Title: Secondary sulfur metabolism in cellular signalling and oxidative stress responses

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Running Title: *Secondary S metabolites in stress cellular signalling*

Highlight

Secondary sulfur metabolism produces several metabolites which regulate various aspects of cellular signalling and homeostasis in response to environmental perturbations.

Abstract

The sulfur metabolism pathway in plants produces a variety of compounds that are central to the acclimation response to oxidative stresses such as drought and high light. Primary sulfur assimilation provides the amino acid cysteine, which is utilized in protein synthesis and as a precursor for the cellular redox buffer glutathione. In contrast, the secondary sulfur metabolism pathway produces sulfated compounds such as glucosinolates and sulfated peptides, as well as a corresponding by-product 3'-phosphoadenosine 5'-phosphate (PAP). Emerging evidence over the past decade has shown that secondary sulfur metabolism also has a crucial engagement during oxidative stress. This occurs across various cellular, tissue and organismal levels including chloroplast-to-nucleus retrograde signalling events mediated by PAP, modulation of

hormonal signalling by sulfated compounds and PAP, control of physiological responses such as stomatal closure, and potential regulation of plant growth. In this review, we examine the contribution of the different components of plant secondary metabolism to oxidative stress homeostasis, and how this pathway is metabolically regulated. We further outline the key outstanding questions in the field that are necessary to understand how and why this 'specialized' metabolic pathway plays significant roles in plant oxidative stress tolerance.

Key words

Sulfur, metabolism, 3'-phosphoadenosine 5'-phosphate, retrograde signalling, sulfotransferase, APK, SAL1, drought, oxidative stress

Abbreviations

ABA	abscisic acid
APK	Adenosine Phosphosulfate Kinase
APS	adenosine phosphosulfate
GSH	glutathione
JA	jasmonic acid
OH-JA	hydroxyl-jasmonic acid
PAP	3'-phosphoadenosine 5'-phosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PAPST	3'-phosphoadenosine 5'-phosphosulfate Transporter
ROS	reactive oxygen species
SA	salicylic acid
SOT	Sulfotransferase
TPST	Tyrosyl Protein Sulfotransferase
XRN	exoribonuclease

Introduction

Plant responses to environmental conditions that invoke reactive oxygen species (ROS) over-accumulation leading to photo-oxidative stress in leaves, such as drought and high light, and combinations thereof, involve coordinated acclimation processes across biochemical, cellular and physiological levels. Processes that are activated include the synthesis and downstream signalling of hormones (Galvez-Valdivieso *et al.*, 2009), alterations in metabolism of nutrients such as sulfur (Malcheska *et al.*, 2017; Rizhsky *et al.*, 2004), chloroplast-to-nucleus retrograde signalling (Chan *et al.*, 2016b), secondary messenger signalling involving reactive oxygen species (ROS) and Ca^{2+} (Steinhorst and Kudla, 2013). Each of these processes impinge on physiological responses such as stomatal closure (Murata *et al.*, 2015) and regulation of plant growth (Bechtold and Field, 2018). Intriguingly, multiple facets of the plant response to oxidative stress are regulated by components of sulfur metabolism (Estavillo *et al.*, 2011; Speiser *et al.*, 2018; Zechmann, 2014).

Plants assimilate sulfur in the form of sulfate anions, which are first taken up into root cells and then transported between and within plant cells *via* four groups of sulfate transporters (SULTRs) with differing subcellular localizations and substrate affinities (Takahashi *et al.*, 2011). The sulfate is first activated in the cytosol and chloroplasts to adenosine phosphosulfate (APS) through the action of ATP sulfurylases (ATPSs) (**Figure 1**). APS contains a high-energy P-S bond which enables it to act downstream as an S-donor in the contrasting branches of a bifurcated metabolic pathway. In the primary branch, the sulfate moiety of APS is successively reduced and integrated into the carbon skeleton of O-acetyleserine for the synthesis of cysteine. Cysteine serves as a substrate for biosyntheses of methionine, as well as the cellular redox buffer glutathione (GSH) (Takahashi *et al.*, 2011), which has well-established important roles in redox control during growth and development and oxidative stress homeostasis (Mhamdi and Van Breusegem, 2018). Alternatively, APS can enter the secondary sulfur metabolism pathway to be phosphorylated by APS kinase (APK) enzymes in the cytosol and chloroplasts to produce 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Mugford *et al.*, 2009). The chloroplastic PAPS can be transported to the cytosol by at least two chloroplast envelope-localized PAPS Transporters (PAPSTs) (Ashykhmina *et al.*, 2018; Gigolashvili *et al.*, 2012), while cytosol-to-

Golgi PAPS is hypothesized to occur though the transporter is yet to be identified. The activated sulfur in PAPS is then transferred to various acceptor molecules including hormones, xenobiotics and growth-regulating peptides by cytosolic and Golgi-resident sulfotransferases (SOTs) (Hirschmann *et al.* (2014)). As a by-product, 3'-phosphoadenosine 5'-phosphate (PAP) is formed, which is dephosphorylated by the SAL1 phosphatase to adenosine monophosphate (AMP) in chloroplasts and mitochondria (Estavillo *et al.*, 2011). (**Figure 1**).

Interestingly, plastidial APS is utilized by both primary and secondary sulfur metabolism (**Figure 1**), which necessitates coordination between these pathways at multiple levels, although the mechanism(s) are not fully understood (Kopriva *et al.*, 2012). For example, loss of APK activity at the entry point into secondary sulfur metabolism re-directs sulfur flux into cysteine and GSH synthesis (Mugford *et al.*, 2011). Conversely, loss of SAL1 activity leads to decreased levels of both primary and secondary sulfur metabolites, as well as that of sulfate (Lee *et al.*, 2012). Therefore, the control of sulfur partitioning between primary and secondary sulfur metabolism still remains to be elucidated, particularly during oxidative stress conditions such as drought stress when multiple sulfur metabolites participate in the cellular response (Chan *et al.*, 2013). Indeed, evidence over the past decade increasingly point towards crucial roles for secondary sulfur metabolites in oxidative stress signalling and responses.

APS Kinases: Facilitators of sulfur flux into secondary sulfur metabolites

The APK enzymes constitute a branching point of sulfur flux into secondary sulfur metabolism, as they direct sulfur away from primary sulfur assimilation through conversion of APS into PAPS. Three of the four APK isoforms in *Arabidopsis* (APK1, APK2, APK4) are localized to chloroplasts. APK1 and APK2 are the major isoforms with partially overlapping tissue expression patterns and functional redundancy (Mugford *et al.*, 2009). This is evidenced by lack of visible growth phenotypes in any of the single *apk* genetic lesions and a dwarfed phenotype of *apk1apk2* which possesses significantly lower levels of PAPS-requiring metabolites such as glucosinolates, and correspondingly accumulate the precursor desulfoglucosinolates (Mugford *et al.*, 2009). Plastidial APK4 and cytosolic APK3 play relatively minor roles in the provision of PAPS (Mugford *et al.*, 2010), since analysis of higher-order *apk* mutant combinations show that *apk3apk4* is aphenotypic while *apk1apk2apk3* and *apk1apk2apk4* phenotypes are additive to *apk1apk2*. The decreased growth in plants with low APK activity is hypothesized to be caused at least in part by loss of sulfated peptides with growth-promoting roles (Mugford *et al.*, 2009), but this has not been confirmed. Importantly,

these findings indicate that APK dependent PAPS production is rate-limiting for the production of downstream secondary sulfur metabolites catalyzed by the SOTs. Indeed, APK2 alone is not sufficient for PAPS provision since *apk1apk3apk4* is embryo-lethal (Mugford *et al.*, 2010) The contribution of APKs to oxidative stress tolerance has not been tested. Intriguingly, APK1 is inactivated by oxidation *in vitro* (Ravilious *et al.*, 2012) (discussed in more detail below), and *apk1apk2* accumulates higher levels of the redox buffer GSH. These findings may indicate that APK inactivation could be favoured under oxidative stress conditions. However, it can also be expected that severe limitation of PAPS supply can be deleterious since many of the SOT products can play important functions in response to oxidative stress as discussed below.

Regulation of hormones, growth and stress homeostasis by sulfur-containing secondary metabolites and sulfotransferases

In mammalian systems, the physiological functions of SOTs are relatively well-characterized, with known substrates including carbohydrates, proteoglycans, proteins, xenobiotics, and various steroid hormones (Gamage *et al.*, 2006; Mueller *et al.*, 2015). Diseases caused by loss-of-function of specific SOTs in humans are also described (Mueller *et al.*, 2015). In contrast, plant SOT research still presents many unknowns. For example, there are 71 predicted SOTs in *Brassica napus*, but they remain poorly characterized with a few notable exeptions (Hirschmann and Papenbrock, 2015). Of the 22 SOTs encoded by the *Arabidopsis* genome, only ten have known *in vitro* substrates or *in vivo* physiological roles (**Figure 2**) (Hirschmann *et al.*, 2014). Their substrates include desulfoglucosinolates, hormones or hormone derivatives (brassinosteroids, salicyclic acid, hydroxy-jasmonic acid), flavonoids, xenobiotics, and peptides (Hirschmann *et al.*, 2014).

Glucosinolates – defense compounds with additional roles in oxidative stress

In *Brassicaceae*, the bulk of sulfur flux *via* the SOTs under steady-state conditions is utilized for the synthesis of glucosinolates, which are anti-herbivory defence compounds (reviewed by Kliebenstein *et al* in this issue). The final step of glucosinolate biosynthesis involves the transfer of the sulfate moiety from PAPS to desulfoglucosinolates by SOT16, SOT17 and SOT18 (Piotrowski *et al.*, 2004). Interestingly, both foliar and root glucosinolate accumulation have been observed in several *Brassica* species subjected to abiotic stresses such as drought and salt stress (Del Carmen Martínez-Ballesta *et al.*, 2013), and deletion of two MYB transcription factors which abolish accumulation of aliphatic glucosinolates was correlated with increased salt stress sensitivity (Martínez-Ballesta *et al.*, 2015). The downstream breakdown products of

glucosinolates might be involved in the oxidative stress responses. The myrosinase enzyme TGG1 which degrades glucosinolates into active isothiocyanates is highly abundant in guard cells, its mutation led to loss of ABA sensitivity (Zhao *et al.*, 2008), and exogenous application of isothiocyanates also closed stomata (Khokon *et al.*, 2011). Exogenous isothiocyanate also increased NADPH oxidase activity and nitric oxide levels in guard cells (Khokon *et al.*, 2011), and stimulates large-scale transcriptomic changes associated with oxidative stress responses (Kissen *et al.*, 2016). It can be hypothesized that the effects of isothiocyanate is due to either the activation of specific signalling proteins, or the stimulation of cellular responses to the increased ROS and NO levels, or both. In animal systems the proposed modes of action for ingested isothiocyanates include direct binding to reactive thiol groups of proteins (Nakamura *et al.*, 2018). Whether such mechanisms also hold true in plants will need further investigation.

Growth regulating peptides

Whereas the sulfation of desulfoglucosinolates by SOT16, 17 and 18 produce glucosinolates which are themselves inert until further activation by myrosinases, sulfation processes by other SOTs have been linked to direct modulation of bioactivity of their substrates. This is best examplified by the Tyrosyl Protein Sulfotransferase (TPST) (Komori *et al.*, 2009). TPST-mediated sulfation of peptides phytosulfokine (PSK), plant peptide containing sulfated tyrosine 1 (PSY1) and root growth factor (RGF) is critical for the growth-promoting functions of these peptides (Komori *et al.*, 2009; Matsuzaki *et al.*, 2010; Zhou *et al.*, 2010). Indeed, loss-of-function of TPST results in severe developmental defects, such as strongly decreased root growth and loss of stem cell identity in roots. The crucial role of TPST is exemplified by its strong evolutionary conservation pre-dating the emergence of land plants, in contrast to cytosolic SOTs such as SOT15 (Zhao *et al.*, 2019). It has been hypothesized that TPST's evolutionary conservation is due not just to its growth promoting effects, but also due to its involvement in stomatal regulation (Zhao *et al.*, 2019) and in balancing the plant's growth and stress responses (**Figure 2**). Its sulfated products, PSK and PSY1, function in both the induction of defense responses to pathogens and as growth factors; these two peptides also function antagonistically (reviewed in Sauter (2015)). This hypothesis is corroborated by the enhanced root growth sensitivity of *tpst* to copper deficiency compared to wild type (Wu *et al.*, 2015). However, it is currently unknown whether TPST also coordinates growth and acclimation to other oxidative stresses such as drought or high light.

Hormones and hormone derivatives

SOTs are also capable of targeting at least four other classes of compounds involved in the coordination of growth and stress responses (**Figure 2**): brassinosteroids (SOT10, SOT12); salicylic acid (SA; sulfated by SOT12), flavonoids (SOT5, SOT8, SOT12, SOT13), and hydroxy-jasmonate (OH-JA; sulfated by SOT15). However, these reported substrates for SOTs have been mostly identified from *in vitro* assays and corresponding *in vivo* validation using genetic mutants have been largely lacking.

Brassinosteroids are regulators of growth and development (Wei and Li, 2016), but can also enhance oxidative stress tolerance through multiple mechanisms when applied exogenously (Sharma *et al.*, 2017). SOT12 can sulfate multiple brassinosteroids *in vitro* but shows a preference for the brassinosteroid precursor 24-epicathasterone (Marsolais *et al.*, 2007). Surprisingly, it is not known whether *sot12* loss of function mutants display defects in brassinosteroid signalling. SOT10 prefers the biologically active end products of brassinosteroid biosynthesis such as 24-epibrassinolides and the naturally occurring (22R, 23R)-28-homobrassinosteroids. Interestingly, sulfation of 24-epibrassinolides can lead to suppression of its bioactivity (Rouleau *et al.*, 1999). However, brassinosteroid-related phenotypes were not observed in *sot10* loss-of-function or SOT10-overexpressing plants (Sandhu and Neff, 2013). These findings parallel that for the *Brassica napus Sulfotransferase 3* (BnST3) and BnST4 enzymes, which are capable of targeting multiple brassinosteroids (Rouleau *et al.*, 1999) but did not lead to BR-related phenotypes when over-expressed in *Arabidopsis* (Marsolais *et al.*, 2004). Conjugation of polar moieties (e.g. through sulfation) to the relatively non-polar brassinosteroids has also been proposed to improve intracellular movement of brassinosteroids from their site of synthesis at the endoplasmic reticulum to their site of perception at the plasma membrane (Symons *et al.*, 2008). Thus, it is suggested that brassinosteroid sulfotransferases regulate brassinosteroid activity, mobility and/or perception, although the exact mechanism(s) remain unknown.

Salicylic acid (SA) is a regulator of pathogen responses and cell death (Seyfferth and Tsuda, 2014). Baek *et al.* (2010) reported that SOT12 is able to transfer sulfate to SA. The authors proposed that sulfation of SA by SOT12 may be a key regulatory point for SA induction in response to pathogens, since s*ot12* sensitivity to the pathogen *Pseudomonas syringae* is accompanied by an inability to accumulate SA. The *sot12* mutant is also more sensitive to some abiotic stresses such as salt stress. In rice, the *Resistance to Rice Stripe Virus on Chromosome 11* (*STV11*) gene encodes a SOT that sulfates SA (Wang *et al.*, 2014). STV11 mediates SA

accumulation *in vivo* and *Japonica* rice varieties lacking functional STV11 are sensitive to Rice Stripe Virus (Wang *et al.*, 2014).

Another known substrate for SOTs in *Arabidopsis* is hydroxyl-jasmonic acid (OH-JA), which functions synergistically and antagonistically to SA in regulating pathogen responses and cell death (Caarls *et al.*, 2015; Tamaoki *et al.*, 2013). Signalling by the biologically active JA can be controlled stepwise through enzymatic conjugation to amino acids, methylation, and hydroxylation to give rise to 12-hydroxy-JA (reviewed in Wasternack and Strnad (2016)). Interestingly, 12-hydroxy-JA and its related compound, 11-hydroxy-JA, can be further modified *via* sulfation by SOT15 to give rise to 12- and 11-hydroxy-JA sulfate respectively (Gidda *et al.*, 2003). Both 12-hydroxy-JA and 12-hydroxy-JA sulfate appear to be inactivated forms of JA, and they also down-regulate the expression of genes encoding for enzymes in JA biosynthesis (Miersch *et al.*, 2008). This suggests that 12-hydroxy-JA sulfate, and by extension SOT15, may constitute part of an "off" switch in JA signalling (Miersch *et al.*, 2008).

Other stress-associated metabolites

Flavonoids have diverse roles including UV protection, growth regulation and plant-microbe interactions (Falcone Ferreyra *et al.*, 2012). At least four SOTs are capable of sulfating a variety of flavonoids but with subtly different substrate preferences and enzymatic rates (Hashiguchi *et al.*, 2013; Hashiguchi *et al.*, 2014). Both SOT13 and SOT5 showed strong preference for the flavonol galangin, although the latter also has significant affinity for kaempferol. In contrast, the most preferred substrate for SOT12 is the flavonone naringenin, although it also processes galangin and kaempferol at similar rates to SOT13 and SOT5 (Hashiguchi *et al.*, 2013). Significantly, SOT12 is also active against quercetin, with a specific activity that is approximately 50% of that against its preferred substrate naringenin (Hashiguchi *et al.*, 2013). This raises the question whether SOT12 might also participate in auxin homeostasis *in vivo*, since quercetin-3-sulfate is proposed to regulate auxin transport in *Flaveria bidentis* (Ananvoranich *et al.*, 1994). However, auxin-related phenotypes have not been reported yet for *sot12*.

SOT12 is also capable to detoxify xenobiotic compounds produced by soil bacteria, such as the protein translation inhibitor cycloheximide (Chen *et al.*, 2015). While xenobiotic compounds are not regulators of plant growth and stress responses *per se*, these metabolites are also

produced endogenously in plants during high light stress and hence require detoxification (D'Alessandro *et al.*, 2018).

Biochemical basis for diversity of SOT substrates and functions

The high number and variety of SOT substrates can be explained by the structural features of the SOT active site. Analysis of the SOT18 crystal structure reveals that plant SOTs have highly conserved catalytic residues and PAPS binding region which are also shared with orthologues from many different species (Hirschmann *et al.*, 2017). In contrast, the binding site of the sulfate acceptor substrate along with three flexible loops gating the entrance to the active site are poorly conserved, thus providing the means for substrate specificity and specialization between SOTs (Hirschmann *et al.*, 2017). Indeed, a small number of amino acid changes can lead to different activity levels and substrate preference of the same SOT enzyme from different *Arabidopsis* ecotypes (Klein and Papenbrock, 2009; Luczak *et al.*, 2013); although these polymorphisms have not been mapped to the recent structural data. These findings could provide the basis for understanding the function and regulation of multi-substrate SOTs such as SOT12 during oxidative stresses. For example, the reported K_M of SOT12 against 24-epicathasterone (6.9 µM) is several times lower compared to salicylic acid (440 µM), and the SOT12 specific activity against different flavonoids varies across two orders of magnitude (Baek *et al.*, 2010; Hashiguchi *et al.*, 2013; Marsolais *et al.*, 2007). It will be interesting to explore the structural basis for these substrate preferences with respect to its protein structure.

PAP-mediated chloroplast-to-nucleus retrograde signalling and physiological responses to abiotic stress

While the different SOTs might process substrates with diverse chemical structures and roles in stress and growth homeostasis, one commonality shared between these enzymes is that they all consume PAPS, thereby producing PAP as a by-product (**Figure 2**). Early work on PAP-accumulating mutants showed that constitutively high PAP impedes auxotrophic growth in yeast and *E. coli* (Glaser *et al.*, 1993; Masselot and De Robichon-Szulmajster, 1975; Neuwald *et al.*, 1992). PAP most likely blocks sulfate assimilation through the inhibition of PAPS reductase in these organisms, which have a different sulfate assimilation pathway to plants and lack sulfotransferases (Murguia *et al.*, 1995, 1996). In mammals, which lack a cysteine biosythesis pathway but do possess sulfotransferases (Gamage *et al.*, 2006; Mueller *et al.*, 2015; Stipanuk, 2004), PAP accumulation inhibits sulfotransferase reactions and also interferes with protein translation through a sulfotransferase-independent mechanism (Frederick *et al.*, 2008;

Hudson *et al.*, 2013). PAP-accumulating mice are non-viable (Frederick *et al.*, 2008; Hudson *et al.*, 2013). In contrast, in plants PAP is not simply a toxic metabolite: although *sal1* mutants in *Arabidopsis* show a dwarfed phenotype, PAP accumulation is not lethal and the mutants are also drought-tolerant (Estavillo *et al.*, 2011; Phua *et al.*, 2018a; Phua *et al.*, 2018b; Wilson *et al.*, 2009). Indeed, PAP acts as a chloroplast-to-nucleus retrograde signal during oxidative stresses such as drought and high light. Under these conditions, intracellular PAP accumulation and its perception in the nucleus activates stress-responsive gene expression, leading to acclimation responses and drought tolerance (Estavillo *et al.*, 2011; Pornsiriwong *et al.*, 2017).

Degradation, intracellular transport, and nuclear function of PAP

Unstressed plants maintain very low (almost undetectable) levels of PAP through the action of the PAP catabolic phosphatase SAL1, which hydrolyses PAP to AMP in chloroplasts and mitochondria (Estavillo *et al.*, 2011; Quintero *et al.*, 1996). During oxidative stress, however, PAP accumulates up to 30-fold higher under drought and by 50% higher under excess light (Estavillo *et al.*, 2011). This accumulation is achieved at least in part *via* an oxidative post-translational inactivation of SAL1 (Chan *et al.*, 2016a). Biochemical and structural analysis of the SAL1 protein show that formation of inter- and intra-molecular disulfide bonds in the SAL1 protein under oxidizing conditions decreases its capacity to degrade PAP. Modification of the redox-sensitive cysteines on SAL1 through glutathionylation similarly decreases its activity against PAP (Chan *et al.*, 2016a). Redox regulation of SAL1 was also observed *in vivo*. Therefore, at least in chloroplasts, SAL1 functions as an oxidative stress and redox sensor by regulating PAP levels (Chan *et al.*, 2016a; Estavillo *et al.*, 2011) (**Figure 3**).

PAP is relocalised between organelles and the cytosol via the PAPS/PAP transporter 1 (PAPST1) and PAPST2, two antiporters which are able to exchange any two of PAPS, PAP, ATP and ADP (Ashykhmina *et al.*, 2018; Gigolashvili *et al.*, 2012). PAPST1 is localized to chloroplasts whereas PAPST2 is dual-targeted to both chloroplasts and mitochondria (**Figure 3**). Non-aqueous organelle fractionation experiments showed that PAP is present in the cytosol and the plastids, with lesions in PAPST2 causing altered intracellular distribution of PAP (Ashykhmina *et al.*, 2018). Complementation experiments targeting SAL1 separately to either the chloroplast, cytosol or the nucleus in a *sal1* loss-of-function mutant demonstrated that the different transgenic lines had almost equal reversion to the wild type phenotype (Estavillo *et al.*, 2011). These results indicate that while PAPS and PAP are present predominantly in plastids and the cytosol, PAP can also move into the nucleus (**Figure 3**). The movement of PAP

between the nucleus and the cytosol is assumed to occur *via* passive diffusion through the nuclear membrane pores (Estavillo *et al.*, 2011).

In the nucleus, PAP primarily targets 5'-3' exoribonucleases XRN2 and XRN3, which have roles in the degradation of uncapped transcripts, removal of RNA Polymerase II (Pol II) from DNA to terminate transcription, and maintenance of gene silencing (Crisp *et al.*, 2018; Gy *et al.*, 2007). Inhibition of XRNs by PAP (Dichtl *et al.*, 1997), leads to the activation of 25 % of the high light stress transcriptome, including genes encoding antioxidant enzymes such as *Ascorbate Peroxidase 2* (*APX2*) (Estavillo *et al.*, 2011). Recent work by Crisp *et al.* (2018) and Krzyszton *et al.* (2018) provided valuable insights into the paradox of how specificity in the regulation of nuclear gene expression by PAP is achieved, given that the XRNs are not transcription factors which bind to specific DNA motifs. PAP-mediated inhibition of XRNs leads to inefficient removal of Pol II upon completion of transcription a particular gene, thereby facilitating polymerase read-through to the downstream gene and increasing transcription of this downstream gene. Indeed, the majority of the highest up-regulated genes in *sal1* mutants are located downstream of an endogenously highly expressed gene. The effect of read-through on transcriptional up-regulation is also correlated with the distance between the upstream and downstream genes (Crisp *et al.*, 2018). Of the genes up-regulated in *sal1*, 23% are directly up-regulated by read-through. Therefore, it is possible that genes up-regulated by Pol II read-through then lead to further up- and down-regulation of other genes *via* feedback effects or downstream signalling. Significantly, transcriptional read-through events were also observed in wild type plants during drought stress, indicating that Pol II regulation by PAP-XRN can be one of the mechanisms modulating gene expression for oxidative stress homeostasis (Crisp *et al.*, 2018).

The characteristics of the PAP-responsive transcriptome are correlated with the increased oxidative stress tolerance phenotype in both *sal1* and *xrn2 xrn3* mutants. Constitutive up-regulation of stress homeostasis genes in these mutants lead to decreased hydrogen peroxide accumulation in response to high light stress, decreased ion leakage in response to osmotic stress, and accumulation of various osmoprotectants (Estavillo *et al.*, 2011; Wilson *et al.*, 2009). These biochemical alterations are in turn correlated with physiological outcomes such as increased drought tolerance, enhanced resistance to cadmium stress, and suppression of cell death (Bruggeman *et al.*, 2016; Estavillo *et al.*, 2011; Xi *et al.*, 2016). Collectively, these results indicate that chloroplastic PAP-mediated signalling, *via* redox-inactivation of SAL1 and

intracellular PAP movement to the nucleus, can activate multiple molecular and physiological acclimation responses to oxidative stress.

Beyond retrograde signalling: connections between PAP and other pathways

The intersection(s) between PAP-mediated signalling and other stress signalling pathways are only just beginning to be defined. Pornsiriwong *et al.* (2017) showed that PAP complements, and participates in, the abscisic acid (ABA) signalling pathway in guard cells, which regulate water loss through stomata. Constitutive accumulation of PAP in *sal1* mutants rescued ABA sensitivity, stomatal closure and drought tolerance in ABA-insensitive mutants such as *open stomata 1* (*ost1*), which lacks a key activator kinase downstream of ABA perception. Exogenous PAP is also able to close stomata in both wild type and *ost1*. The *ost1 xrn2 xrn3* triple mutant also had restored ABA sensitivity and stomatal closure, indicating that PAP is restoring ABA sensitivity *via* inhibition of the XRNs. Activation of PAP-XRN signalling in guard cells up-regulates the expression of multiple genes involved in ABA signalling, including transcription factors and calcium signalling proteins such as *Calcium Dependent Protein Kinases 32* (*CPK32*) and *CPK34*. Importantly, both CPK32 and CPK34 can phosphorylate, and activate, the SLAC1 anion channel whose activity is necessary for stomatal closure (Pornsiriwong *et al.*, 2017; Vahisalu *et al.*, 2008). Given that SLAC1 is also a phosphorylation target for the major ABA signalling kinase OST1 and other CPKs (Geiger *et al.*, 2010; Geiger *et al.*, 2009), these findings provide one mechanism by which PAP-mediated retrograde signalling complements hormonal signalling by converging upon common protein targets to regulate physiological responses to drought stress (Pornsiriwong *et al.*, 2017). Significantly, exogenous PAP application induces stomatal closure in representative species of all land plant clades, and PAP influences guard cell ion fluxes and ROS production in the same manner across multiple plant species (Zhao *et al.*, 2019). These findings, together with the targeting of SAL1 to plastids predating the emergence of stomata, indicate that the PAP-mediated retrograde signalling network had the capacity to be integrated with multiple cellular signalling networks throughout plant evolution (Zhao *et al.*, 2019).

The connections between PAP-mediated chloroplast communication and other signalling pathways still require extensive elucidation. Prolonged PAP accumulation exerts additional impacts on hormonal homeostasis and signalling. This was recently reviewed in Phua *et al.* (2018b) and thus will not be discussed in detail here. Nevertheless, it is intriguing to note that PAP accumulation alters JA metabolism and SA levels, and PAP also decreases glucosinolate

levels (Ishiga *et al.*, 2017; Lee *et al.*, 2012; Rodríguez *et al.*, 2010). These alterations are linked to decreased plant immunity (Bruggeman *et al.*, 2016; Ishiga *et al.*, 2017), but have not been explored in the context of oxidative stress tolerance. PAP accumulation also increases tolerance to cadmium stress (Xi *et al.*, 2016). The tolerance might be associated to altered levels, or signalling, of stress in the endoplasmic reticulum (ER), since *sal1* shows lower induction of ER Unfolded Protein Response (UPR) marker genes in response to cadmium (Xi *et al.*, 2016). However, direct links between chloroplastic PAP and ER signalling have not been demonstrated.

Co-operativity between secondary sulfur metabolites and intracellular signalling pathways

The substrates and/or products of the different sulfotransferases discussed above have partially overlapping functions in different cellular and physiological responses to oxidative stress. (**Figure 2**). In particular, it is noteworthy that PAP is involved in multiple physiological responses and would be produced regardless of which of the sulfotransferase(s) are activated, for example in the coordination of stress responses and growth (**Figure 2**). TPST and its sulfated peptide products are known regulators of growth especially in roots, while the putative SOT10/SOT12 substrate, brassinosteroids, are well-established regulators of root and shoot development. Similarly, sulfation of SA and 12-OH-JA by SOT12 and SOT15 respectively might affect the balance between SA and JA signalling in the regulation of stress responses and cell death. PAP accumulation concurrently up-regulates stress homeostasis genes and suppresses growth (Estavillo *et al.*, 2011; Phua *et al.*, 2018b; Rossel *et al.*, 2006; Wilson *et al.*, 2009). One possible mechanism by which PAP regulates growth and metabolism over short periods of drought stress is by extending the circadian period (Litthauer *et al.*, 2018). Both *sal1* and *xrn* mutants have extended circardian period and application of osmotic stress also prolongs the circardian clock (Litthauer *et al.*, 2018). Primary metabolism and growth are strongly associated with circadian regulation; thus PAP together with other signal(s) might contribute to the regulation of these processes during oxidative stress (Jones, 2018). For instance, the effect of PAP on circadian rhythm is most pronounced under blue light (Litthauer *et al.*, 2018). Enhanced activity of the blue light receptor proteins, cryptochromes, inhibits rosette growth (Lin *et al.*, 1996); and cryptochromes have been implicated in responses to various oxidative stresses (D'Amico-Damião and Carvalho, 2018). PAP accumulation can suppress the abundance and/or downstream signalling of growth-promoting hormones such as gibberelic acid (GA) and auxin while up-regulating signalling by stress-responsive hormones such as

ABA (Phua *et al.*, 2018b). Furthermore, the effects of PAP on growth can vary depending on its intracellular concentration. While strong PAP accumulation impedes growth, a small increase in cytosolic PAP pools can actually promote growth although the mechanism is currently unknown (Ashykhmina *et al.*, 2018).

The involvement of secondary sulfur metabolites in guard cell regulation also highlights an interesting potential divergence in the roles of primary and secondary sulfur metabolism during drought. Various reports indicate that primary sulfur metabolism largely influences ABA synthesis; whereas secondary sulfur metabolism components are more prominent in ABA signalling. During drought, sulfate is actively loaded into xylem sap in a manner associated with ABA synthesis (Ernst *et al.*, 2010). Indeed, sulfate up-regulates expression of ABA biosynthesis genes, and blocking sulfate transport into guard cells attenuates stomatal closure in leaves (Ernst *et al.*, 2010; Malcheska *et al.*, 2017). Sulfate and cysteine availability are important for ABA synthesis, with decreased sulfate and cysteine reducing ABA levels and impacting on stress tolerance (Cao *et al.*, 2014). Direct feeding of cysteine to guard cells stimulates expression of the rate-limiting ABA biosynthesis gene *NCED3*, induces ABA accumulation, and closes stomata via the canonical ABA signalling pathway (Batool *et al.*, 2018; Rajab *et al.*, 2019). Accumulation of glutathione also leads to enhanced ABA levels, up-regulation of ABA synthesis and signalling genes at the translational level, and improved drought tolerance (Chen *et al.*, 2011; Cheng *et al.*, 2015). In contrast, PAP accumulation has variable impacts on ABA accumulation (Pornsiriwong *et al.*, 2017; Rossel *et al.*, 2006) but clearly activates multiple components of downstream ABA signalling via a parallel pathway that is independent of the canonical ABA signalling proteins (Pornsiriwong *et al.*, 2017). The putative SOT substrates such as brassinosteroids and SA are also known to influence ABA-mediated guard cell responses (Ha *et al.*, 2016; Prodhan *et al.*, 2018).

Collectively, understanding the precise roles of PAP and other sulfotransferase substrate(s) and product(s) in stress and growth regulation networks may help to uncover how drought-stressed plants manage the dual problem of increased ROS production and diminished supply of water and carbon (Bechtold and Field, 2018).

Regulatory mechanisms in secondary sulfur metabolism

Secondary sulfur metabolism is tightly regulated at various levels **(Figure 3**). This regulation is important for at least two reasons. Metabolically, sulfur consumption through cysteine and

glutathione synthesis in primary assimilation needs to be coordinated with that of the aforementioned secondary sulfur metabolites (Chan *et al.*, 2013; Mugford *et al.*, 2011). In the context of cellular signalling and oxidative stress tolerance, metabolites such as PAP can play critical roles but also have strong impacts on various aspects of plant physiology (Phua *et al.*, 2018b); thus their synthesis and degradation require tight control.

Regulation of APKs catalyzing PAPS synthesis

The biosynthesis of PAPS by the APK enzymes is subject to transcriptional and postranslational control (**Figure 3**). The levels of *APK1* and *APK2* transcripts are regulated by MYB transcription factors which also regulate the glucosinolate biosynthesis genes (Yatusevich *et al.*, 2010). In contrast, *APK3* seems to be only weakly activated, while *APK4* is not regulated, by these MYBs (Yatusevich *et al.*, 2010). Low sulfur availability down-regulates *APK* expression alongside those of the glucosinolate genes, presumably to conserve sulfur for primary assimilation (Yatusevich *et al.*, 2010). This down-regulation is mediated by the central transcription factor Sulfur Limitation 1 (SLIM1); as well as nuclear-localized transcriptional regulator proteins Sulfur Deficiency Induced 1 (SD1) and SDI2 which directly bind to, and inactivate, MYB28 (Aarabi *et al.*, 2016; Maruyama-Nakashita *et al.*, 2006). The APK enzymes are redox-sensitive *in vitro*; with cysteine disulfide-mediated dimerization strongly decreasing PAPS biosynthetic activity (Ravilious *et al.*, 2012). This redox regulation appears to have evolved during the transition from cyanobacteria to land plants (Herrmann *et al.*, 2015); and involves disulfide bonding between a cysteine present on a land plant-specific N-terminal domain with a cysteine located on the catalytic core. Disulfide bond formation inverts the binding affinities at the ATP/ADP and APS/PAPS sites (Ravilious and Jez, 2012; Ravilious *et al.*, 2013). This altered order of adenosine binding decreases APK activity because binding of APS prior to ATP to the APK active site traps the enzyme in a dead-end complex (Ravilious and Jez, 2012; Ravilious *et al.*, 2013). Due to the technical challenge to measure APK activity in leaf extract, the redox control of APK enzymes still awaits experimental validation *in vivo* (Mugford et al., 2009). During oxidative stress, it can be expected that APKs retain a degree of activity since the metabolites downstream of PAPS, such as glucosinolates and PAP, accumulate during drought (Estavillo *et al.*, 2011; Mewis *et al.*, 2012).

Regulation of SOTs

The transcriptional and posttranslational control of SOTs are still under-explored in the context of oxidative stress responses. Most SOTs have very low expression levels at unstressed

conditions (Hirschmann et al., 2014). However, the expression of some SOTs can change significantly in response to oxidative stress and hormonal treatments (Baek *et al.*, 2010; De Clercq *et al.*, 2013; Ng *et al.*, 2013).

The best-characterized SOTs at the level of transcriptional regulation are the glucosinolate-producing SOTs. Expression of *SOTs 16*, *17* and *18* is regulated by a group of six R2R3 MYB transcription factors including MYB28 and MYB29 (Burow *et al.*, 2015; Frerigmann and Gigolashvili, 2014; Gigolashvili *et al.*, 2009; Sønderby *et al.*, 2010) (**Figure 3**). Similar to *APK1* and *APK2*, the transcriptional repression of *SOT17* and *SOT18* under sulfur-deficient conditions is mediated by SDI1 and SDI2 through physical interaction with MYB28 (Aarabi *et al.*, 2016). However, these SOTs do not seem to be targeted by the SLIM1 transcription factor since their repression by sulfur deficiency is unchanged in *slim1* (Maruyama-Nakashita *et al.*, 2006). Expression of *SOT16* is also regulated through feedback regulation mediated by the glucosinolate biosynthesis gene *2-oxo acid-dependent dioxygenase 2* (*AOP2*) independent of MYB28 and MYB29 (Burow *et al.*, 2015).

During drought stress, aliphatic glucosinolates were increased while indolic glucosinolates decreased in *Arabidopsis* phloem sap. The significance of these glucosinolate changes with regards to PAP levels is unclear, and it is currently not known whether and how the central transcriptional regulators SDI1/2 and SLIM1 might be involved in the drought stress response. Whether this process also involves differential regulation of *SOTs 16*, *17* and *18* will need to be investigated since SOT16 is specific for tryptophan-derived indolic desulfoglucosinolates whereas SOT17 and SOT18 process methionine-derived aliphatic desulfoglucosinolates (Klein and Papenbrock, 2009; Piotrowski *et al.*, 2004). A recent survey of the sulfenylated plastid proteome shows that many amino acid metabolism enzymes, including those involved in tryptophan biosynthesis, contain oxidized cysteines after hydrogen peroxide treatment (De Smet *et al.*, 2018). This raises the question whether GL synthesis and diversity could also be regulated upstream at the level of amino acid availability during oxidative stresses.

The expression of *SOT12* is up-regulated in response to multiple abiotic stresses and hormonal treatments (Baek *et al.*, 2010). Interestingly, *SOT12* expression is also highly responsive to mitochondrial oxidative stress, for example in response to antimycin A treatment which blocks mitochondrial respiration (De Clercq *et al.*, 2013; Ng *et al.*, 2013). The *SOT12* promoter contains a mitochondrial dysfunction motif (MDM) which is targeted by a group of ANAC

transcription factors responsive to mitochondrial oxidative stress (**Figure 3**). Mutation of the MDM in *SOT12* abolishes its up-regulation under antimycin A treatment (De Clercq *et al.*, 2013). Interestingly, the ANAC proteins regulating *SOT12* expression are in turn regulated through protein-protein interactions by Radical-Induced Cell Death 1 (RCD1), a redox-sensitive WWE domain-containing protein which is suppressed by PAP accumulation (Shapiguzov *et al.*, 2019). One recent hypothesis is that the *SOT12* up-regulation could be linked to PAP signalling for maintaining mitochondrial homeostasis and suppression of cell death (Shapiguzov *et al.*, 2019; Van Aken and Pogson, 2017). There is limited evidence for regulation of SOTs at the protein level through post-translational modifications, although enzymatic activity of SOTs is feedback-inhibited by high PAP levels (Hirschmann *et al.*, 2017).

Regulation of SAL1 and PAP degradation

In contrast to *SOTs*, *SAL1* transcript is readily detectable in multiple tissue types and developmental stages in unstressed conditions (Hruz *et al.*, 2008). There is strong up-regulation of *SAL1* during seed imbibition (Hruz *et al.*, 2008) which also corresponds with the relatively higher SAL1 protein abundance in leaf tissue compared to dry seed (Pornsiriwong *et al.*, 2017). Inducible or strong constitutive promoter-driven artificial micro RNA and RNA interference silencing lines of *SAL1* show up to 90% reduction in *SAL1* transcript abundance, but do not have a corresponding increase in PAP levels (Phua *et al.*, 2018a). Furthermore, while PAP accumulation extends the circadian period, the expression of *SAL1* is diurnal rather than circadian (Litthauer *et al.*, 2018). Therefore, there is limited evidence for transcriptional regulation of *SAL1* being a mechanism for regulation of PAP levels during stress. It is likely that the regulation of PAP is primarily driven by SAL1 enzymatic activity, which is consistent with SAL1's relatively high affinity for PAP (K_M < 10 μM) and its inactivation *via* redox regulation (Chan *et al.*, 2016a).

Under oxidative stress conditions, the regulation of SAL1 activity is largely mediated at the post-translational level through redox regulation rather than changes in protein abundance. The redox regulation of SAL1 is already discussed above in the context of PAP retrograde signalling, but two additional points are worth discussing. First, protein modelling through molecular dynamics simulations suggest that formation of the disulfide bonds in oxidized SAL1 decreases flexibility of Loop 1, a flexible protein loop that overhangs the active site (Chan *et al.*, 2016a). Rigidification of Loop 1 is proposed to inhibit accessibility of the active site for substrate entry or product release (Chan *et al.*, 2016a), and could represent an additional

regulatory target for modulation of SAL1 activity. Secondly, the redox midpoint potential (E_m) of SAL1 is approximately -308mV and -284mV at pH 7.5 for monomeric and dimeric SAL1 respectively. These values overlap with those of oxidation-inhibited APKs (-286mV at pH 7.5) and oxidation-activated primary sulfur metabolism enzymes (−330 mV at pH 8.0 for APR and −318 mV at pH 7.0 for the glutathione biosynthesis enzyme GSH1) (Chan *et al.*, 2016a; Hicks *et al.*, 2007; Ravilious *et al.*, 2012). This suggests that under oxidative stress, the redox status in the chloroplast may simultaneously regulate the activities of all three enzymes towards a coordinated sulfur consumption for protein and GSH synthesis, PAPS synthesis, and PAP accumulation.

Transporters as enigmatic gatekeepers of organellar PAPS/PAP flux

The coordination of PAP intracellular localization and movement *via* transporters is an emerging extra level of regulation for the secondary sulfur metabolism (**Figure 1** and **Figure 3**). The localization of the PAPST1 and PAPST2 is consistent with the chloroplastic localization of APK1 and APK2 which are the major sources of PAPS for sulfation reactions (and most likely, PAP production); and with the chloroplastic-mitochondrial dual localization of SAL1 for PAP degradation. A simplistic model would have envisaged both transporters facilitating the export of PAPS from chloroplasts to the cytosol and import of cytosolic PAP into plastids for degradation. I*n vitro* characterization of PAPST1 and PAPST2 show that both transporters are capable of transporting any two of ADP, ATP, PAP and PAPS in antiport mode. However, while recombinant PAPST1 shows a clear trend of substrate preference (ATP > PAPS > PAP >> ADP) when the counter-substrate is ATP; PAPST2 appears to have almost equal preference for PAP and ATP, with ADP and PAPS being only slightly less preferred. PAPST1 also shows an approximately nine-fold higher affinity for PAPS compared to PAPST2 (Ashykhmina *et al.*, 2018; Gigolashvili *et al.*, 2012).

Importantly, genetic data indicate that PAPST1 and PAPST2 play different roles *in vivo* (Ashykhmina *et al.*, 2018). The *papst1* mutant shows a significant decrease in glucosinolates, strong accumulation of desulfo-glucosinolates, up-regulation of glucosinolate biosynthetic genes, and accumulation of the primary sulfur metabolites cysteine and GSH. These features indicate that PAPST1 is important for the shuttling of PAPS produced in the chloroplast into the cytosol for utilization by SOTs for example in glucosinolate biosynthesis, and that its absence leads to re-direction of sulfur flux into primary sulfur metabolism (Gigolashvili *et al.*, 2012). In contrast, *papst2* only shows a small reduction in glucosinolates and correspondingly

low accumulation of desulfo-glucosinolates, with no up-regulation of glucosinolate biosynthetic genes. The accumulation of GSH and cysteine is also less marked in *papst2*. Therefore, PAPST2 is less important in provision of PAPS to the cytosol. Organelle fractionation experiments show higher cytosolic PAP accumulation in *papst2*, but not in *papst1*, compared to wild type plants (Ashykhmina *et al.*, 2018). Importantly, crossing *papst2*, but not *papst1*, to the *sal1* mutant allele *fry1* exacerbates cytosolic PAP accumulation and the *fry1* phenotype; and the *fry1papst2* phenotype cannot be rescued by complementation with mitochondrial-targeted SAL1. Collectively, these results indicate that PAPST2 has a greater role in the transport of cytosolic PAP into organelles for degradation by SAL1, although PAPST1 is still able to transport PAP into the chloroplast. Indeed, targeting PAPST1 to the mitochondria also fails to rescue the *papst2* phenotype, indicating independent roles for these two transporters (Ashykhmina *et al.*, 2018). . Therefore, it is possible that under oxidative stress conditions PAPST1 and PAPST2 contribute to the control of PAPS availability for sulfation and PAP localization for signalling respectively.

There are several unanswered questions with respect to PAPS/PAP transport (**Figure 3**). First, given the multiple possible substrates for PAPST1 and PAPST2, the kinetics and direction of transport for PAPS and PAP mediated by these two transporters during oxidative stress is difficult to predict (Ashykhmina *et al.*, 2018). Second, PAPS is clearly needed in the Golgi for TPST-mediated sulfation reactions (Komori *et al.*, 2009), but a PAPS/PAP transporter has not been identified for the Golgi. Third, that both PAPST2 and SAL1 co-localize to the mitochondria would suggest that either PAP has a metabolic role in this organelle, or that SAL1-PAP are involved in mitochondrial retrograde signalling (Van Aken and Pogson, 2017; Van Aken and Whelan, 2012). Fourth, low cytosolic PAP accumulation in *papst2* enhances growth (Ashykhmina *et al.*, 2018) (possibly through stimulation of SOTs) whereas high cytosolic PAP accumulation in *sal1* mutants suppresses growth (Phua *et al.*, 2018b). Whether this growth suppression in *sal1* is due to the primed stress responses (Estavillo *et al.*, 2011; Wilson *et al.*, 2009), suppression of circadian rhythm (Litthauer *et al.*, 2018), sulfur deficiency (Lee *et al.*, 2012), alteration of hormonal signalling (Phua *et al.*, 2018b), or all of the above, will need to be addressed. Since *papst2* does not share the abovementioned *sal1* phenotypes such as low sulfate and GSH content but both mutants do have lower glucosinolates (Ashykhmina *et al.*, 2018; Lee *et al.*, 2012), it is possible that different levels of cytosolic PAP activate different signalling pathways. The contribution of the PAPST proteins in regulating these different pathways during oxidative stress conditions will need to be critically addressed.

The road forward: how do we uncover new insights into secondary sulfur metabolism?

Some key questions in secondary sulfur metabolism (also indicated in **Figure 3**) that are still awaiting resolution include: 1) What are the functions and *in vivo* substrates of the SOTs? Would SOT functions dramatically differ in non-*Brassicaceae* species which lack glucosinolates? 2) What is the identity of the elusive Golgi PAPST(s)? 3) How is secondary sulfur metabolism coordinated across PAPS synthesis, PAP production and degradation, and intracellular shuttling of metabolites in response to different oxidative stresses? 4) Are there additional regulatory mechanisms beyond transcriptional control and redox modulation of protein activity? 5) What is the wider intersection network with other oxidative stress signalling pathways for plant acclimation to stress?

The plant sulfur metabolism pathways are extensively studied using a combination of biochemistry, protein structural biology and mutant characterization. These approaches provide a high degree of detail on individual protein(s) but are less helpful in identifying new pathway components. Most likely, the latter will be facilitated by utilization of "big data" and next-generation 'omics technologies (reviewed in Kopriva *et al.* (2015)). For example, PAPST1 was identified through co-expression analysis of publically available transcriptome data for putative transporters strongly co-expressed with glucosinolate metabolism (Gigolashvili *et al.*, 2012). The increasing availability of genome sequences, for example through the 1000 Plants (1KP) project (Matasci *et al.*, 2014), will allow investigation of how SOTs have diversified and identify "core" SOTs with key roles throughout evolution. A recent analysis shows that only TPST and SOTs 19-21 are evolutionarily conserved from lycophytes (moss) to higher land plants, whereas most of the cytosolic SOTs arose much later in evolution (Zhao *et al.*, 2019); suggesting that SOTs 19-21 play (unknown) conserved roles in plant function. Mining of unbiased proteomics data could also reveal new questions. Our quick survey of SOTs 16-18 using the PTMViewer database (https://dev.bits.vib.be/ptm-viewer/index.php) (Willems *et al.*, 2018) shows that these enzymes can be acetylated and/or contain reversibly oxidized cysteines: how do these posttranslational modifications affect protein function and overall regulation of the metabolic pathway? A complement of "new" and "old" strategies can help to reveal new insights into a pathway that is of secondary importance by classical definitions of metabolism, but that is clearly playing primary roles in plant acclimation to oxidative stresses and beyond.

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Figures

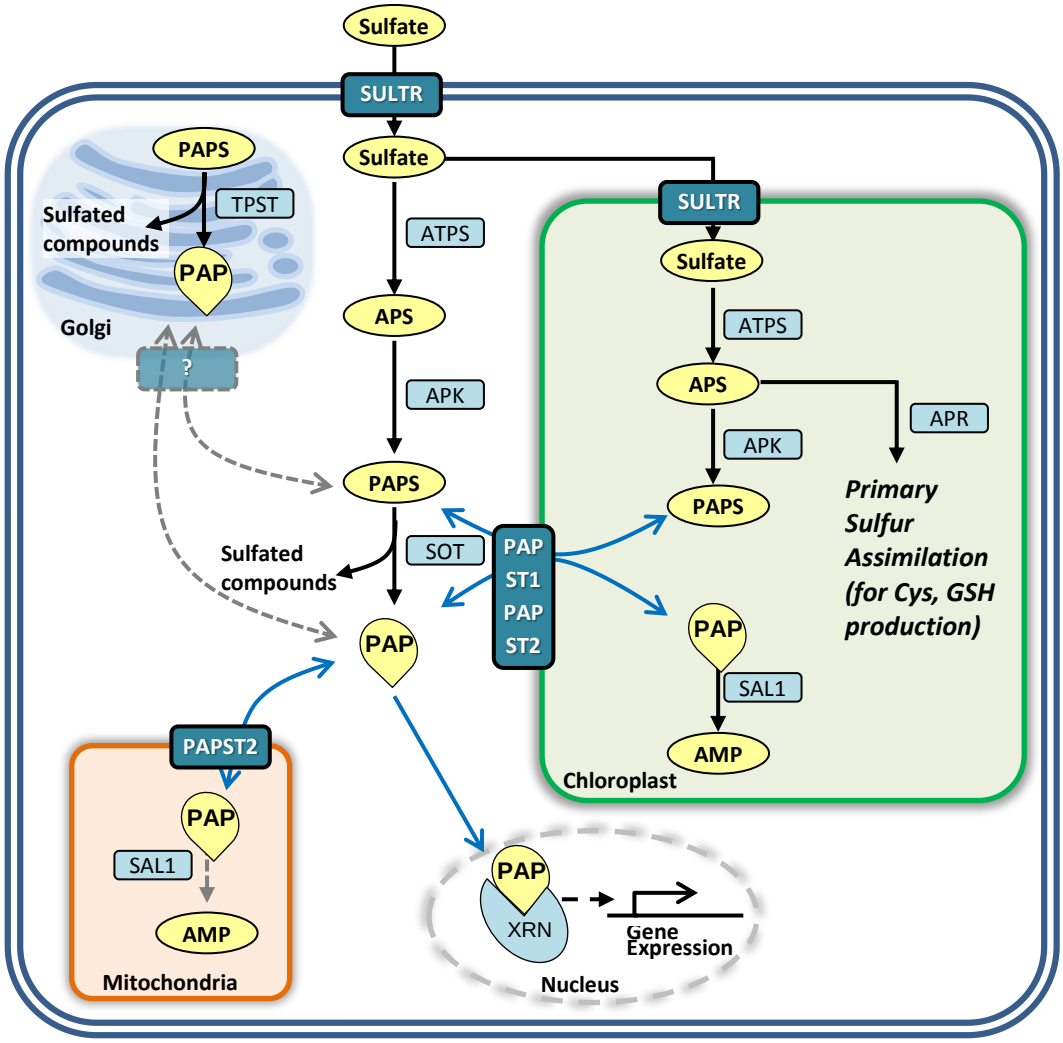

Figure 1. **The secondary sulfur metabolism pathway in *Arabidopsis*.**
Inorganic sulfate anions taken up from the soil are transported into the cell *via* SULTR transporter proteins. ATP-consuming sulfate activation by ATPS isoforms in chloroplasts and the cytosol provides APS which feeds into either primary sulfur assimilation via APR for cysteine and glutathione synthesis, or into secondary sulfur metabolism through APK-mediated phosphorylation to PAPS. SOT-catalyzed reactions in the cytosol and Golgi transfer the S moiety from PAPS to acceptor compounds, thereby producing PAP as a by-product. PAP is degraded by SAL1 enzymes which are localized to both chloroplasts and mitochondria. The shuttling of PAPS and PAP between plastids and the cytosol is mediated by PAPST1 and PAPST2. Accumulated PAP can also traverse to the nucleus to alter RNA metabolism and gene expression. Transporters are shown as dark blue boxes, enzymes as light blue boxes, and metabolites as light yellow ovals. Black arrows indicate enzymatic reactions while blue arrows indicate metabolite movement. The un-identified Golgi PAPS/PAP transporter(s) and transport mechanism are indicated by a blue box with dashed outline and dashed blue lines respectively. Abbreviations: SULTR, sulfate transporter; ATPS, ATP sulfurylase; APS, adenosine phosphosulfate; APK, APS Kinase; APR, APS Reductase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SOT, sulfotransferase; TPST, tyrosyl protein sulfotransferase; PAP, 3'-phosphoadenosine 5'-phosphate; PAPST, PAPS/PAP transporter; AMP, adenosine monophosphate; XRN, exoribonuclease; Cys, cysteine; GSH, glutathione.

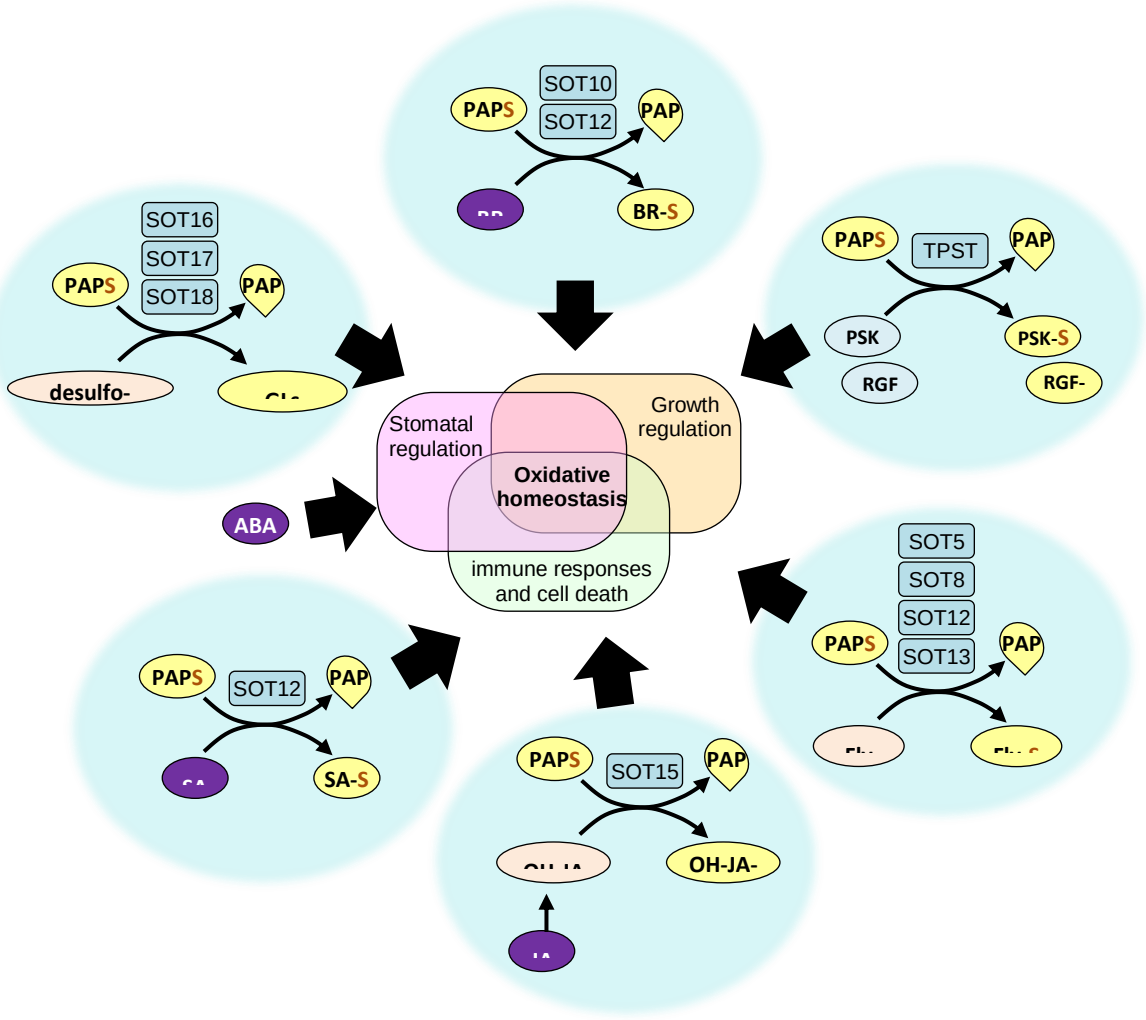

Figure 2. **Inter-cooperativity between secondary sulfur metabolism components and hormonal signalling for cellular homeostasis during oxidative stress .**
The different secondary sulfur metabolism metabolites are able to intersect with different facets of oxidative stress signalling pathways or physiological responses mediated by hormonal signalling such as ABA, SA and JA. In several cases the putative substrates or products of SOTs converge on the same process. Note that a commonality shared between the diverse SOT reactions is that they all produce PAP as a by-product. PAP accumulation also impacts on the three aspects of oxidative homeostasis outlined above (see text). Metabolite and protein colour schemes are the same as for **Figure 1**, except that phytohormones are shown as purple ovals. Abbreviations: ABA, abscisic acid; GLs, glucosinolates; SA, salicylic acid; JA. Jasmonic acid; OH-JA, hydroxy jasmonic acid; BR, brassinosteroids; Flv, flavonoids, PSK, Phytosulfokine; RGF, Root Growth Factor

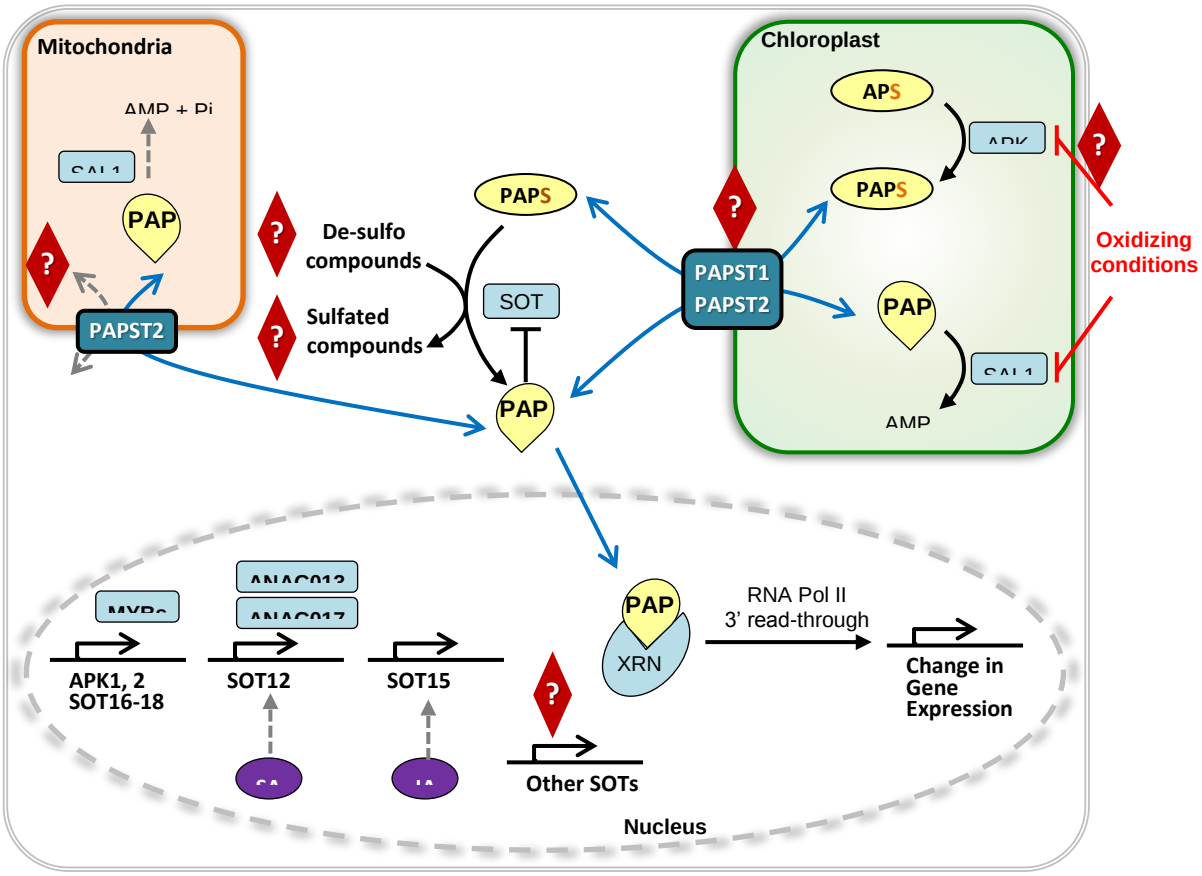

Figure 3. Regulation of secondary sulfur metabolism components and SAL1-PAP retrograde signalling during oxidative stress.
During conditions of oxidative stress such as drought and high light, a shift in the chloroplast redox poise decreases SAL1 activity against PAP, and is expected to have similar effects on APK activity. The accumulated PAP is able to travel intracellularly and inhibit XRNs in the nucleus, causing RNA Pol II read-through and activation of gene expression. PAP is also able to feedback-inhibit SOT activity. Red diamonds indicate the outstanding questions relating to transcriptional regulation of SOTs, the identity and role of SOT substrates and products, redox regulation of APKs, and transport mechanisms of PAPS and PAP during oxidative stress.