stress, whereas those overexpressing WT ScHAL2 did not (Fig. 8C). Thus, addition of the cysteine residues alone is sufficient to induce enhanced redox sensitivity to the yeast SAL1 21 ortholog. 22

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#### 1 Discussion

#### 2 Redox regulation of AtSAL1 involves multiple structural mechanisms

A diverse range of proteins are specifically regulated by disulfide formation (23). The Cys167-3 4 Cys190 intramolecular disulfide in AtSAL1 is intriguing because it occurs across two antiparallel beta-strands immediately adjacent to one another, at the base of a hairpin loop connecting these 5 6 strands (Fig. 3A). Formation of a disulfide across adjacent beta strands is a form of Cross-Strand 7 Disulfide (CSD) that have been termed 'forbidden disulfides'(23), since they disobey the established rules of protein stereochemistry (43, 44) and introduce strain into the protein 8 structure that may be energetically and structurally unfavorable (44). However, recent findings 9 10 indicate that strain in local areas of a protein are tolerated for regulation of protein function (23). 11 An increasing number of proteins have been characterized that contain CSDs; in most cases, these disulfides regulate function (23). These examples include proteins involved in chloroplast 12 redox control such as thioredoxins (TRXs), which have a canonical CSD-containing motif that is 13 strongly conserved across evolution (23, 45). CSD formation directly blocks catalytic cysteines 14 in thioredoxins (46, 47), whereas the CSD decreases protein flexibility in AtSAL1 (Fig. 3). 15 Formation of the intramolecular Cys167-Cys190 disulfide in response to oxidizing conditions is 16 17 dependent upon dimerization (Fig. 4C), which stabilizes the protein conformation (Fig. 3). The dimerization interface between AtSAL1 monomeric subunits is relatively small (Fig. 3A), which 18 may result in a relatively weak or transient interaction in vivo and explain the monomer-dimer 19 equilibrium in solution under reducing conditions, until the interface is locked together through 20 the Cys119-Cys119 intermolecular disulfide bonding during oxidative stress. Interestingly, the 21 22 inactivation of both monomeric and dimeric AtSAL1 by GSH/GSSG, and formation of the

Cys167-Cys190 disulfide by glutathionylation *via* thiol-disulfide exchange (Fig. 5), reveals an
 additional mechanism for redox regulation of AtSAL1.

### AtSAL1 redox regulation allows both metabolic control of sulfur assimilation and oxidative stress signaling

The flux of sulfur in plants is regulated partly *via* redox control of key enzymes in the sulfur assimilation pathway (48-50). Oxidative stress is expected to increase sulfur flux into sulfur reduction for production of the redox buffer GSH/GSSG, as two key enzymes in this primary branch of sulfur metabolism, APS reductase (APR) and Glutamate-Cysteine Ligase (GCL) are more active when oxidized (49, 50). Conversely, PAP biosynthesis in the parallel, secondary pathway should be down-regulated by oxidative stress since enzymatic synthesis of the PAP precursor, PAPS, by APS kinase (APK) is decreased by oxidation (48).

Within the present study we show that AtSAL1 is significantly less active when oxidized (Figs. 12 1-6), thus providing a mechanism for the 30-fold accumulation of PAP seen in WT plants during 13 drought (9), without necessitating increased sulfur allocation into secondary sulfur metabolism 14 and PAP synthesis. We also show that metabolites from one sulfur metabolism branch can 15 16 influence biosynthesis of metabolites in the parallel pathway, since GSH/GSSG directly regulates AtSAL1 activity (Fig. 5) and hence PAP levels. Significantly, the redox midpoint 17 potential ( $E_m$ ) of AtSAL1 (-308 ± 2 mV for monomer and -284 ± 5 mV for dimer at pH 7.5; Fig. 18 19 5C) overlaps with those determined for the oxidation-activated primary sulfur metabolism (GSH/GSSG) enzymes (-330mV  $\pm$  10mV at pH 8.0 for APR and -318  $\pm$  11 mV at pH 7.0 for 20 GCL) (49, 50) and oxidation-inhibited secondary sulfur metabolism (PAPS/PAP) APK enzymes 21  $(-286 \pm 18 \text{ mV at pH } 7.5)$  (48). Therefore, the same redox state in chloroplasts can concomitantly 22

regulate multiple sulfur metabolism enzymes including AtSAL1. This could allow coordination
 of flux through the sulfur pathway for redox buffering (GSH/GSSG) concomitant to stress
 signaling (PAP).

The redox-responsive cysteine residues in AtSAL1 are conserved in plant species that lack glucosinolate biosynthesis, which constitute a major sink for sulfur (and source of PAP) in *Arabidopsis* and its relatives in the *Brassicaceae* (14) (Fig. S5). Indeed, the redox regulation was conserved in OsSAL1 (Fig. 7), despite *O. sativa* being monocotyledonous and lacking glucosinolates. Another indication that the SAL1-PAP pathway functions independently of glucosinolates is that it also mediates stress tolerance in wheat (16). Therefore, the redox regulation of SAL1 can be uncoupled from sulfur metabolism.

### SAL1 acts as a general redox sensor in the chloroplast for retrograde signaling and PRANG regulation

13 Our results indicate that SAL1 activity in plants is sensitive to the overall redox state of the chloroplast and not to a specific stimulus or sensor protein (Fig. 1). The SAL1 protein is 14 sensitive to ROS production and redox state of PSI, where O<sub>2</sub> is produced and detoxified via 15 16 Superoxide Dismutase (SOD)-mediated dismutation to  $H_2O_2$ , which is further detoxified by thylakoidal and stromal APXs (tAPX and sAPX). Deficiency in sAPX compromises H<sub>2</sub>O<sub>2</sub> 17 degradation and concomitantly increases  $O_2^-$  abundance at PSI due to SOD inhibition (1); this 18 increased the effect of HL stress on suppression of AtSAL1 activity (Fig. 1B). Indeed, direct 19 induction of O<sub>2</sub> production at PSI by methyl viologen inhibited AtSAL1 (Fig. 1A). Perturbing 20 multiple aspects of chloroplast redox homeostasis also influenced AtSAL1 activity (Fig. 1B). 21 This includes the major oxidized protein regeneration pathway in chloroplasts involving 22

1 NADPH-dependent Thioredoxin Reductase C (NTRC) (51), and the redox buffers NADPH

2 /NADP<sup>+</sup> and GSH / GSSG. Mutants in these pathways are unsurprisingly hypersensitive to

- 3 oxidative stress (52, 53), but also deregulated in PRANG expression during stress (54). The latter
- 4 probably reflects the influence of redox poise on AtSAL1 activity and PAP accumulation.

The convergence of photosynthetic ROS and redox cues on SAL1 has key implications for stress 5 retrograde signaling and regulation of PRANGs. Our results here demonstrating the coupling of 6 SAL1 activity and PAP accumulation to chloroplastic redox poise presents this enzyme as an 7 unconventional oxidative stress sensor and hub for the convergence of  $H_2O_2$ ,  $O_2^-$  and redox cues 8 in the chloroplast that alter nuclear gene expression (4, 6, 7). That is, the primary function of 9 SAL1 is not to sense ROS and redox state, yet its sensitivity to these chloroplast cues enables 10 them to be sensed by the nucleus via PAP, providing capacity for fine-tuning responses. Such a 11 hypothesis does not preclude parallel pathways that also sense or respond to ROS or redox state 12 in the chloroplast. For example, recent evidence shows that projections from chloroplasts called 13 14 stromules increase in abundance during stress and can enable transport of ROS from chloroplasts 15 to the nucleus (55, 56). The extent to which PAP and other ROS communication pathways overlap is a subject of future research. 16

The conserved secondary redox sensing by SAL1 suggests that these enzymes may be considered 'moonlighting' proteins (57). Many moonlighting proteins are evolutionarilyubiquitous enzymes that have secondary functions in diverse processes including metabolism and disease (57). Intriguingly, to our knowledge SAL1 would constitute the first moonlighting oxidative stress sensor described in plants. The sensitivity of the sensor may be fine-tuned differently across evolution to fulfill kingdom-specific functions, since Arabidopsis and rice SAL1 appears more responsive to ROS and redox state than yeast HAL2 due to presence of the
 intramolecular Cys167-Cys190 disulfide (Figs. 3A, 8B-C, Table S3).

3 Whether chloroplast communication necessitates additional layers of complexity in regulation of SAL1 in plants requires further elucidation. Regardless, there is precedent for the evolution of 4 increased redox sensitivity in PAP/PAPS metabolism in plants. For instance, the adenosine 5'-5 phosphosulfate kinase (APK) enzyme catalyzing PAPS production evolved increased redox 6 sensitivity in the transition from cyanobacteria to higher plants (58). It is also fascinating that 7 WT ScHAL2 shares some conservation of redox-sensitivity (Fig. 8, Table S3), albeit to a lesser 8 extent, with AtSAL1 despite lacking the AtSAL1 redox-responsive cysteine residues. Instead, 9 ScHAL2 may be regulated *via* a different surface-exposed cysteine residue (Fig. S6). It may be 10 11 that PAP phosphatases in other kingdoms are redox-regulated via analogous mechanisms targeting different cysteine residues, and this will be interesting to explore. 12

In summary, transient elevation of PAP levels as an oxidative stress signal in plants is coupled to 13 the redox state perceived by SAL1. That at least four different ROS/redox couples (H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub>, 14 GSH/GSSG and DTT<sub>red</sub>/DTT<sub>ox</sub>) regulate SAL1 enables a fine-tuning of SAL1 activity and thus 15 retrograde signaling to communicate the different fluctuations of chloroplast ROS balance/redox 16 poise in response to environmental stimuli. It is intriguing that a similarity in regulation of SAL1 17 orthologs relates more to the secondary function of redox sensing and stress signaling than 18 sulfation, and raises the question as to which function was the primary driver for evolutionary 19 conservation of this protein. Our results suggest that dual-function SAL1 orthologs may be 20 uniquely positioned as single-component integrators of sensing and signaling of aspects of 21 oxidative stress in plants. 22

#### 1 Materials and Methods

#### 2 Plant material and growth conditions

3 *Arabidopsis* seeds were germinated on soil and kept at 4 °C for 3 days to synchronize

4 germination. Plants were grown at 100-150  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>, 12 h photoperiod, 21-23 °C

5 and 50-55 % humidity. Five-week old plants were used for all stress assays (SI Text). T-DNA

6 insertion lines for the redox homeostasis mutants *sapx* (1), *ntrc* (52), *phs1* (53), and *cos1* (59)

7 (Table S1) were obtained from the Arabidopsis Biological Resource Centre (ABRC). The

8 amiRNA silencing line for tAPX (1) was provided by Prof. Christophe Laloi (Aix-Marseille) and

9 *rax1-1* (54) was obtained from Prof. Phil Mullineaux (Essex) (**Table S1**).

#### 10 **Protein purification from biological samples**

11 *Arabidopsis* leaf native proteins were extracted using a protocol modified from Murguia *et al.* 

12 (60) (SI Text). Native proteins were kept on ice and used immediately in Clear-Native PAGE

13 and activity assays, as described later.

14 For detection of the Cys167-Cys190 intramolecular disulfide in endogenous AtSAL1, native

15 proteins were incubated with 10 mM iodoacetamide in the dark for 1 h to prevent oxidation of

16 any cysteines that were reduced *in vivo*. Leaf protein was then precipitated in TCA / acetone,

- 17 washed twice with cold acetone and resuspended in solubilisation buffer (9 M urea, 4 % (w/v)
- 18 CHAPS, 1 % (w/v) DTT, 35 mM Tris base) before SDS-PAGE and western blotting (SI Text).

#### 19 Recombinant protein purification

- 20 Recombinant WT AtSAL1 protein was expressed in *Escherichia coli* BL21 DE3 cells (New
- 21 England BioLabs, USA) and purified using standard metal affinity purification (SI Text). The

purified recombinant proteins were stored in storage buffer [50 mM Tris-HCl pH 8.0, 150 mM
 NaCl, 20 mM KCl, 1 mM MgCl<sub>2</sub>, 15% glycerol] at -80 °C.

#### **3 Protein gel electrophoresis**

4 Recombinant protein, 0.5-1  $\mu$ g, was incubated in degassed storage buffer [50 mM Tris-HCl pH

5 8.0, 150 mM NaCl, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 15% glycerol] in the presence of either 5 mM

6 DTT<sub>red</sub> (reducing conditions) or 5mM DTT<sub>ox</sub> (trans-4,5-Dihydroxy-1,2-dithiane; oxidizing

7 conditions) for 1 hour at RT, then resolved by SDS-PAGE and stained as described in **SI Text**.

8 To visualize effects of GSH/GSSG, proteins were incubated in degassed storage buffer

9 containing 20mM GSH or 20mM GSSG, then run on gels and stained as before.

10 For Clear-Native PAGE, proteins were incubated with redox agents as above but resuspended in

11 Native Sample Loading buffer [100 mM Tris-HCl, 10 % glycerol, 0.0025 % bromophenol blue,

12 pH 8.6) and resolved on a 3-12 % Novex NativePAGE gel (Life Technologies, USA) in Native

13 Running Buffer [25 mM Tris, 192 mM Glycine, pH 8.3] without denaturing agents.

#### 14 Activity assays

15 Recombinant protein activity against PAP was assayed by incubating 0.2 µg protein in degassed

16 Activity Buffer [100 mM Tris-MES pH 7.5, 1 mM Mg acetate] in presence of either reducing or

17 oxidizing equivalents of  $DTT_{red}/DTT_{ox}$  (5 mM) or GSH/GSSG (20mM) for 1 hour at 25 °C, then

increasing concentrations of PAP was added to a final volume of 150 µL and initial activity

19 assayed at 25 °C (AtSAL1 and OsSAL1) or 30 °C (ScHAL2). The reaction was stopped by flash-

20 freezing in liquid N<sub>2</sub>. AMP produced from degradation of PAP was quantified using the method

21 for derivatization and detection of adenosines *via* High Performance Liquid Chromatography as

22 previously described (9). All Michaelis-Menten kinetics parameters were calculated using

20

1	GraphPad Prism (GraphPad Software Inc., USA). For redox titration of activity, SAL1 protein
2	was incubated as above with different ratios of $DTT_{red}$ : $DTT_{ox}$ (final total concentration of 5 mM)
3	or GSH:GSSG (final concentration of 20 mM). Values for redox midpoint potential, $E_m$ , was
4	calculated by fitting titration data to the Nernst equation using GraphPad Prism:
5	$E_h = E_m + (RT/nF)(ln ([GSSG]/[GSH]^2))$ for glutathione
6	And
7	$E_h = E_m + (RT/nF)(ln ([DTT_{ox}]/[DTT_{red}]))$ for DTT
8	with an RT/F of 25.7mV and n=2 (49, 61)
9	For activity of AtSAL1 in native protein extracts from Arabidopsis, 10 µg of total native protein
10	extract was incubated in the same Activity Buffer as above with increasing concentrations of
11	PAP at 25 $^{\circ}$ C without any redox agents
11	The at 25° C without any redox agonts.
12	Crystallization, data collection and refinement
11 12 13	Crystallization, data collection and refinement AtSAL1 crystals were grown by vapor-diffusion in hanging-drops. Crystals formed at a protein
11 12 13 14	Crystallization, data collection and refinement AtSAL1 crystals were grown by vapor-diffusion in hanging-drops. Crystals formed at a protein concentration of 20 mg/ml in 20-30 % PEG 2000-MME, 0.2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M HEPES pH
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112 13 14 15 16 17 18	<b>Crystallization, data collection and refinement</b> AtSAL1 crystals were grown by vapor-diffusion in hanging-drops. Crystals formed at a protein concentration of 20 mg/ml in 20-30 % PEG 2000-MME, 0.2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M HEPES pH $8.0 - 8.6$ . Diffraction data were collected at the Australian Synchrotron at the MX2 beamline ( $\lambda = 0.9537$ Å). The resolutions limits of the data were assessed on the basis of the significance of the CC <sub>1/2</sub> at the <i>P</i> =0.001 level (62, 63). Diffraction data were integrated using XDS (64) and scaled using <i>SCALA</i> from the CCP4 program suite (65). The crystals belonged to the <i>P</i> 6 <sub>1</sub> space
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112 123 131 141 151 161 171 181 192 201	<b>Crystallization, data collection and refinement</b> AtSAL1 crystals were grown by vapor-diffusion in hanging-drops. Crystals formed at a protein concentration of 20 mg/ml in 20-30 % PEG 2000-MME, 0.2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M HEPES pH $8.0 - 8.6$ . Diffraction data were collected at the Australian Synchrotron at the MX2 beamline ( $\lambda = 0.9537$ Å). The resolutions limits of the data were assessed on the basis of the significance of the CC <sub>1/2</sub> at the <i>P</i> =0.001 level (62, 63). Diffraction data were integrated using XDS (64) and scaled using <i>SCALA</i> from the CCP4 program suite (65). The crystals belonged to the <i>P</i> 6 <sub>1</sub> space group and significant merohedral twinning was identified by xtriage (66)(twin operator h,-h-k,-l). Phases were obtained by molecular replacement in Phaser (67) using the yeast ortholog of SAL1
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#### 1 Simulation of the oxidized AtSAL1 dimer

The intermolecular (Cys119-Cys119) and intramolecular (Cys167-Cys190) disulfides were 2 modelled using the GROMACS package (69) in conjunction with the GROMOS 53a7 force-3 field for condensed phases (70). To prepare the model, the crystallographic coordinates of the 4 5 AtSAL1 dimer were immersed in a cubic shaped box of solvent with a minimum of 1 nm between the protein and the box edge. The simple point charge model was used to represent 6 water (71) and the protein's charge was neutralized by the addition of sodium ions. Electrostatic 7 energy was calculated using the particle mesh Ewald (PME) method (72) and cut-off distances 8 for the calculation of van der Waals and Coulomb interactions were set at 0.9 and 1.4 nm, 9 10 respectively. To model the disulfide bonds between Cys167-Cys190 and Cys119-Cys119 the AtSAL1 topology file was edited to include a bond between the sulfur atoms of the cysteines. 11 The resulting system was energy minimized via steepest descent to the limit of machine 12 13 precision. During energy minimization the disulfide bond length decreased to an appropriate length  $(2 \pm 0.2 \text{ Å})$ . For comparison the reduced AtSAL1 monomer was modelled. To prepare the 14 model the crystallographic coordinates of each monomer in the AtSAL1 asymmetric unit were 15 energy minimized. The same procedure was used to energy minimize the monomeric structure as 16 the dimeric structure, except disulfide bonds (Cys167-Cys190) were not included in the 17 monomer. To obtain an estimate of the flexibility of the energy minimized structures the 18 structures were submitted to the iMOD normal mode analysis webserver (73). To analyze the 19 20 coupling of AtSAL1 motions the covariance of  $C_{\alpha}$  motions were plotted (74).

21

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9	Manuscript preparation: K.X.C., P.D.M., C.J.J., B.J.P. All authors discussed the results
10	and commented on the manuscript.
11	Author information: Crystal structure data is deposited at PDB (PDB ID: 5ESY). The authors
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13	should be addressed to B.J.P. (barry.pogson@anu.edu.au).
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#### 1 Figure Legends

#### 2 Fig. 1: In vivo AtSAL1 activity is down-regulated by oxidative stress.

3 (A) In vivo AtSAL1 activity is down-regulated by oxidative stress with negligible change in protein abundance (WW: Well-watered, MD: Mid-Drought, LD: Late-Drought, HL: High-light, 4 MV: Methyl Viologen, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide). Activity was measured without any reducing 5 agent, while protein electrophoresis and western blotting were performed under reducing 6 7 conditions for optimal protein transfer from gel to membrane. Similar results were obtained from two independent experiments. Means and standard error for three to four biological replicates per 8 9 treatment are shown. a,b,c (p < 0.05) show significant differences between treatments. (B) In vivo 10 AtSAL1 activity is sensitive to redox state. Disrupting redox homeostasis at Photosystem I water-water cycle, ascorbate detoxification of ROS, cellular redox buffers or regeneration of 11 oxidized proteins (also see Table S1) result in significantly greater (\*: p<0.05, \*\*: p<0.01) down-12 regulation of AtSAL1 activity compared to WT under high-light stress. Some mutants only show 13 14 a trend of down-regulation in activity but the differences were not significant. Means and 15 standard error for averaged relative activities of two biological replicates at three different concentrations of PAP per genotype are shown. 16

### Fig. 2: Regulation of AtSAL1 activity by redox state *via* its intramolecular Cys167-Cys190 disulfide.

(A) Down-regulation of AtSAL1 activity by oxidation requires oxidation of cysteines, as
mutagenesis of cysteines to alanine in AtSAL1 abrogated redox sensitivity. The redox sensitivity
correlates with a band directly beneath the full length reduced protein (black arrow), which was
determined to be a Cys167-Cys190 intramolecular disulfide (see Fig. 2C). Vertical dashed lines
indicate splicing and truncation of the gel shown in full in Fig. 2C. Asterisk indicates p<0.1.</li>

1 Activity of all proteins were assaved in the presence of 13.4µM PAP. Means and standard error of two independent experiments are shown. (B) Formation of disulfides in AtSAL1 by oxidation 2 is rapidly reversed by returning the redox state to reducing conditions. Vertical dashed lines 3 indicate splicing of the gel to show these three samples side-by-side; all samples were run on the 4 same gel. (C) Determination of Cys-Cys disulfide pairs observed in WT AtSAL1 using cysteine 5 to alanine substitution mutants of AtSAL1 under oxidation. Oxidized AtSAL1 proteins migrate at 6 different rates to reduced AtSAL1 protein. The different Cys-Cys disulfide pairs were identified 7 by cross-comparison to cysteine mutants: AtSAL1 containing a Cys167-Cys190 intramolecular 8 9 disulfide (black triangles) migrates closest to reduced AtSAL1. The oxidized form is absent in all AtSAL1 mutants lacking either or both of Cys167 and Cys190. Other combinations such as the 10 Cys21-Cys167 and Cys21-Cys190 disulfide did not correlate with the down-regulation of 11 AtSAL1 activity by oxidation (Fig. 2A). These are likely non-specifically formed during protein 12 denaturation and SDS-PAGE. AtSAL1 containing the Cys167-Cys190 intramolecular disulfide is 13 the only oxidized AtSAL1 species detected in endogenous plant protein samples pretreated with 14 iodoacetamide to block reduced cysteines during protein extraction to prevent non-specific 15 disulfide formation (Fig. 6). Experiments were performed twice, with identical results. 16

#### 17 Fig. 3: Structural basis for redox regulation of AtSAL1 activity.

(A) Structural elucidation of AtSAL1 reveals a dimerization interface, and three potentially redox-sensitive cysteine residues. Middle inset shows a view of the 2mFo-dFc map (blue lines, contoured at 1.0  $\sigma$ ) centered on Cys119 which is located at the interface between chain A (orange sticks) and chain B (green sticks), or a view of the 2mFo-dFc map centered on Cys167 and Cys190. Right inset shows the disulfide bonds present in an energy minimized model of the oxidized AtSAL1 dimer. (B) Closure of Loop 1 of AtSAL1, as predicted by normal mode

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analysis (NMA). The lowest frequency normal mode is shown. Positions of  $C_{\alpha}$  atoms are shown as coloured spheres, from the crystal structure (blue) to the most closed conformation (31). **(C)** Dimerization and disulfide formation reduces the mobility of key loops (loops 1, 3, 4, 8) in AtSAL1. Energy minimized models of the oxidized AtSAL1 dimer (left) or the reduced AtSAL1 monomer (middle) are colored according to mobility (blue least mobile to red most mobile) for details of energy minimization and normal mode analysis see methods. Right: plot of normal mode analysis (NMA) mobility by residue for the oxidized dimer and reduced monomer.

#### 8 Fig. 4: AtSAL1 is also regulated via dimerization involving Cys119

(A) AtSAL1 in monomer-dimer equilibrium detected during size exclusion chromatography of 9 10 purified recombinant protein. Dimers were detected in at least three independent purification runs. Inset:SDS-PAGE gel of monomeric and dimeric AtSAL1 indicating the proteins were of 11 similar purity. The masses of the monomer and dimer were confirmed by SEC-MALLS (Fig. 12 S1). (B) The monomer-dimer equilibrium can be shifted by an intermolecular disulfide under 13 oxidizing conditions resulting in increased dimer abundance, or reduction of the disulfide by 14 15 DTT dissociating the dimer. Whereas DTT is sufficient to achieve dimer separation, GSH is not. This is consistent with the relative redox potentials of these compounds: -264 mV at pH 7.4 for 16 GSH compared to DTT (-360 mV) and the redox potential of disulfide bonds (ranging from -330 17 to -95 mV in thiol-disulfide oxidoreductases). Oxidation (DTT<sub>ox</sub>, GSSG) increased dimer 18 19 abundance to 100%, indicating formation of an intermolecular disulfide dimer under oxidation. Reversing the oxidation with reductant ( $DTT_{ox} + DTT$  and GSSG + DTT lanes) that breaks 20 disulfide bonds shifts the equilibrium to monomer. The low resolution and fuzzy appearance of 21 the higher MW bands are likely due to the type of gel (Tris-Glycine) as well as lack of detergent 22 (SDS) and reductant (DTT) that inhibit resolving native, folded proteins that are also oxidized. 23

Similar results were obtained in two independent experiments. (C) Under redox titration by
DTT<sub>ox</sub> in *vitro*, which induces formation of the Cys167-Cys190 disulfide, only dimeric AtSAL1
showed significant down-regulation of activity. Identical results were obtained from two
independent experiments. Inset: Dimerization is required for formation of the Cys167-Cys190
intramolecular disulfide that regulates AtSAL1 activity.

#### 6 Fig. 5: AtSAL1 can be regulated by glutathionylation at redox-sensitive cysteines.

7 (A) Glutathionylation of AtSAL1 with oxidized glutathione (GSSG, yellow arrows) results in formation of the intramolecular C167-C190 disulfide (black arrows), presumably via the thiol-8 9 disulfide exchange mechanism (34). Identical results were obtained in two independent 10 experiments. (B) m/z spectrum of AtSAL1 treated with GSSG and an untreated AtSAL1 sample, showing a shift in mass consistent with glutathionylation of the redox-sensitive Cys119 (red). 11 Charge is indicated in brackets. In AtSAL1 treated with GSSG, glutathionylation was also 12 detected on the Cys190 residue involved in intramolecular disulfide formation (not shown). (C) 13 14 Both monomeric and dimeric AtSAL1 are sensitive to glutathionylation, with decrease in activity 15 in redox titration with GSH/GSSG (a less negative redox potential indicates more oxidizing conditions). The redox midpoint potential (E<sub>m</sub>) was close to physiological GSH/GSSG redox 16 potential of Arabidopsis chloroplasts (35). Although activity of dimeric AtSAL1 is only 17 decreased to 40% under fully oxidizing conditions compared to 10% for monomeric AtSAL1, the 18 19 basal activity of dimeric AtSAL1 is already significantly lower than monomeric AtSAL1 under the same redox state (Table 1). Measurements were performed twice. 20

## Fig. 6: AtSAL1 is redox-regulated *via* intramolecular disulfide formation and dimerization *in vivo*, and it is sensitive to the chloroplast redox state.

30

1	(A) Down-regulation of AtSAL1 activity and concomitant PAP accumulation correlates with
2	formation of the Cys167-Cys190 intramolecular disulfide (black triangles) in endogenous
3	AtSAL1 during drought stress. Means and standard error are shown for n=4 biological replicates
4	for well-watered and n=3 for drought. In contrast to Fig. 1A, leaf protein extracts were blocked
5	with iodoacetamide and then protein electrophoresis and western blotting were performed under
6	non-reducing conditions to visualize the Cys167-Cys190 disulfide. Loading control was
7	Coomassie Blue staining. Similar results were obtained in two independent experiments. (B) The
8	monomer-dimer equilibrium of AtSAL1 in vivo is shifted in favor of the dimer during oxidative
9	stress, suggesting formation of the Cys119-Cys119 intermolecular disulfide to stabilize the
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11	Native-PAGE, immunoblotted and the relative quantities of dimeric to monomeric AtSAL1
12	estimated by image analysis on ImageJ.
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### Fig.7: Biochemical and structural evidence for conservation of redox sensitivity in a rice SAL1 ortholog.

(A) Redox titration on OsSAL1 shows that the protein is redox sensitive and has a redox
midpoint potentials (E<sub>m</sub>) in the physiologically-relevant range. A less negative redox potential
indicates more oxidizing conditions. (B) Oxidation of AtSAL1 and OsSAL1 with GSSG
similarly result in glutathionylation of the proteins, increasing their apparent molecular weight
when resolved on non-reducing SDS-PAGE (yellow triangles). Vertical dashed lines indicate
splicing and truncation of the gel to remove additional lanes not relevant to this result. (C)

Comparison between redox-sensitive cysteine residues detected in structures of AtSAL1 and
 modelling of OsSAL1. Unlike AtSAL1 which contains both surface-exposed and intramolecular
 disulfide cysteines, OsSAL1 is predicted to contain surface exposed cysteines (marked in
 yellow). Both Cys203 and Cys221 of OsSAL1 are strongly conserved in *Poaceae* SAL1
 orthologs (see Fig. S5).

### Fig. 8: Enhancement of redox sensitivity in yeast ScHAL2 by introduction of the AtSAL1 intramolecular disulfide

(A) Structural alignment-guided introduction of the intramolecular disulfide from AtSAL1 8 (orange) into yeast ScHAL2 (gray) by the Tyr176Cys mutation. Thiol groups are indicated in 9 yellow. (B) Introduction of additional disulfide in ScHAL2+3C results in increased redox 10 sensitivity in vitro compared to WT ScHAL2. Means and standard error from two independent 11 experiments for specific activity at 3.35µM PAP are shown. For full results, see Table S3. 12 Asterisks indicate significant differences (p<0.05). (C) Introduction of additional disulfide in 13 14 ScHAL2+3C results in increased redox sensitivity and faster initiation of PAP accumulation in *vivo* when expressed in yeast  $\Delta hal2$  cells under mild oxidative stress. Significant differences are 15 indicated by a,b (p<0.05). Error bars indicate standard error; n=3 independent cultures for all 16 17 experiments. n.s. = no significant difference



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A Protein structure of AtSAL1 with potential intermolecular and intramolecular disulfides

B Closure of loop 1 in AtSAL1





C Effect of dimerization and disulfide bonding on flexibility of AtSAL1 protein loops



#### Fig. 3: Structural basis for redox regulation of AtSAL1 activity.

(A) Structural elucidation of AtSAL1 reveals a dimerization interface, and three potentially redox-sensitive cysteine residues. Middle inset shows a view of the 2mFo-dFc map (blue lines, contoured at 1.0  $\sigma$ ) centered on Cys119 which is located at the interface between chain A (orange sticks) and chain B (green sticks), or a view of the 2mFo-dFc map centered on Cys167 and Cys190. Right inset shows the disulfide bonds present in an energy minimized model of the oxidized AtSAL1 dimer. (B) Closure of Loop 1 of AtSAL1, as predicted by normal mode analysis (NMA). The lowest frequency normal mode is shown. Positions of C<sub>a</sub> atoms are shown as coloured spheres, from the crystal structure (blue) to the most closed conformation (31). (C) Dimerization and disulfide formation reduces the mobility of key loops (loops 1, 3, 4, 8) in AtSAL1. Energy minimized models of the oxidized AtSAL1 dimer (left) or the reduced AtSAL1 monomer (middle) are colored according to mobility (blue least mobile to red most mobile) for details of energy minimization and normal mode analysis see methods. Right: plot of normal mode analysis (NMA) mobility by residue for the oxidized dimer and reduced monomer.



Α

Detection of AtSAL1 monomers and dimers



C Effect of dimerization on redox sensitivity and formation of the Cys167-Cys190 intramolecular disulfide



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#### B Effect of redox state on *in vitro* AtSAL1 monomer dimer equilibrium



#### В

Glutathionylation of redox-sensitive Cys119 in AtSAL1





(A) Glutathionylation of AtSAL1 with oxidized glutathione (GSSG, yellow arrows) results in formation of the intramolecular C167-C190 disulfide (black arrows), presumably via the thiol-disulfide exchange mechanism (34). Identical results were obtained in two independent experiments. (B) m/z spectrum of AtSAL1 treated with GSSG and an untreated AtSAL1 sample, showing a shift in mass consistent with glutathionylation of the redox-sensitive Cys119 (red). Charge is indicated in brackets. In AtSAL1 treated with GSSG, glutathionylation was also detected on the Cys190 residue involved in intramolecular disulfide formation (not shown). (C) Both monomeric and dimeric AtSAL1 are sensitive to glutathionylation, with decrease in activity in redox titration with GSH/GSSG (a less negative redox potential indicates more oxidizing conditions). The redox midpoint potential ( $E_m$ ) was close to physiological GSH/GSSG redox potential of Arabidopsis chloroplasts (35). While dimeric AtSAL1 activity was only decreased to 40% under fully oxidizing conditions compared to 10% for monomeric AtSAL1, the basal activity of dimeric AtSAL1 was already significantly lower than monomeric AtSAL1 under the same redox state (Table 1). Measurements were performed twice.



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WW: Well-watered, MD: Mid-Drought, LD: Late-Drought, HL: High-light, MV: Methyl Viologen, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide.



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#### Fig. 7



B Effect of introducing AtSAL1 Cys167-Cys190 intramolecular disulfide on redox sensitivity of yeast ScHAL2 *in vitro* 



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#### Table 1

Kinatia paramatara	Monomeric AtSAL1		Dimeric AtSAL1		
Killetic parameters	Reduced	Oxidized	Reduced	Oxidized	
<i>К</i> <sub>М</sub> (µМ РАР)	9.9 ± 3.3	8.3 ± 1.2	8.7 ± 5.2	4.2± 1.8	
k <sub>cat</sub> (min <sup>-1</sup> )	123 ± 18	119 ± 13	24.4 ± 1.1	4.3 ± 1.3	
$k_{\rm cat}$ / $K_{\rm M}$ ( $\mu { m M}^{-1}$ min <sup>-1</sup> )	12.4	14.2	2.8	1.0	

Table 1: Effect of dimerization on redox sensitivity and enzyme kinetics of AtSAL1 under different redox states. All enzymatic assays were performed with 0.2 $\mu$ g recombinant protein in the presence of 5mM DTT (reduced) or DTT<sub>ox</sub> (oxidized) at 25°C. Results shown are means and standard error from two independent experiments.

#### Table 2

Kinetic parameters	OsS	OsSAL1		
	Reduced	Oxidized		
<i>К</i> <sub>М</sub> (µМ РАР)	20 ± 6.9	61 ± 23.6		
k <sub>cat</sub> (min <sup>-1</sup> )	44 ± 3.9	11 ± 1.5		
k <sub>cat</sub> / K <sub>M</sub> (μM <sup>-1</sup> min <sup>-1</sup> )	2.2	0.2		

#### Table 2: Effect of oxidation on enzyme kinetics of the riceSAL1 ortholog, OsSAL1.

Enzymatic assays were performed with 0.2 µg recombinant protein in the presence of 20mM GSH (reduced) or GSSG (oxidized) at 25°C. Experiment was performed twice, with similar results.

Fig. S1



#### Fig. S1: AtSAL1 exists in a monomer-dimer equilibrium.

Purified AtSAL1 was exchanged into assay buffer and analysed by MALLS following separation by size exclusion chromatography. Differential refractive index is shown as a blue line (right axis) and molecular mass derived from light scattering data as shown as red points (left axis). The expected masses of AtSAL1 monomer (37.5 kDa) and dimer (75 kDa) are indicated by dashed lines.



#### Fig. S2: Prediction of coupled motions in AtSAL1.

Covariance maps of  $C_{\alpha}$  motions (74) calculated for the reduced AtSAL1 monomer and oxidized AtSAL1 dimer. Residues with coupled motions are shown in red, residues with anti-correlated motions are shown in blue. The positions of the mobile loops described in Fig. 3C (loops 1,3,8) are indicated.



Formation of intramolecular disulfide via glutathionylation and thiol-disulfide exchange

Fig. S3: Formation of an intramolecular disulfide via a thiol-disulfide exchange initiated by glutathionylation of a cysteine residue. See ref 34 for review of this mechanism.



Effect of substituting redox-sensitive cysteines in AtSAL1 on protein stability and abundance in the soluble fraction when heterologously expressed in *E. coli* 

**Fig. S4: Cys-Ala mutations negatively affect AtSAL1 protein stability and abundance.** The yield of soluble AtSAL1 protein is drastically decreased when the redox-sensitive Cys residues were mutagenized to Ala. The SDS-PAGE gel shows semi-purified recombinant WT or mutated AtSAL1 proteins after a soluble protein fraction from 7 mL of induced *E. coli* cells was incubated with Ni-NTA beads in a 1.5mL Eppendorf tube, washed with 20mM imidazole, and the bound AtSAL1 + 6X His-Ub fusion proteins (black triangles) eluted with 250mM imidazole. The abundance of soluble AtSAL1 protein decreased with increasing number of Cys-Ala substitutions. The negative effect of the Cys-Ala mutations was reproducible in 2 independent transformed bacterial colonies.



**Fig. S5: Conservation of redox-sensitive cysteines in SAL1 orthologs in plants**. The Cys119 involved in dimerization of AtSAL1 is moderately conserved in eudicots and non-*Poaceae* monocot plants, but is absent in other lineages. The intramolecular disulfide pair Cys167-Cys190 is invariant across bryophytes, lycophytes, primitive angiosperm, eudicots and non-Poaceae monocots. Cys190 is C-terminally shifted by seven amino acids in *Poaceae* monocot plants. Additionally, another cysteine is strongly conserved in the *Poaceae* family of monocots (Cys221 in OsSAL1; see Fig. 7). In contrast to plant SAL1 orthologs (green), the fungal SAL1 ortholog in *Saccharomyces cerevisiae* HAL2 (blue) lacks Cys119 and Cys190.



**Fig. S6: WT and engineered ScHAL2. (A)** Presence of a surface-exposed Cys349 in the ScHAL2 crystal structure (1KA1), which may explain the redox sensitive activity observed in Fig. 8B. **(B)** The WT ScHAL2 and ScHAL2+3C proteins are equally active *in vivo* when expressed in yeast cells deficient in ScHAL2 ( $\Delta hal2$ ), as both proteins complement PAP levels in  $\Delta hal2$  to similar levels, albeit still about 10-fold higher than WT. Similar results were obtained in two independent experiments.

#### Table S1

Name	Gene Identifier	Type of mutation	Biological pathway affected	Ref.
tAPX (thylakoidal Ascorbate Peroxidase)	AT1G77490	amiRNA- knockdown	Detoxification of H <sub>2</sub> O <sub>2</sub> in the chloroplast thylakoid, detoxification of superoxide by superoxide dismutase in water-water cycle at Photosystem I (PSI).	1
sAPX (stromal Ascorbate Peroxidase)	AT4G08390	T-DNA insertion, homozygous	Detoxification of $H_2O_2$ in the chloroplast stroma, where AtSAL1 is also located	1
phs1 (photosensitive 1)	AT3G47390	T-DNA insertion, homozygous	Involved in riboflavin and FAD synthesis. Mutant allele has increased oxidative stress due to reduced NADPH/NADP+ ratios and overproduction of ROS at PSI under high light.	53
cos1 (coronatine insensitive1 suppresor)	AT2G44050	T-DNA insertion, heterozygous	Involved in the same metabolic pathway as <i>phs1</i> (riboflavin and FAD synthesis).	59
rax1-1 (regulator of Ascorbate Peroxidase 2 1-1)	AT4G23100	Point mutation, homozygous	Rate-limiting step of glutathione synthesis. GSH:GSSG redox ratio may be altered under oxidative stress.	54
ntrc (NAPDH-dependent Thioredoxin Reductase C)	AT2G41680	T-DNA insertion, homozygous	Reduction of 2-Cys peroxiredoxins and redox control within the chloroplast. Deficiency causes hypersensitivity to abiotic stress	52

Table S1: Summary of redox homeostasis-deficient mutants used in Fig. 1.Loss-of-function mutants deficient in various aspects of redox homeostasis in the chloroplast were chosen for analysis ofAtSAL1 activity under abiotic stress. The mutants are deficient in ROS detoxification, redox control at PSI, or chloroplast redox buffer pathways.

#### Table S2

AtSAL1 (PDB 5ESY)				
Data Processing				
Space group	<i>P</i> 6 <sub>1</sub>			
Cell dimensions (Å) a,b,c	137.14 137.14 74.64			
$\alpha, \beta, \gamma$ (°)	90, 90, 120			
Resolution range (Å)	38.247 - 3.05 (3.24 - 3.05) <sup>1</sup>			
Total number of reflections	125243 (20978)			
Number of unique reflections	15341 (2522)			
Multiplicity	8.2 (8.3)			
Completeness (%)	99.63 (92.8)			
Mean I/ $\sigma(I)$	15.23 (2.2)			
Wilson B factor (Å <sup>2</sup> )	83.6			
<sup>2</sup> CC <sub>1/2</sub>	0.999 (0.620)			
R <sub>merge</sub>	0.1042 (1.110)			
Refinement				
R <sub>work</sub> /R <sub>free</sub>	0.220/ 0.264 (0.324/0.409)			
Total number of atoms	5095			
Protein	5095			
Ligand/ion	0			
Water	0			
RMSD for bonds (Å)	0.011			
RMSD for angles (deg)	1.47			
Ramachandran favored (%)	87			
Ramachandran outliers (%)	3			
Clashscore	13			
Average B factor $(Å^2)$	106.0			

Table S2: Data collection and refinement statistics for crystal structure of AtSAL1

<sup>1</sup> Values in parenthesis are for the highest resolution shell. <sup>2</sup> Pearson's correlation coefficient calculated from two half-sets of the data (63, 64).

Protein	[PAP] (µM)	Specific Activity (pmol µg prot <sup>-1</sup> min <sup>-1</sup> )		Decrease in Activity (%)	
		Reduced	Oxidized		Average
ScHAL2	3.35	124 ± 0.9	77 ± 16	38	
	13.4	414 ± 66	220 ± 31	46	$46 \pm 4.8^{a}$
	26.8	597 ± 150	270 ± 35	54	
ScHAL2+3C	3.35	53 ± 10	17 ± 2	67	67 ± 1.4 <sup>b</sup>
	13.4	72 ± 24	25 ± 5	65	(p<0.005 relative to ScHAL2)
	26.8	81 ± 29	24 ± 0.8	70	

Table S3: Effect of introducing AtSAL1 Cys167-Cys190 disulfide pair on redox sensitivity of ScHAL2.Enzymatic assays were performed with 0.2  $\mu$ g recombinant protein in the presence of 20mM GSH (reduced) or GSSG (oxidized) at 30°C. Means and standard error from two independent experiments are shown. The decrease in activity for ScHAL2+3C by oxidation is significantly greater (a,b; p<0.005) than that in WT ScHAL2.