Dissection of the signalling function of O-acetylserine in plants

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ABSTRACT

Sulfur is an essential element for plant growth, development, defense and many other physiological processes. It is taken up by the roots, reduced and incorporated into organic compounds. Sulfate assimilation is well described in plants, however the regulation and sensing are not yet fully understood. O-acetylserine (OAS) is the acceptor of the reduced sulfur and the precursor of cysteine. OAS has been extensively discussed as a signalling molecule in sulfur metabolism, as it accumulates during sulfur starvation and induces the expression of sulfur marker genes. A systems biology approach established a group of 6 genes whose expression correlated to OAS accumulation, the so-called OAS cluster genes. In this study, we recreated the experimental set-up that led to the discovery of the cluster in combination with gene expression and metabolite analyses with different *Arabidopsis thaliana* mutants in order to identify the mechanisms leading to OAS accumulation and dissect the regulation of the OAS cluster genes.

Our study revealed that every SERAT isoform can contribute to the induction of the OAS cluster genes, with SERAT2;2 taking up a major role. Moreover, our deficiency experiments point towards OAS as a long term signal adjusting and coordinating the carbon, nitrogen and sulfur pathways. Regarding the OAS cluster genes, we identified SLIM1 (key transcription factor in sulfur deficiency response), RVE1 and RVE8 (circadian clock related transcription factors) as essential elements for their transcriptional activation. The 3 transcription factors were found to be involved in the control of OAS accumulation as well. Their binding to the promoters of the OAS cluster genes was confirmed and their role was tested in different conditions. SLIM1 emerged as the main regulator of the cluster, with RVE1 and RVE8 taking a more context-dependent function. Furthermore, our study directly and indirectly established new connections between the circadian clock and sulfur metabolism. We confirmed the circadian expression pattern of the OAS cluster genes and their transcriptional regulation by RVE1 and RVE8. We also demonstrated that the early sulfur deficiency response at different times of the day differs in its magnitude. Additionally, we provided new information about SLIM1, showing that it acts as a repressor of the OAS transcriptional signal and that it is not involved in the early response to sulfur starvation. These findings contribute to a better understanding of the regulation of sulfur homeostasis but also show that the regulation is more complex than initially believed.

TABLE OF CONTENT

1 INTRODUCTION

1.1 The importance of sulfur

Sulfur is a vital nutrient for plant growth and development, playing critical roles in various physiological processes. In fact, sulfur is considered to be the fourth most important nutrient for plant growth after nitrogen, phosphorus and potassium. One of the primary functions of sulfur in plants is its role in protein synthesis. Sulfur is a crucial component of amino acids such as methionine, the translational start of every protein, and cysteine, essential for the formation of disulfide bonds and consequently maintaining protein structure, stability and activity (Duke & Reisenauer, 1986). Moreover, these two amino acids cannot be synthesized *de novo* in animal cells (Wu, 2014), therefore an appropriate supply from plants is fundamental for a healthy diet in animals and thus a major concern in food security. Sulfur is also involved in the production of chlorophyll and its deficiency can lead to chlorosis (Lunde et al., 2008).

Additionally, sulfur is present in vitamins like thiamine and biotin (Goodrich & Garrett, 1986) as well as in Iron-Sulfur clusters, which are necessary for photosynthesis, respiration, nitrogen fixation, and DNA synthesis (Lu, 2018). Processes like plant defense mechanisms against biotic and abiotic stresses also require sulfur. It participates in the synthesis of defense-related compounds such as the phytoalexins camalexin and brassinin, involved in protecting plants against pathogen attack (Koprivova and Kopriva, 2014) and glucosinolates, which act against general pathogens and herbivores (Halkier and Gershenzon, 2006; Bednarek et al., 2009). In redox homeostasis, sulfur-containing compounds such as glutathione, thioredoxins and glutaredoxins are key components, as they coordinate a high number of essential processes like carbon metabolism, reactive oxygen species (ROS) scavenging or post-translational modifications (Meyer et al., 2008).

1.2 Sulfate assimilation in plants

Sulfate is the main source of sulfur for plants. It is taken up into the root (Figure 1) and distributed within the plant by a network of four groups of sulfate transporters (SULTR1, 2, 3 and 4; reviewed in Maruyama-Nakashita & Ohkama-Ohtsu, 2017). Uptake from the soil is achieved by two high affinity sulfate transporters of group 1, SULTR1;1 and SULTR1;2. SULTR1;1 is

sulfur sensitive, therefore induced at sulfur deficiency, whereas SULTR1;2 is constitutively active (Yoshimoto et al., 2002). Low-affinity sulfate transporters of subgroups 2, 3 and 4 mediate xylem and phloem loading for long-distance transport, plastid import, and vacuole export, respectively (Maruyama-Nakashita & Ohkama-Ohtsu, 2017). Thus, sulfate is provided to the main sites of assimilation, sink organs and storage tissues, and can be re-mobilized for the plant metabolism (Maruyama-Nakashita & Ohkama-Ohtsu, 2017).

For its assimilation into bioorganic molecules, sulfate is first activated by adenylation to adenosine 5-phosphosulfate (APS). This is carried out by ATP sulfurylases (ATPS), a family of four isoforms in plastids (ATPS1, ATPS2, ATPS3, ATPS4) and cytosol (ATPS2) (Rotte and Leustek, 2000; Saito 2004; Maruyama-Nakashita and Ohkama-Ohtsu, 2017). APS represents the branching point for the partitioning of sulfur into primary and secondary assimilation pathways (Kopriva et al., 2012).

In the primary sulfate assimilation pathway, APS is reduced in two steps to sulfide, which is used as a precursor for cysteine biosynthesis. Firstly, APS reductase (APR) reduces sulfate to sulfite by transferring two electrons, subsequently, six electrons are added by the ferredoxin-dependent sulfite reductase (SiR) to yield sulfide (Saito, 2004; Takahashi et al., 2011). Although in Arabidopsis only one SiR isoform is present in comparison to three APRs, the first reduction step catalyzed by APR is the rate-limiting step of the pathway (Vauclare et al., 2002; Kopriva 2006; Kopriva et al., 2009).

After reduction, sulfide is used for the biosynthesis of cysteine, the first compound containing organic sulfur. It is incorporated into the amino acid skeleton of O-acetylserine (OAS) by O-acetylserine (thiol) lyase (OAS-TL) to form cysteine (Maruyama-Nakashita and Ohkama-Ohtsu, 2017). Cysteine biosynthesis takes place in plastids as well as in mitochondria and cytosol (Maruyama-Nakashita & Ohkama-Ohtsu, 2017). OAS-TL acts together with serine acetyltransferase (SERAT), the enzyme activating serine to OAS, in the cysteine synthase complex (CSC) (Wirtz et al., 2004). There are 9 OAS-TL-like genes (Heeg et al., 2008), and among them 3 major isoforms of OAS-TL (a, b and c); regarding SERAT, there are 5 isoforms (1;1, 2;1, 2;2, 3;1 and 3;2). Both families are distributed through the cytosol, plastids and mitochondria (Takahashi et al., 2011). Cysteine then has structural and regulatory functions in many proteins; however, it can be also used for the synthesis of methionine (Met), co-enzymes and glutathione

(GSH). Glutathione has various roles in redox regulation, detoxification, conjugation and transport of several metabolites, as well as acting as a sulfur donor for compounds like glucosinolates. It is the most important antioxidant providing protection against reactive oxygen species and has potential further signalling functions (Noctor et al., 2002).

In the secondary assimilation pathway, four isoforms of APS kinase (APK) phosphorylate APS to yield 3-phosphoadenosine-5-phosphosulfate (PAPS) (Kopriva et al., 2009;). PAPS is the active form of sulfate for the sulfation of peptides or secondary metabolites catalyzed by sulfotransferases (SOT) (Takahashi et al., 2011; Koprivova and Kopriva, 2014). One group of acceptors are the amino acid-derived glucosinolates, which represent a potent mechanism in plant defense against pathogens and herbivores (Halkier and Gershenzon, 2006; Bednarek et al., 2009). Myrosinases break these compounds down into glucose and an unstable aglycone via hydrolysis, resulting in bioactive, toxic compounds such as isothiocyanates, nitriles, or thiocyanates (Halkier and Gershenzon, 2006).

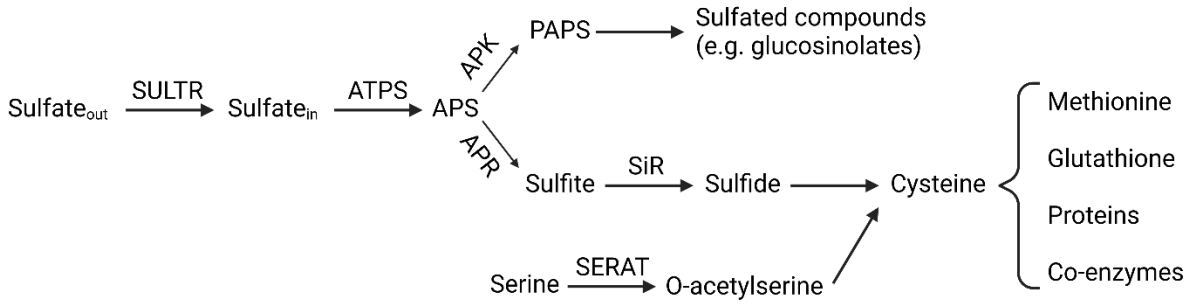

Figure 1: Sulfate assimilation pathway. Adapted from Koprivova & Kopriva, 2014.

1.2.1 Serine acetyltransferase (SERAT) family

As described before, the SERAT family in Arabidopsis includes 5 isoforms: SERAT1;1 in the cytosol, SERAT2;1 in the plastids, SERAT2;2 in the mitochondria, SERAT3;1 and SERAT3;2 in the cytosol. This multigene family has also been found in other species, suggesting that its formation precedes the monocot/dicot divergence (Watanabe et al., 2008b). A possible advantage of five SERAT isoforms in Arabidopsis participating in OAS formation is a robust and efficient maintenance of cysteine homeostasis. Furthermore, gene redundancy could serve as a source for evolutionary and biochemical innovations (e.g., different expression patterns depending on

development stages), and protection against the mutational loss of other SERAT isoforms (Watanabe et al., 2008b).

Previous research showed a redundancy of function between SERAT isoforms as no visible phenotypic differences were observed in single SERAT mutants compared to wild type under normal conditions, sulfur deficiency and cadmium stress. The functional relevance of each isoform is supported by the fact that a quintuple SERAT mutant is lethal, also indicating that there is no alternative formation for cysteine than via SERAT (Watanabe et al., 2008b, 2018).

Further investigation of the role of SERAT isoforms was possible using quadruple SERAT mutants. Quadruple mutants *q2;1* (with only SERAT2;1 left) and *q3;1* showed a dwarf phenotype in both agar plates and pots (Watanabe et al., 2008b, 2018). However, the dwarfism of *q2;1* and the non-significant changes in *s2;1* (single mutant for SERAT2;1) regarding OAS and thiol accumulation were surprising, as plastids were considered to be dominant for cysteine formation (Wachter et al., 2005). Therefore, Watanabe and colleagues (2008b) suggested that cysteine formation does not predominantly occur in plastids. These findings are consistent with the knowledge about plastidic OAS-TL mutants showing smaller changes in thiol contents compared to cytosolic and mitochondrial OAS-TL (Heeg et al., 2008; Watanabe et al., 2008a). Quadruple mutants *q2;2* and *q1;1* did not suffer from dwarfism and in fact, SERAT2;2 is considered to be the most efficient isoform as SERAT enzyme activity significantly decreased in *s2;2*, as well as in silencing lines for this isoform (Haas et al., 2008), and no significant changes were observed in *q2;2* compared to wild type (Watanabe et al., 2008b).

It is also noteworthy, that the expression of mitochondrial SERAT decreases and cytosolic isoforms increases during seed development and germination, indicating a precise regulation of SERAT and OAS formation during plant development in Arabidopsis (Watanabe et al., 2008b). Moreover, cadmium stress, sulfur deficiency and loss of equivalent isoforms lead to an increase of *SERAT3;2* expression in wild type, indicating abiotic factors also regulate OAS homeostasis (Kawashima et al., 2005; Watanabe et al., 2008b). Furthermore, only cytosolic SERAT1;1 and SERAT3;2 are sensitive to feedback regulation by cysteine (Noji et al., 1998; Kawashima et al., 2005), supporting the idea of predominant cytosolic cysteine synthesis, as the dominant OASTL isoform is also localized in cytosol (Hooper et al., 2017).

1.3 Regulation of sulfate assimilation

Several metabolites (products and intermediates) have been assigned important roles in the regulation of sulfate assimilation. It is known that upon sulfur starvation, uptake and reduction of sulfate are highly upregulated. This also happens after treatment with OAS, light, carbohydrates and reduced nitrogen compounds. In contrast, the pathway is inhibited by the presence of reduced sulfur, as well as by limitation of available nitrogen and carbon (reviewed in Kopriva, 2006). Phytohormones such as jasmonate, abscisic acid, salicylate (reviewed in Takahashi et al., 2011) and cytokinins (Pavlů et al., 2022) have also been connected to sulfur metabolism, adding an extra layer to the regulatory network. Target of rapamycin (TOR) kinase, a central regulator and integrator of multiple pathways, has also been linked to sulfur and it is suggested that it mediates sulfur signalling, adapting the metabolic network of the plant in response to sulfur supply (Yu et al., 2022).

Unlike other major nutrients like nitrogen or phosphorus, and despite its importance for plants and animals, there are still many open questions regarding sulfur sensing and regulation (reviewed in Ristova & Kopriva, 2022). Which molecule(s) act(s) as the sensor for sulfur is still unknown, and only a few regulatory elements have been identified and studied so far. Among them, the transcription factor SULFUR LIMITATION 1 (SLIM1) appears to be the key transcription factor in sulfur deficiency response. It was identified during a mutagenesis screen of transgenic Arabidopsis, expressing GFP under control of the sulfur deficiency inducible SULTR1;2 promoter in a Col-0 background. A single base substitution led to mutants with impaired sulfur deficiency response, showing reduced sulfate uptake, growth and transcriptional changes (Maruyama-Nakashita et al., 2006). Recently, the transcription factor ETHYLENE-INSENSITIVE3-LIKE 1 (EIL1), which belongs to the same family as SLIM1, has also been identified as a regulator of sulfur deficiency, working in an additive manner. Similarly, miRNA395 also controls processes like sulfur uptake under the control of SLIM1.

Although core processes in the sulfur pathway such as sulfate uptake, translocation, activation, glucosinolate degradation and cysteine synthesis, among others, have been linked to SLIM1, the whole picture is not complete. Transcript levels of the APR isoforms seem to be independent of SLIM1 and EIL1 (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020), implying that other factors participate in the regulation.

1.3.1 OAS and OAS cluster genes

OAS has been for a long time considered to be an important molecule in sulfur signaling. When plants are grown under sulfur deficiency, OAS accumulates (Hirai et al., 2003; Nikiforova et al., 2003). Besides, the transcriptome of plants subjected to exogenous OAS overlaps substantially with that of sulfur-starved plants (Hirai et al, 2003). However, there is also controversy, since other studies showed that the increase in transcripts precedes the OAS accumulation (Hopkins et al., 2005). Furthermore, OAS restored sulfate assimilation in nitrogen-starved plants more rapidly than any other nitrogen sources, so it has also been considered a link between sulfur and nitrogen metabolism.

In a systems biology approach, and using the data generated in two previous studies (Espinoza et al., 2010; Caldana et al., 2011), Hubberten and colleagues in 2012 identified a group of genes whose expression showed high correlation exclusively to OAS but not to any sulfur-containing metabolites (Figure 2). This group, termed the OAS cluster genes, includes the following genes: *APR3* (*APS REDUCTASE 3*), *GGCT2;1* (*γ -GLUTAMYL-CYCLOTRANSFERASE 2;1*) *SDI1* (*SULFUR DEFICIENCY-INDUCED 1*), *SDI2* (*SULFUR DEFICIENCY-INDUCED-2*), *SHM7* (*SERINE HYDROXYMETHYL TRANSFERASE 7*) and *LSU1* (*LOW SULFUR-INDUCED 1*). The individual functions of some of them are yet unclear. APR3 is one of the 3 isoforms of this family but not the major one, since the knockout of *APR2* reduces the enzymatic activity by 80% (Loudet et al., 2007). Despite catalyzing the same reaction, every APR isoform is regulated differently and might be involved in various processes. For example, the transcripts of every isoform are distinctively regulated in response to light, nitrogen, salt and oxidative stress (Kopriva et al., 1999; Koprivova et al., 2000; Koprivova et al., 2008). Therefore, despite the obvious role in sulfate assimilation, it is likely that this isoform participates in specific processes, perhaps adjusting the sulfate assimilation rate in response to other variables such as nitrogen availability. GGCT2;1 is an interesting member of the γ-glutamyl-cyclotransferase family, which has a major role in glutathione degradation and recycling. This process is key in sulfur metabolism since glutathione acts as one of the largest sources of cysteine, which can then be mobilized and used as a nutrient. Specifically, GGCT2;1 has been connected to several processes since its mRNA levels strongly increase during pollen tube growth, heavy metal stress, sulfur starvation, salinity stress and cytokinin treatment (Paulose et al., 2013; Joshi et al., 2019; Dietzen et al., 2020; Pavlů et al., 2022). Additionally, glutathione concentration and GGCT2;1 activity are essential for proper root growth

in response to sulfur deficiency (Joshi et al., 2019). It is then logical to claim that the role of GGCT2;1 goes far beyond than just nutrient availability in response to the sulfur status of the plant, as altering the glutathione pool can lead to profound changes in the redox state of the cell and therefore a severe impact in homeostasis. SDI1 and SDI2, whose sequences share high similarity, are highly induced under sulfur deficiency (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020). They are known to interact with MYB transcription factors and downregulate the synthesis of glucosinolates and coordinate the different sulfur pools under sulfur deficiency (Aarabi et al., 2016). Furthermore, a recent *in silico* analysis suggests that the functions of SDI1 and SDI2 are not redundant and also not restrained to sulfur metabolism (Rakpenthai et al., 2022). The case of SHM7 is similar to the ones described before since its function goes beyond the production of S-adenosylmethionine. SHM7 is important for DNA methylation in response to sulfur deficiency, and many sulfur marker genes are confirmed targets such as *SULTR1;1*, *SULTR1;2*, *APR3*, *ATPS4* and several glucosinolate genes (Huang et al., 2016). The fact that this gene is upregulated in other contexts (Ristova & Kopriva, 2022) again suggests a role in controlling sulfur metabolism that is not just limited to sulfur deficiency, but many other conditions. LSU1 is part of the LSU family, which constitutes a network hub for proteins in response to biotic and abiotic stresses (Sirko et al., 2015; Garcia-Molina et al., 2017). *LSU1* transcript levels are strongly upregulated under sulfur deficiency (Maruyama-Nakashita et al., 2006; Garcia-Molina et al., 2017; Dietzen et al., 2020) even though its role in the context of sulfur metabolism is not clear. Several other conditions induce the expression of *LSU1*, such as Fe deprivation, Cu excess, and salt stress and it is also involved in ROS production and plant immunity (Garcia-Molina et al., 2017). Besides, LSU1 (and the rest of the LSU family) is known to interact with components of the Abscisic acid (ABA), ethylene and jasmonate signaling pathways (Sirko et al., 2015; Niemiro et al., 2020).

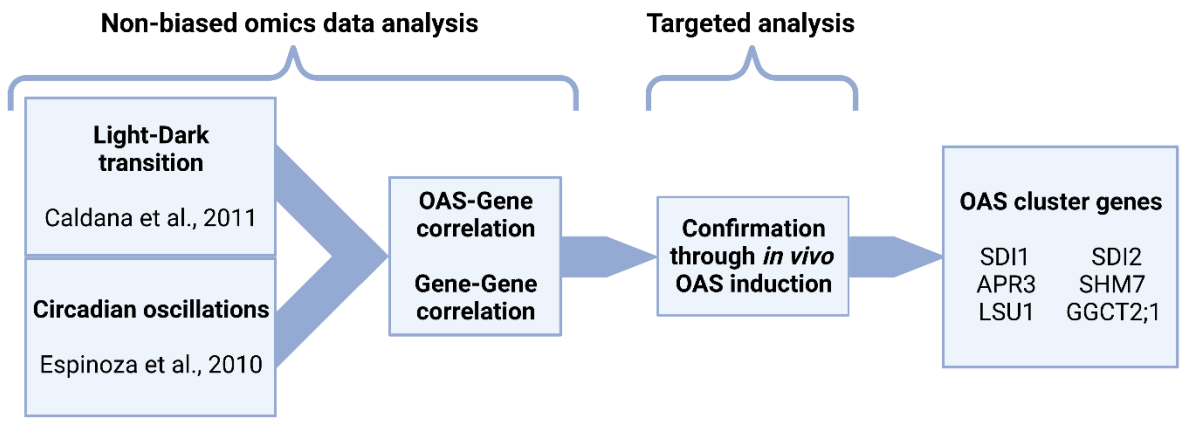

Figure 2: Workflow of the strategy used to identify and confirm the OAS cluster genes. Adapted from Hubberten et al., 2012. Two publicly available sets of data were obtained (Espinoza et al., 2010; Caldana et al., 2011). A computational approach was used to identify OAS-Gene and Gene-Gene correlation and the candidate genes were confirmed via a targeted experimental approach using SERAT inducible expression.

All six genes are strongly upregulated by sulfur deficiency (Hirai et al., 2003; Maruyama-Nakashita et al., 2006; Dietzen et al., 2020) and interestingly also share a SLIM1 binding site in their promoters (Ran et al., 2020), but their transcript levels also rise with endogenous and exogenous OAS at normal sulfate nutrition (Hirai et al., 2003; Hubberten et al., 2012), suggesting that the signaling function of OAS and the OAS cluster genes might go beyond the sulfur status of the plant. This idea is further supported considering that 4 of the OAS cluster genes might be seen as multiplicators of the signal, since their products either interact with and modulate other proteins (SDI1, SDI2 and LSU1) or they affect general cell functions like DNA methylation (SHM7). The experimental set-ups that led to the discovery of the OAS cluster genes (Hubberten et al., 2012, Figure 2), a transient increase in OAS after a sudden transition from light to darkness (Caldana et al., 2011) and OAS accumulation during the night (Espinoza et al., 2010), together with the correlated increase in OAS cluster genes transcript levels, point towards a more general and conserved function that is not necessarily just connected to sulfur. Given the central position of OAS in primary metabolism and the results from previous studies, it is possible that OAS might function in coordinating the assimilatory pathways with general processes. In fact, a comparable co-regulated cluster is present in two other species: *Oryza sativa* and *Populus trichocharpa* (Hubberten et al., 2012), further supporting the biological relevance of this network and indicating similar regulatory mechanisms. Besides, these genes are not only co-expressed under sulfur starvation and the aforementioned conditions, they are also co-regulated in response to oxidative

and heat stress (Ristova & Kopriva, 2022), once again providing evidence that their role is not simply connected to sulfur metabolism.

1.3.2 Sulfur deficiency response

Despite showing growth impairment when grown under sulfur deficiency, plants build a more extensive root system than under control conditions. The root-to-shoot mass ratio is significantly higher and lateral roots development is enhanced in search for the lacking sulfate, a process in which auxins seem to be involved (Nikiforova et al., 2003; Nikiforova et al., 2005).

The metabolic status of sulfur starved plants is very different from control plants. The total sulfur level of the plant decreases, an effect which is more severe after a long period of starvation (Nikiforova et al., 2003). Glucosinolates and sulfate content is decreased (Hirai et al., 2003, Hirai et al., 2004) and cysteine and glutathione levels fall. In the case of glutathione, not only the pool is smaller, but the oxidation ratio is higher (Lunde et al., 2008). On the other hand, internal concentrations of O-acetylserine, serine and tryptophan are significantly increased (Hirai et al., 2003; Nikiforova et al., 2003), as well as the content of flavonoids (Lunde et al.¸2008). Although levels of methionine remain almost unchanged, S-adenosylmethionine decreases, therefore, hindering many metabolic pathways, including photosynthesis (Nikiforova et al., 2005; Hoefgen & Nikiforova, 2008). One of the most impactful changes in sulfur-starved plants is the reduction of lipid content, not only sulfolipids, but also glycolipids and phospholipids, which is also connected with the photosynthesis impairment (Nikiforova et al., 2008). Taken together, plants grown under sulfur deficiency conditions show an enhanced catabolism of sulfur-containing compounds and an accumulation of their precursors.

Many of these metabolic changes can be explained by transcriptional changes, mainly controlled by SLIM1 (Maruyama-Nakashita et al., 2006), but post-translational regulation may also play an important role in controlling the activity and stability of the enzymes involved. For example, the levels of mRNA for high affinity transporters *SULTR1;1* and *SULTR1;2* are upregulated under sulfur deficiency (Yoshimoto et al., 2002), as well as those for *SULTR2;1* and *SULTR3;5* (Kataoka et al., 2004), but the changes in protein levels, much more relevant for the sulfate uptake increase, do not reach such an extent (Yoshimoto et al., 2007). The role of microRNA-395 should also be considered, as it accumulates during sulfur deficiency and targets *ATPS1*, *ATPS3*, *ATPS4* and

SULTR2;1, however it is under the control of SLIM1 (Kawashima et al., 2009). In case of APR, the activity changes correspond to mRNA and protein levels, transcripts for the three isoforms are accumulated under sulfur starvation (Vauclare et al., 2002; Maruyama-Nakashita et al., 2006), but at the same time are also redox-regulated (Bick et al., 2001). Regarding APK, while considering that it is the enzyme that controls the sulfur flux to the secondary pathway, its regulation should be very precise (Martin et al., 2005). In fact, a reduction of APK activity leads to a decrease in glucosinolates levels and an increase in the content of cysteine and glutathione (Mugford et al., 2009). *APK1* and *APK2* transcript levels are, together with other genes involved in glucosinolate synthesis, coordinately reduced by sulfur deficiency-induced proteins SDI1 and SDI2, which repress the MYB factors controlling glucosinolate biosynthesis. Thus, the amount of sulfate to be used for the secondary pathway is diminished and plants redirect sulfur resources to the primary pathway (Aarabi et al., 2016).

1.4 Circadian clock

The circadian clock is a vital regulator of physiological and biochemical processes, enabling living beings to synchronize their activities with the rhythmic patterns of the environment. For plants, more specifically, it allows them to anticipate and adapt to daily and seasonal changes, optimizing photosynthesis, energy utilization, growth, and defense mechanisms. This internal timekeeper grants plants a competitive advantage by ensuring optimal resource allocation, enhancing fitness, and facilitating survival in a dynamic environment (Green et al., 2000; Venkat & Muneer, 2022). Basically, the system consists of input signals (e.g. light, temperature), the internal clock and outputs. Over the last years, many clock elements have been identified in *A. thaliana* and it has been revealed how the feedback loops of activators and repressors and their interconnections are essential for a robust clock network (Nohales & Kay, 2016). In a simple way (Figure 2), the MYB-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) together with LATE ELONGATED HYPOCOTYL (LHY) work together at dawn and bind to the so-called evening element (EE), a cis-element motif found in the promoters of clock genes but also many other output genes, to repress their targets. Among the target clock genes, we can find PSEUDO-RESPONSE REGULATOR 5 (PRR5), PRR7, and PRR9 and their homolog TIMING OF CAB EXPRESSION 1 (TOC1; also known as PRR1). This group of proteins represses *CCA1*

and *LHY*, creating the first feedback loop and restricting their expression and function in time. The proteins LUX ARRHYTHMO (LUX), EARLY FLOWERING 3 (ELF3), and ELF4 associate and work together in the evening complex (EC). The EC can repress *PRR7* and *PRR9*, therefore indirectly activating *CCA1* and *LHY*. Another important family of clock regulators is the REVEILLE (RVE) MYB-like transcription factors. They have sequence and binding specificity similarities with CCA1 and LHY. In the afternoon, RVE8 and likely its homologs RVE4 and RVE6, bind to EE motifs and activate the expression of *PRR* and EC genes, while the PPR proteins, in turn, repress *RVE8* expression, creating yet another feedback loop (reviewed in Shalit-Kaneh et al., 2018).

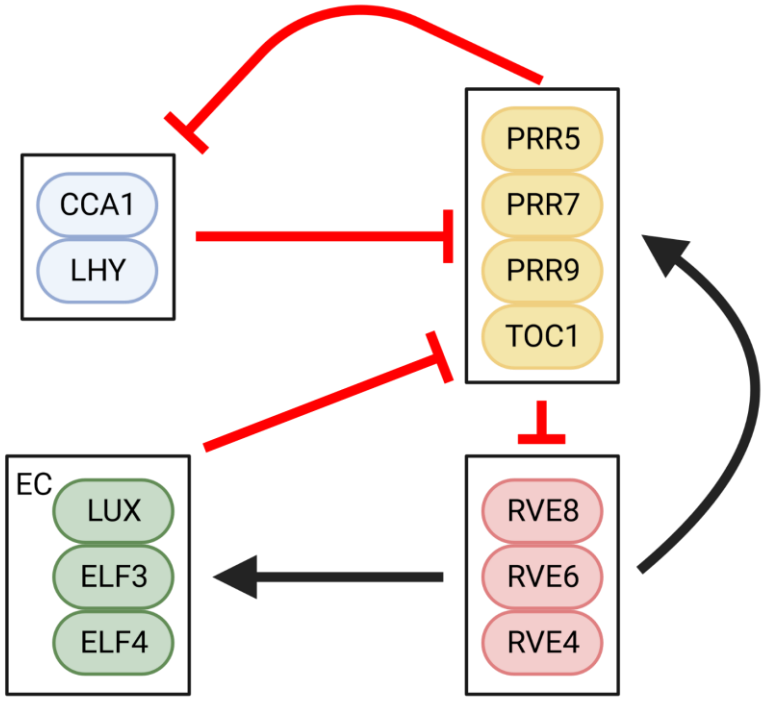

Figure 3: Circadian clock in *Arabidopsis thaliana*. Simplified scheme of the circadian clock feedback loops in *A. thaliana*. Black arrows indicate activation and red bars indicate repression. Description in the text.

The complexity of the circadian clock enables plants not only to react to predictable changes, daily or seasonal, but also provides an advantageous adaptive tool that can lead to enhanced fitness or the possibility to improve crop traits and yield (Green et al., 2002; McClung, 2021). In fact, many physiological processes, such as stress acclimation, hormone signaling, morphogenesis, carbon metabolism, and defense response, are currently being investigated for their interactions with the circadian clock (reviewed in Venkat & Muneer, 2022). An optimized interplay between

metabolism and the circadian clock might lead to an improvement in resource allocation and productivity. Two of the best examples are photosynthesis and carbon metabolism. Light is one of the main inputs for the internal clock and the driving force of photosynthesis, therefore it is logical that they are tightly connected. Many genes in photosynthesis and carbon metabolism possess a rhythmic expression pattern, ensuring that the light-dependent phase of photosynthesis is taking part during the day and sugars are accumulated during the night period so that they can be used for processes like respiration (Haydon et al., 2013). Irrespective of the length of the day, starch degradation is also under circadian control to ensure that almost all the starch is used by dawn (Graf et al., 2010). Defense mechanisms have also been associated with the circadian clock. Processes like production of reactive oxygen species or the signaling cascade of hormones like salicylic acid and jasmonate are directly regulated by clock genes (Lai et al., 2012; Zhang et al., 2019), not only improving their immunity performance but also maintaining a proper balance between defense and growth. Interestingly, the plant shows a stronger defense response at dawn rather than at dusk (Griebel & Zeier, 2008).

Regarding sulfur metabolism, there is already evidence about how light and circadian oscillations affect core elements in the pathway such as sulfate transporters, APR (Kopriva et al., 1999; Hornbacher et al., 2019) and the flux into thiols, proteins and glucosinolates (Huseby et al., 2013). Moreover, the OAS cluster genes have been reported to follow a circadian expression pattern, based on the data from two independent studies (Espinoza et al., 2010; Hubberten et al., 2012; Bonnot & Nagel, 2021). In addition, the transcription factors RVE1 and RVE8 are predicted to bind to the promoters of all the OAS cluster genes (Ran et al., 2020), further supporting the connection between sulfur metabolism and the circadian clock. The transcription factor RVE8 is, as stated before, a MYB-like transcription factor that shares a high degree of sequence identity with CCA1 and LHY (Farinas & Mas, 2011). It is part of the clock core, its expression follows a circadian pattern and it is involved in flowering time control (Farinas & Mas, 2011; Rawat et al., 2011). RVE1, on the other hand, works as an output in the clock network. Despite its circadian expression pattern, it is not necessary to maintain rhythmicity and its function is connected to plant development. In association with other proteins, RVE1 has been associated with seed dormancy, germination and auxins. However, no clear connection has been established so far between these 2 transcription factors and sulfur metabolism.

1.5 Aims

Despite the importance of sulfur in plants and animals, there are still many open questions about sulfur signalling. Over the last few decades, OAS has been considered to act as a potential signal in sulfur metabolism, however recent studies may suggest that its function could also be independent of the sulfur status of the plants. Therefore, unraveling the factors leading to OAS accumulation and the regulation of the OAS cluster genes, as well as how the downstream processes are affected, needs to be dissected. In particular, the aims of this thesis are:

1) To study the mechanisms of OAS accumulation, find the responsible SERAT isoform, and analyze how they are affected by disturbances in C, N and S assimilation pathways.
2) To investigate the transcriptional regulation of the OAS cluster genes and identify transcription factors involved in OAS signalling.
3) To explore the connection between the circadian clock and sulfur metabolism.

To address these objectives, mutants of SERAT, the enzyme producing OAS, as well as mutants of transcription factors predicted to regulate the OAS cluster genes were used in a series of gene expression and metabolite analyses, including nutrient deficiency experiments, OAS feeding and transition to dark.

2 MATERIAL AND METHODS

2.1 Plant material

Arabidopsis thaliana ecotype Col-0 was used as wild type (WT) in this study while Landsberg erecta (L*er*) was used only for cell culture. The mutant lines used in this study can be found in Table 1, with the SERAT mutants kindly provided by Dr Hoefgen (Watanabe et al., 2008b).

Table 1: *A. thaliana* mutants used in this study

Mutant	Gene locus	Line
serat1;1	AT5G56760	SALK_05021
serat2;1	AT1G55920	SALK_099019
serat2;2	AT3G13110	Kazusa_KG752
serat3;1	AT2G17640	SALK_030223
serat3;2	AT4G35640	SALK_030011
q1;1 (*serat2;1 serat2;2 serat3;1 serat3;2*)	AT1G55920, AT3G13110, AT2G17640, AT4G35640	
q2;1 (*serat1;1 serat2;2 serat3;1 serat3;2*)	AT5G56760, AT3G13110 AT2G17640, AT4G35640	
q2;2 (*serat1;1 serat2;1 serat3;1 serat3;2*)	AT5G56760, AT1G55920, AT2G17640, AT4G35640	
q3;1 (*serat1;1 serat2;1 serat2;2 serat3;2*)	AT5G56760, AT1G55920, AT3G13110, AT4G35640	
q3;2 (*serat1;1 serat2;1 serat2;2 serat3;1*)	AT5G56760, AT1G55920, AT3G13110, AT2G17640	
rve1	AT5G17300	SALK_025754
rve8	AT3G09600	SALK_053482C

slim1-1	AT1G73730	Point mutation (Maruyama-Nakashita et al., 2006)

2.2 Growth conditions

Prior to every experiment, seeds were surface sterilized using chlorine gas under the fume hood for 3 hours by adding 2.5 mL of concentrated 37 % (V/V) HCl to 125 mL sodium hypochlorite in a desiccator. Seeds were then sown under sterile conditions on the corresponding system using 0.1% agarose.

2.2.1 Growth conditions: agar plates

Sterilized seeds were placed on modified Long Ashton Medium agarose plates (Table 2) containing either full nutrient supply (**Control**), low sulfur supply (**-S**; 0.75 mM $MgCl_2x6H_2O$ and 0.015 mM $MgSO_4x7H_2O$) or low nitrogen supply (**-N**; 1.5 mM $CaCl_2$ in exchange for $Ca(NO_3)_2x4H_2O$). Plates were kept in darkness at 4ºC for 3 days (stratification) and later incubated for 18 days in Panasonic light chambers at 22ºC under long day conditions (16 h light/8 h darkness; 150 $\mu E*m^{-2}*s^{-1}$). Samples from shoots and roots were then collected in 1.5 mL tubes from different plates and immediately frozen in liquid nitrogen, securing at least 4 biological replicates for each treatment.

Table 2: Modified Long Ashton Medium composition, pH was adjusted to 5.7.

Macroelements	**Final concentration**
$Ca(NO_3)_2x4H_2O$	1.5 mM
KNO_3	1 mM
KH_2PO_4	0.75 mM
$MgSO_4x7H_2O$	0.75 mM
Fe-EDTA	1 mM
Microelements	**Final concentration**
$MnCl_2x4H_2O$	10 µM

H_3BO_3	50 µM
$ZnCl_2$	1.75 µM
$CuCl_2$	0.5 µM
Na_2MoO_4	0.8 µM
KI	1 mM
$CoCL_2x6H_2O$	0.1 mM
Additives	
Sucrose	5 g/L
MES hydrate (Sigma-Aldrich)	0.8g/L
Low EEO agarose (Biozym Scientific Gmbh)	8 g/L

2.2.2 Growth conditions: hydroponic plates

For this system, 12-well plates were used and 1 mL of liquid modified Long Ashton Medium (containing all the components except agarose; Table 2) was pipetted in every well. Seeds were placed on a sterile square polypropylene mesh, the plates were wrapped in aluminum foil and stored in darkness at 4ºC for 3 days (stratification). They were then moved to a Percival growth chamber under long day conditions (as described before) and the aluminum foil was removed after 3 days to allow etiolation. Plants were grown for 2 weeks and the media was replaced after every week. On day 15 the respective treatment was applied and shoots and roots were collected as described before.

2.3 Genotyping

To obtain homozygous mutants, seeds of T-DNA line were sown on ½ Murashige and Skoog (MS) media (Sigma-Aldrich) containing 0.5% sucrose. After stratification, a leaf from one-week old plants was placed in a 1.5 mL tube containing 3 glass beads and 300 µL of Magic Buffer

(Tris/HCl ph 7.2 50 mM; NaCl 300 mM; Sucrose 10%). Samples were homogenized using a Bead Ruptor 24 3D (Omni International, USA) and the genomic DNA (gDNA) obtained was used as a template for PCR reactions with the respective primer pairs for wildtype and mutant alleles. PCR products were then visualized after electrophoresis (1% gel, 130 V, 20 min) and homozygous seedlings were transferred into soil and grown in the greenhouse in order to collect seeds. Specific primer pairs were designed with the T-DNA Primer Design tool from SALK (Salk Institute Genomic Analysis Laboratory (SIGnAL), San Diego, USA) and can be found in Table 6.

To genotype the crosses between T-DNA lines (*rve1* and *rve8*) and *slim1-1*, specific primers were designed (Table 6) taking advantage of the point mutation to introduce a cutting site for the restriction enzyme SpHI (New England BioLabs) in the WT allele but not in the mutant. After the PCR reaction, the product was incubated with the restriction enzyme and reaction buffer at 37º C overnight according to the manufacturer instructions and then visualized after electrophoresis (4% gel, 80 V, 2 hours).

2.4 Metabolite analysis

2.4.1 Isolation and quantification of anions

Frozen samples were homogenized as described in section 3. Genotyping, 1 mL of sterile water was added to each tube and they were incubated at 4ºC and 1500 rpm for 1 hour. Next, samples were kept at 95ºC for 15 minutes, vortexed and centrifuged at maximum speed for 15 min. The supernatant was transferred to plastic vials and diluted with water when necessary. Anions were measured and separated using a Dionex ICS-1100 chromatography system with a Dionex IonPac AS22 RFIC 4x 250 mm analytic column (Thermo Scientific, Germany). External standards of nitrate, phosphate and sulfate were prepared as 0.5mM, 1 mM and 2 mM mix of KNO_3, KH_2PO_4 and K_2SO_4 and 4.5 mM $NaCO_3$/1.4 mM $NaHCO_3$ was used as running buffer.

2.4.2 Isolation and quantification of low-molecular-weight thiols

Frozen samples were homogenized as described before and a 10-fold amount (from fresh weight) of 0.1 M HCl was added to each tube and the tubes were vortexed. Samples were centrifuged at maximum speed at room temperature and 60 µL supernatant was transferred to a

new tube. At this point, standards containing 0 µM, 20 µM, 50 µM and 100 µM of reduced L-cysteine and reduced glutathione were added to the process. 100 µL of 0.25 mM CHES-NaOH pH 9.4 were added to each tube, together with 35 µL of 10 mM DTT and then samples were vortexed and incubated for 40 min at room temperature. Afterwards, 5 µL of 25 mM Monobromobimane were added and the extracts were again vortexed and incubated at room temperature for 15 min. The reaction was stopped with the addition of 110 µL of 100 mM methanesulfonic acid and samples were vortexed and centrifuged at 4ºC for 20 min. 200 µL of supernatant were transferred to plastic vials and measured by HPLC (Dionex UltiMate^{TM} 3000 system (Thermo Fisher Scientific) using a LC column 250 x 4.6 mm (Spherisorb^{TM} ODS2, 5 µm). Low-molecular-weight thiols were detected fluorometrically (excitation: 390 nm, emission: 480 nm). Two eluents were used in a linear gradient from 95 to 82% A (10% methanol, 0.25% acetic acid, pH 4.1) in B (90% methanol, 0.25% acetic acid, pH 4.1) with a constant flow rate of 1 mL/min.

2.4.3 Isolation and quantification of O-acetylserine

Frozen samples were homogenized as described before and extracted with 400 µL of 80% ethanol (in 2.5mM HEPES pH 6.8), shaken at 2000 rpm for 20 min at room temperature and centrifuged at 4ºC for 12 min at maximum speed. Supernatants were collected in new tubes and samples were re-extracted as described before with 400 µL of 50% ethanol (in 2.5 mM HEPES pH 6.8) and once again with 200 µL of 80% ethanol (in 2.5 mM HEPES pH 6.8). After centrifuging for 5 min at room temperature at maximum speed, 200 µL of the supernatant were transferred to plastic vials and measured by HPLC (Dionex UltiMate^{TM} 300 system (Thermo Fisher Scientific) using a LC column 150 x 4.6 mm (HyperClone, 3 mm, ODS (C18), 120 A) and detected fluorometrically (emission: 330 nm; excitation: 450 nm) after reaction with *O*-phtalaldehyde (Dr. Maisch GmbH, Germany). Two eluents A (964 mL H_2O, 33 mL 0.4 M $NaPO_4$ (pH 6.8), 3 mL tetrahydrofuran) and B (225 mL H_2O, 110 mL acetonitrile, 175 mL MeOH, 25 mL 0.4 M $NaPO_4$ (pH 6.8)) were used for separation (0-2 min, 100% A; 2-21 min, 87% A, 13% B; 21-28.25 min, 85% A, 15% B; 28.25-37.3 min, 50% A, 50% B; 37.3-48.3 min 40% A; 48.3-63.3 min, 100% B; 63.3-65 min, 100% A) with a constant flow rate of 0.8 ml/min. For peak confirmation, 100µL of 1M borate buffer pH 10.7 (Grace Davison, Germany) was added to additional technical replicates to specifically eliminate the OAS peak from the chromatogram.

2.4.4 Isolation and quantification of soluble proteins

Soluble protein content extraction was conducted according to Jones et al. (1989), 1 mL of NaOH was added to the frozen samples, homogenized as described before, followed by a 30 min incubation at room temperature. After a 5 min centrifugation at maximum speed and room temperature, supernatants were transferred to new tubes. Bovine serum albumin was used as an external standard, and 10 µL of each sample were mixed with 790 µL of water and 200 µL of concentrated Bradford dye (Bio-Rad). After 15 min incubation at room temperature, absorbance was measured at 595 nm in a microplate reader (TECAN Infinite® 200 PRO).

2.5 Expression analysis

2.5.1 RNA extraction

Frozen samples were homogenized as described before with 500 µL of extraction buffer (Tris/HCL pH 9.0 80 mM; LiCl 150mM; EDTA 50 mM; SDS 5% W:V). After vortexing, 500 µL of phenol-chloroform-isoamyl alcohol mix (25:24:1; v/v; Sigma-Aldrich) were added and samples were vortexed again and left shaking and rotating at 200 rpm during the process and for 5 additional minutes after the last sample. After centrifuging at max speed for 25 minutes at room temperature, supernatants were transferred to a new set of tubes containing 500 µL phenol-chloroform-isoamyl alcohol mix and vortexed. This process was repeated again, supernatants were transferred to new tubes and mixed with 150 µL of 8 M LiCl. Samples were vortexed and stored at -20ºC overnight. The next day, samples were defrosted, centrifuged at maximum speed for 40 min at 4ºC and supernatant was discarded and replaced with 300 µL of MilliQ water. After vortexing and shaking at 65ºC for 10 minutes, 100 µL of 8 M LiCl were added and tubes were again vortexed and stored at -20ºC overnight. The following day, RNA was pelleted as described for the previous day, but instead washed with 400 µL of 70% ethanol. After centrifugation, supernatants were completely discarded and the tubes were air-dried for 5 min, adding afterwards 25 µL of water and shaking at 65º C for 20 min. RNA concentration and purity was determined using NanoDrop 2000c Spectrophotometer (Thermo Scientific).

2.5.2 Real time quantitative polymerase chain reaction (RT-qPCR)

DNase treatment and reverse transcription were performed with QuantiTect® Reverse Transcription Kit (Qiagen, Germany) with 800 ng of RNA in a 6 µl reaction according to the manufacturer's instruction. qPCR reactions (4 µL primer mix, 1 µL cDNA, and 5 µL GoTaq® qPCR Master Mix (Promega)) were performed in a CFX96 TouchTM Real-Time PCR Detection System (Bio Rad, Germany). Transcript levels were quantified using CFX ManagerTM Software (Bio Rad, Germany) and normalized to *TIP42 INTERACTING PROTEIN OF 41 KDA* (*TIP41*, AT4G34270) using the 2-ΔΔCT method (Pfaffl, 2012).

2.6 Light-Darkness transition (LD) experiment

In order to study OAS accumulation and the transcriptional activation of the OAS cluster genes, a set-up based on Caldana et al. (2011) was used with a few modifications. Seedlings were germinated on modified Long Ashton Medium agarose plates and grown for 5 days. Afterwards, they were transferred to plates containing the same medium but without sucrose and kept until day 18. Then, samples were taken 4 hours after subjective dawn (Time 0) and plants were transferred immediately to darkness. With the help of a green lamp, shoot samples were taken in the dark 5 min, 20 min and 40 min after the transition.

2.7 Night cycle experiment

Following the same goal as described in the previous section, the night cycle set-up used in Caldana et al., (2011) was replicated with a few modifications. Seedlings were grown for 18 days as described before and samples were taken right at the end of the subjective day (Time 0) and then after 30 min, 2 hours, 4 hours, 6 hours and 8 hours (end of subjective night) with the help of a green lamp.

2.8 OAS feeding

To further study the function of OAS in plants, seedlings were grown on hydroponic plates as described before. On treatment day, 1 mM OAS was added to the liquid media and samples were collected after 4 hours.

2.9 Early sulfur deficiency response

Plants were grown hydroponically as previously described and the liquid modified Long Ashton Medium was replaced with a version of the same medium containing no sulfate ($MgCl_2x6H_2O$ was used as the substitute instead of $MgSO_4x7H_2O$) and samples were collected after 4 hours. This experiment was performed at 3 different times of the day: 9 am, 5 pm and 1 am, corresponding to 3 and 11 hours after subjective dawn and 3 hours after onset of subjective night, respectively.

2.10 Nitrogen depletion and resupply

Hydroponically grown plants were either kept in full-nutrient liquid modified Long Ashton Medium or moved to a non-Nitrogen containing version of the medium. Before this change, samples were taken. After 24 hours, plants were again harvested, and N-depleted plants were fed with 1 mM Nitrogen either as Ammonium succinate or KNO_3. Samples were taken again after 2 hours and 24 hours and OAS accumulation was measured in the roots.

2.11 Cloning

Genomic DNA was isolated from WT plants grown on ½ MS agar plates with 200 µL of extraction buffer (Tris 0.2 M pH 8/EDTA 0.025 M/NaCl 0.25 M/SDS 0.5%) and washed with 200 µL isopropanol. Amplification of genes of interest (GOI) and promoters of interest (POI) was done with Phusion polymerase (New England BioLabs) according to the manufacturer's instructions. After electrophoresis, the amplicons were purified using the ReliaPrep™ DNA Clean-up and Concentration System (Promega). Purified fragments were then ligated into pENTRY-D-TOPO vector using the TOPO™ Cloning Kit (Thermo Fisher Scientific). Competent TOP10 *Escherichia coli* bacteria were transformed with the product of this reaction and spread on plates in order to select positive clones. After confirmation of fragment incorporation by colony PCR using GoTaq® DNA Polymerase, positive clones were grown in liquid media and plasmids were extracted and purified using the PureYield™ Plasmid Miniprep System (Promega). Samples were sequenced and checked on ApE Plasmid Editor (Davis & Jorgensen, 2022). Confirmed constructs were introduced into the destination vectors (pGWB2 for GOI and pGWB3 for POI) using the Gateway™ LR Clonase™ Kit (Thermo Fisher Scientific) and the product was used to transform

competent DH5α *E. coli* bacteria. After confirmation via colony PCR and sequencing, plasmids were extracted and purified as described before and used to transform competent *Agrobacterium tumefaciens* LBA4404.pBBR1MCS virGIN 54D. All the bacterial strains and vectors used in this study can be found in Tables 3 and 4, respectively.

Table 3: Bacterial strains used in this study

Species	**Strain**	**Resistance**
Escherichia coli	TOP10	
Escherichia coli	DH5α	
Agrobacterium tumefaciens	RK19	Rif, Kan
Agrobacterium tumefaciens	LBA4404.pBBR1MCS virGIN 54D	Rif, Chl

Table 4: Vectors used in this study

Vector	**Description**	**Resistance**
pENTRY-D-TOPO	Gateway donor vector	Kan
pGWB2	Gateway destination vector, 35S promoter	Kan, Hyg
pGWB3	Gateway destination vector, no promoter, C-GUS tag	Kan, Hyg

2.12 Transactivation assay

A. tumefaciens glycerol stocks containing the constructs of interest, together with RK19 strain, were spread on plates and let grow for 2 days, then a colony was picked for liquid culture and was incubated overnight. Bacteria were pelleted, washed and resuspended in AT-medium (Table 5), also used to dilute the Ler-0 cell culture 1:4. In 6 well plates, 4 mL of diluted cell suspension was pipetted, together with 30 µL of RK19 suspension. Then the different combinations of POI-GOI bacteria were inoculated. *A. tumefaciens* containing pGWB2_pro*35s::GUS* was used as a positive control and a POI without effector was used as a negative control. Plates were covered in aluminum foil and shaken softly at room temperature in a growing chamber for 3 days. After incubation, 50 µL of X-Gluc (10 mg/mL; diluted in dimethylformamide) were added into each well, plates were covered again and incubated at 37ºC.

Positive control wells turned blue after 3-4 hours and the other combinations were incubated overnight.

Table 5: AT-medium composition, pH adjusted to 5.8

Components	**Final concentration**
MS basal salt mixture (Duchefa)	4,4 g/L
Gamborg's vitamin solution (Sigma-Aldrich)	0,5 % (v/v)
Glucose	30 g/L
2,4-Dichlorophenoxyacetic acid	1 mg/L

2.13 Oligonucleotides

Primers were ordered from Sigma-Aldrich and can be found in Table 6.

Table 6: Oligonucleotides used in this study

Genotyping		
Name	**Gene**	**Sequence (5' →3')**
RVE1-LP	AT5G17300	AAGTGGAGATGAATCTCATGCTC
RVE1-RP		CAAAGACCGCAGTTCAGATTC
RVE8-LP	AT3G09600	TTCAGCAAAATCAGGAACACC
RVE8-RP		AGAGCTGGACAGAGGAAGAGC
SLIM1_SPHI_F	AT1G73730	CAAGCTCAGAGGAAG
SLIM1_SPHI_R		CCACTCACAGGCATG
LBb1.3	SALK Lines	ATTTTGCCGATTTCGGAAC
qPCR		
Name	**Gene**	**Sequence (5' →3')**

q-TIP41-F	AT4G34270	GAACTGGCTGACAATGGAGTG
q-TIP41-R		ATCAACTCTCAGCCAAAATCG
q-SDI1-F	AT5G48850	TCCCTGTGGAGACACTCCTT
q-SDI1-R		CCATCTCCGGGTTCTTCTCT
q-SDI2-F	AT1G04770	GTCCTTATGTCCGAGCGAAG
q-SDI2-R		TCTCGCTTTAATCGCTATCCA
q-LSU1-F	AT3G49580	TTAAGTTGTGGCAGCGAACG
q-LSU1-R		CCATGAGGAAGAGCATGCGA
q-APR3-F	AT4G21990	CCAATCAAGTATCCATCAGAGAAG
q-APR3-R		CCGAACAAGATTCAAGAAAGATG
q-GGCT2;1-F	AT5G26220	TCCACCGGAGCTATTTGC
q-GGCT2;1-R		CGTTCCAAGTACTCCATTGCT
q-SHM7-F	AT1G36370	TCTTAGAAGAAAATGCAGACTGG
q-SHM7-R		CACACACTTTCTCGTAGACTTTCC
q-SULTR1.2-F	AT1G78000	ATCCGTTTTCAAAGCAGCTC
q-SULTR1.2-R		TCAAGAATGATGCACCAATGA
q-APR2-F	AT1G62180	AAAAGAGCTCCACGGGCTAT
q-APR2-R		CGACATGAGTGAATCAACATCTC
q-SLIM1-F	AT1G73730	TGACTTGGACTTTGACTATGGTG
q-SLIM1-R		CTATTGCCATGTCCTTTTGAACT
q-RVE1-F	AT5G17300	CACTGTTGGATCAGAAGCAT
q-RVE1-R		AACCAGTGTTTGATCCAGTCG
q-RVE8-F	AT3G09600	GGGAAGCTCAAGCCGAACAGTATC
q-RVE8-R		GGCCTCTCGTTTCAGGATCAAGA

q-NIR1-F	AT2G15620	AGCGATTCCTCTTGATGC
q-NIR1-R		GTTCGTCGATAAGCCACA
q-GDH3-F	AT3G03910	GCAGCTCTAGGGGGAGTCAT
q-GDH3-R		CAGCCTCAGGATCAGTTGGG
q-SAT1;1-F	AT5G56760	TGACTTGGACTTTGACTATGGTG
q- SAT1;1-R		CTATTGCCATGTCCTTTTGAACT
q-SAT2;1-F	AT1G55920	CACTGTTGGATCAGAAGCAT
q-SAT2;1-R		AACCAGTGTTTGATCCAGTCG
q-SAT2;2-F	AT3G13110	GGGAAGCTCAAGCCGAACAGTATC
q-SAT2;2-R		GGCCTCTCGTTTCAGGATCAAGA
q-SAT3;1-F	AT2G17640	AGCGATTCCTCTTGATGC
q-SAT3;1-R		GTTCGTCGATAAGCCACA
q-SAT3;2-F	AT4G35640	GCAGCTCTAGGGGGAGTCAT
q-SAT3;2-R		CAGCCTCAGGATCAGTTGGG
Cloning		
Name	**Gene**	**Sequence (5’ →3’)**
cRVE8-F	AT3G09600	CACCATGAGCTCGTCGCC
cRVE8-R		TTATGCTGATTTGTCGCTTGTTGAG
cRVE1-F	AT5G17300	CACCATGGCGTCGTCTCC
cRVE1-R		TTATAAGTGGAGATGAATCTCATGC
cSLIM1-F	AT1G73730	CACCATGGGCGATCTTGC
cSLIM1-R		CTAAGCTCCAAACCATGAGAAATC
proGGCT2;1-F	AT5G26220	CACCAATTGAATCAGCTAACTTAAT
proGGCT2;1-R		CTTTGATCCTTAGCCTCACAC

proSDI1_2kb-F	AT5G48850	CACCTTCTCTTTGTCTCTCT
proSDI1_2kb-R		CTTTTTTTCCTCTGTTTTTCTCTTT
M13-F	pENTRY-TOPO ligation	GTAAAACGACGGCCAG
M13-R		CAGGAAACAGCTATGAC
B1	pGWB2/3 ligation	ACAAGTTTGTACAAAAAAGCAGGCT
B2		ACCACTTTGTACAAGAAAGCTGGGT

2.14 Data processing and statistical analysis

Data was processed using Microsoft Excel (Office 365; version 16.58) and transferred to GraphPad Prism (version 8.0) for visualization and graphs generation. 4 biological replicates were taken for every measurement, and key experiments were repeated at least twice. Statistical differences were calculated performing a two-way ANOVA in RStudio when there were 2 independent variables and one-way ANOVA/two-tailed, unpaired Student's t-test when there was only one. Respective statistical analyses are indicated in graph legends.

3 RESULTS – CHAPTER 1: Control of OAS accumulation

3.1 Mutations in the SERAT family impact OAS accumulation and affect sulfur and nitrogen metabolism

OAS provides the carbon and nitrogen backbone for cysteine, which is a convergence point for carbon, nitrogen and sulfur metabolism. In *A. thaliana*, there are 5 isoforms of SERAT, the enzyme that synthesizes OAS; they can be found in plastids, mitochondria and cytosol. The presence of these 5 isoforms and their distribution in different cell compartments implies possible extra functions of SERAT apart from the synthesis of OAS. Thus, we used quadruple knockouts of these isoforms (the quintuple knockout is not viable as described by Watanabe and colleagues in 2008b), in which only a single SERAT is active, to study the importance of individual SERAT isoforms during S and N deficiency.

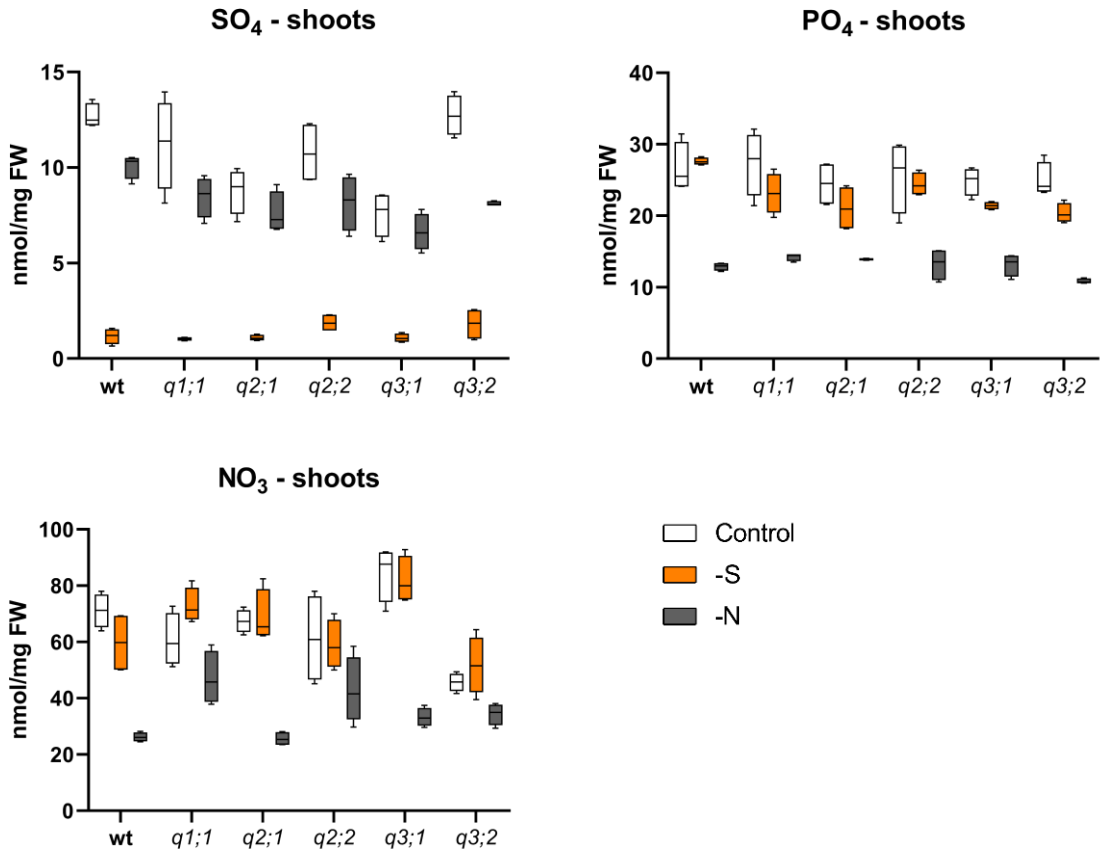

Figure 4: Anion content in shoots of WT and quadruple SERAT mutants. Control conditions (white), sulfur deficiency (-S; orange), and nitrogen deficiency (-N; gray). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and compact letter display can be found in supplemental data.

When the nutrient supply is sufficient, the anions content in the leaves (Figure 4) is quite similar between the mutants and WT. We can observe some differences, for example the mutants *q2;1* and *q3;1* accumulated significantly less sulfate than WT plants. Regarding phosphate, all the lines behaved similarly and almost the same can be seen for nitrate, with only *q3;2* showing significantly less foliar nitrate than WT. Under sulfur deficiency conditions, sulfate levels decreased dramatically in all lines, as expected, however when comparing the mutants to WT only *q2;1* and *q3;1* show significantly lower concentration. Phosphate levels did not change in plants grown on low sulfur media and nitrate showed the same tendency. Interestingly, under nitrogen deficiency sulfate shows a tendency of accumulating to a lesser extent compared to control conditions, however those differences were only statistically significant for *q3;2*. In low nitrate, accumulation of phosphate in the leaves was significantly lower in all lines, pointing towards the tight connection between these nutrients. Nitrate, as expected due to the treatment, was reduced significantly in all lines compared to control conditions but no differences were found among the genotypes.

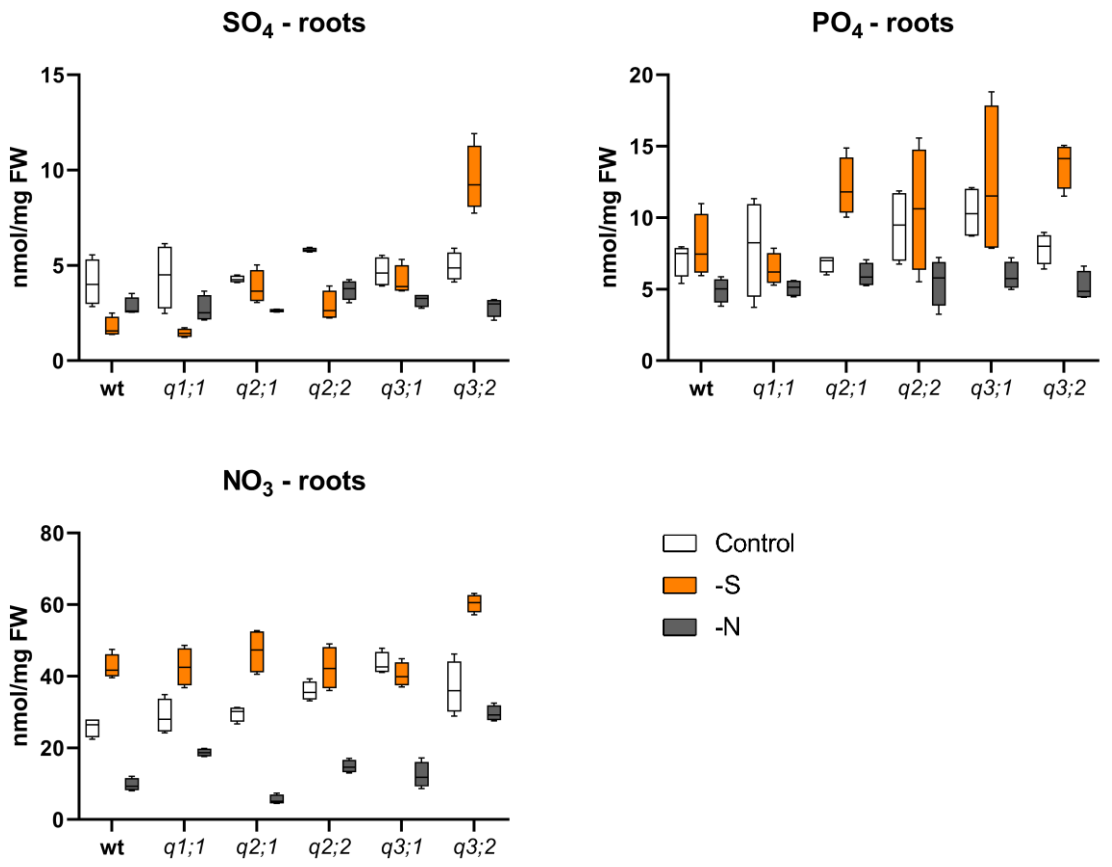

Figure 5: Anion content in roots of WT and quadruple SERAT mutants. Control conditions (white), sulfur deficiency (-S; orange), and nitrogen deficiency (-N; gray). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and compact letter display can be found in supplemental data.

The anion content in roots (Figure 5) was very similar in all lines under control conditions, with the exception of *q3;1* accumulating significantly more nitrate. When dealing with sulfur deficiency, the sulfate content in roots was reduced, but the decrease was only significant in WT, *q1;1* and *q2;2*. Conversely, the mutant *q3;2* showed a significant increase of sulfate when compared to any other line and condition, which was surprising. Phosphate levels were not significantly different when compared to control conditions, however *q3;2* line again showed a different behaviour and accumulated significantly more phosphate under sulfur deficiency in comparison to WT. Nitrate levels in roots tended to increase when the sulfur supply was not sufficient, but this increase was not statistically significant for *q2;2* and *q3;1*. Overall, *q3;2* showed a high content of the 3 anions under sulfur deficiency, which points towards a possible sensing

function of this isoform. Under nitrogen deficiency, sulfate and phosphate concentration remained comparable to control levels in roots, whereas nitrate levels were significantly lower in WT, *q2;1*, *q2;2* and *q3;1*. Once again, the *q3;2* mutant showed higher nitrate content in roots under this condition, being only comparable to *q1;1*.

Altogether, anion contents showed interesting differences among the mutants in different conditions, therefore, since SERAT is the only enzyme responsible for OAS synthesis, the concentration of this metabolite was analyzed in shoot and roots via HPLC (Figure 6)

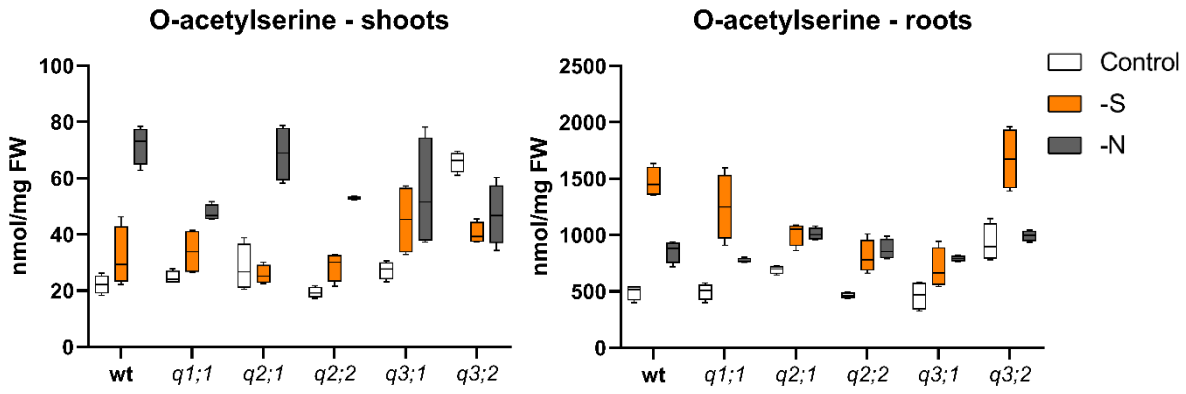

Figure 6: OAS content in shoots and roots of WT and quadruple SERAT mutants. Control conditions (white), sulfur deficiency (-S; orange), and nitrogen deficiency (-N; gray). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and compact letter display can be found in supplemental data.

When knocking out 4 out of the 5 SERAT isoforms in the mutants, it was expected to find a reduction in OAS accumulation. However, all the lines showed similar levels in shoot under control conditions except for *q3;2*, which intriguingly accumulated 3 times more OAS than WT. Under sulfur deficiency, the OAS content in the shoot remained unchanged with the exception of *q3;2*, which showed a significantly lower concentration compared to control conditions. Interestingly, under nitrogen deficiency all the lines but *q3;2* showed a significant increase in OAS concentration when compared to control conditions. In roots, the concentration of OAS was more than 100-fold higher than in shoots under all conditions. Under control conditions, we found no differences among the lines, but the sulfur deficiency treatment caused different responses in the mutants. Low sulfur concentration in the media led to OAS accumulation in the roots, but it was

not significant for *q2;1* and *q3;1*. Also, the mutants *q2;1*, *q2;2* and *q3;1* had a significantly lower OAS content than WT. Similar to the shoots, the concentration of OAS tended to increase under nitrogen deficiency, with *q3;2* acting as an outlier. Despite the unanticipated results found in the *q3;2* mutant, the OAS levels support the previously obtained anions data. Besides, it can be observed how OAS levels change when the levels of sulfur or nitrogen are altered in the media and how every SERAT isoform is able to deal with the deficiencies differently.

Since OAS is the precursor of cysteine and, by extension, of other low-molecular-weight thiols like glutathione, the concentration of these two metabolites was analyzed via HPLC (Figure 7).

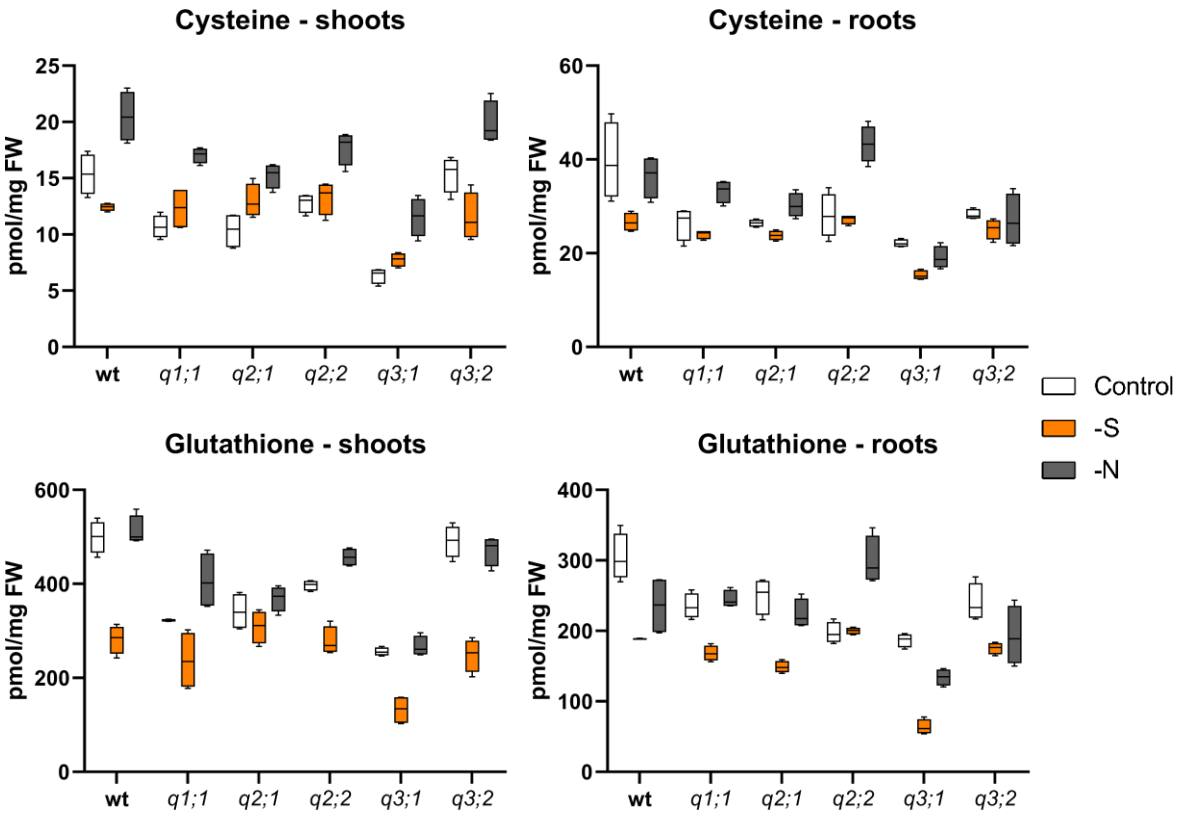

Figure 7: Low-molecular-weight thiols content in shoots and roots of WT and quadruple SERAT mutants. Control conditions (white), sulfur deficiency (-S; orange), and nitrogen deficiency (-N; gray). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and compact letter display can be found in supplemental data.

Foliar cysteine levels in the mutants remained comparable to WT under control conditions except for *q3;1*, which was significantly lower, while in roots no differences were found. Under sulfur deficiency, cysteine showed a tendency to accumulate less, however it was only significant in the roots of WT plants. Under nitrogen deficiency, cysteine accumulated in shoots, possibly because of the plant focusing on the synthesis of primary metabolites, while in roots the concentration was similar to control conditions with the exception of *q2;2* showing an increase. The *q3;1* mutant showed the lowest cysteine concentration in both organs under all conditions.

Regarding glutathione, all the mutants but *q2;2* accumulated a significantly lower amount than WT in shoots, while in roots this was the case only for *q2;2* and *q3;1*. Low sulfur in the media had a negative impact on glutathione content, with significantly lower concentration in all lines and organs with the exception of *q2;1* shoots and *q2;2* roots. Interestingly, the *q3;1* mutant was the most affected one with the lowest glutathione concentration exactly as described before for cysteine. Despite the effect of nitrogen deficiency on cysteine, the consequences on glutathione were not dramatic and similar amounts under control conditions could be found in almost all cases, which shows that while there is a channelling of resources towards cysteine, that cysteine is not mainly used for glutathione synthesis. Remarkably, the mutant *q3;2* mimics WT levels of both low-molecular-weight thiols, just as previously seen for OAS and anions.

Given the differences found in metabolites and the importance of sulfur and nitrogen as components of proteins, we decided to extract and quantify soluble proteins (Figure 8).

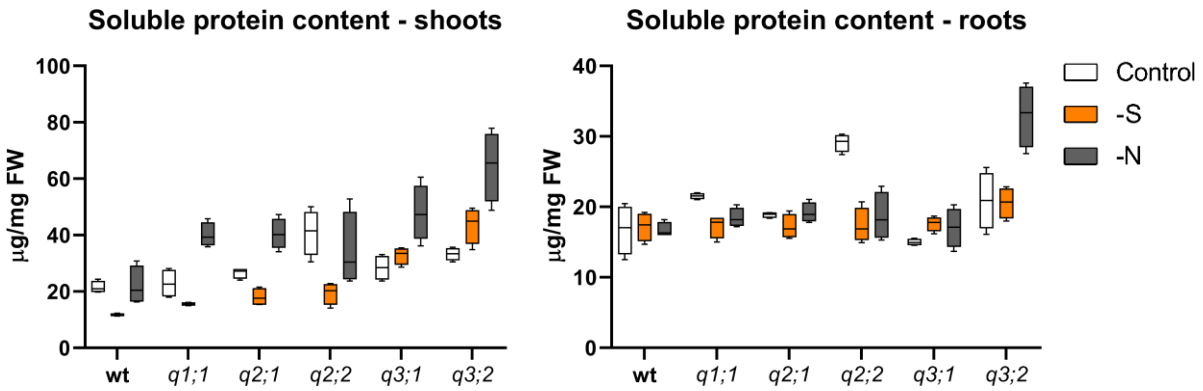

Figure 8: Soluble protein content in shoots and roots of WT and quadruple SERAT mutants. Control conditions (white), sulfur deficiency (-S; orange), and nitrogen deficiency (-N; gray). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean

and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and compact letter display can be found in supplemental data.

The soluble protein content in shoots was very similar among the mutants under control conditions, only the *q2;2* mutant showed a significantly higher concentration than the rest. Under sulfur deficiency, we could observe a tendency of lower protein amount compared to control conditions except for the mutants with a remaining isoform of family III (*q3;1* and *q3;2*), however those differences were not significant. Interestingly, if we compare the levels within this condition then the 2 aforementioned mutants showed higher amounts than the rest of the lines, which also happened under nitrogen deficiency. In roots, there were not many remarkable differences, but it is important to notice that the mutant *q2;2* showed the highest amount of soluble proteins (as seen in shoots) and those levels are comparable to those of the mutant *q3;2* under nitrogen deficiency. Overall, the soluble protein content did not decrease under nitrogen deficiency the way it could be expected, but since we are not measuring total protein content this could be explained by a shift into this specific type.

Many sulfur-related metabolites were altered in the different mutants and their levels did not always match the respective OAS levels, pointing to the possibility that the mutants are also affected in signalling. To investigate it, RT-qPCR was performed in shoots (Figure 9) and roots (Figure 10) to evaluate the relative expression levels of sulfur and nitrogen deficiency marker genes. A large number of genes, known as sulfur marker genes, are upregulated under sulfur deficiency. In this case we tested *SDI1*, *LSU1* and *APR3*, which, as described before, are also part of the OAS cluster genes. *NITRITE REDUCTASE 1 (NIR1)* and *GLUTAMATE DEHYDROGENASE 3 (GDH3)* were used as nitrogen starvation marker genes.

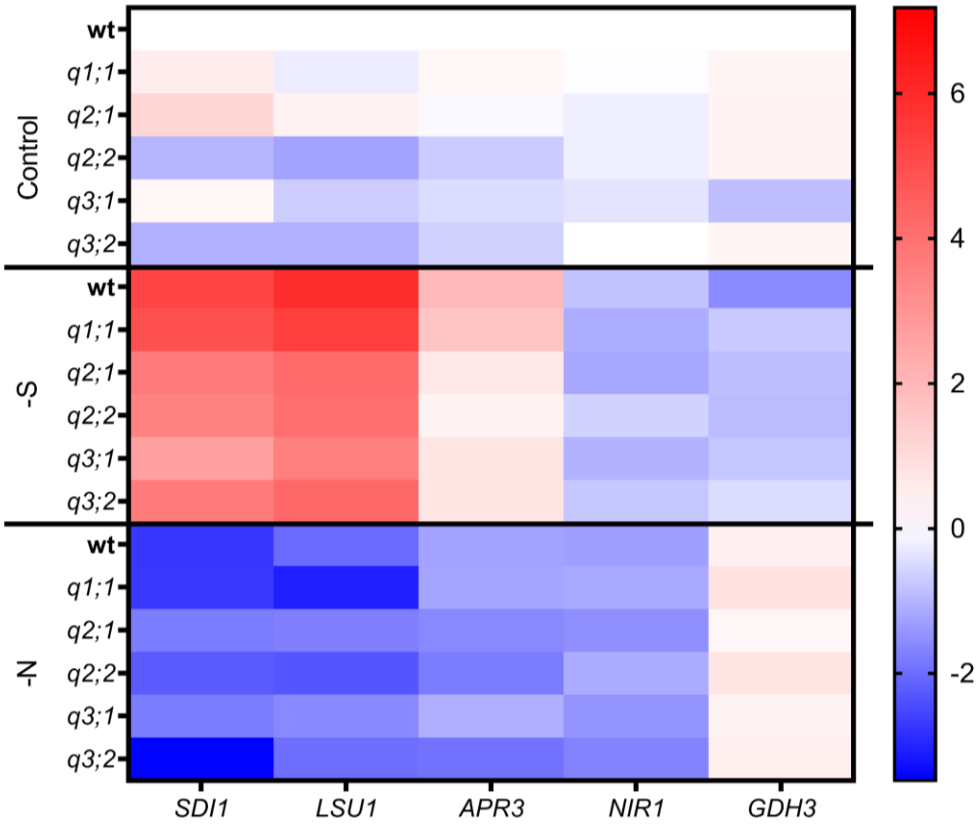

Figure 9: Relative expression of sulfur and nitrogen marker genes in shoots. This heatmap shows the log2 Fold Change of the relative gene expression of wild type and all quadruple SERAT mutants in control condition (control), sulfur deficiency (-S), and nitrogen deficiency (-N) for shoots and roots. Fold Change was calculated against wild type under control conditions and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

Under control conditions, the differences in relative gene expression in the shoots were not substantial. The mutants *q2;2* and *q3;2* showed a slight downregulation of the 3 sulfur marker genes while *q3;1* only of 2 genes, while having on the other hand the lowest relative expression level of *GDH3*. Sulfur starvation led to a strong induction of the sulfur-responsive genes in all lines, with WT and *q1;1* having the highest levels. On the other hand, *q3;1* had the smallest increase. Interestingly, both *SDI1* and *LSU1* responded to sulfur starvation at a higher rate than *APR3*, a pattern that could be observed across all the lines. Under sulfur deficiency, the nitrogen marker genes were slightly downregulated with WT having the lowest levels for *GDH3* while the expression of *NIR1* remained consistent. Under nitrogen deficiency, the expression of *NIR1* was also downregulated although only slightly lower than under sulfur deficiency. *GDH3*, however,

was upregulated consistently among the genotypes. Interestingly, the 3 chosen sulfur marker genes were also downregulated when the nitrogen supply in the media was low.

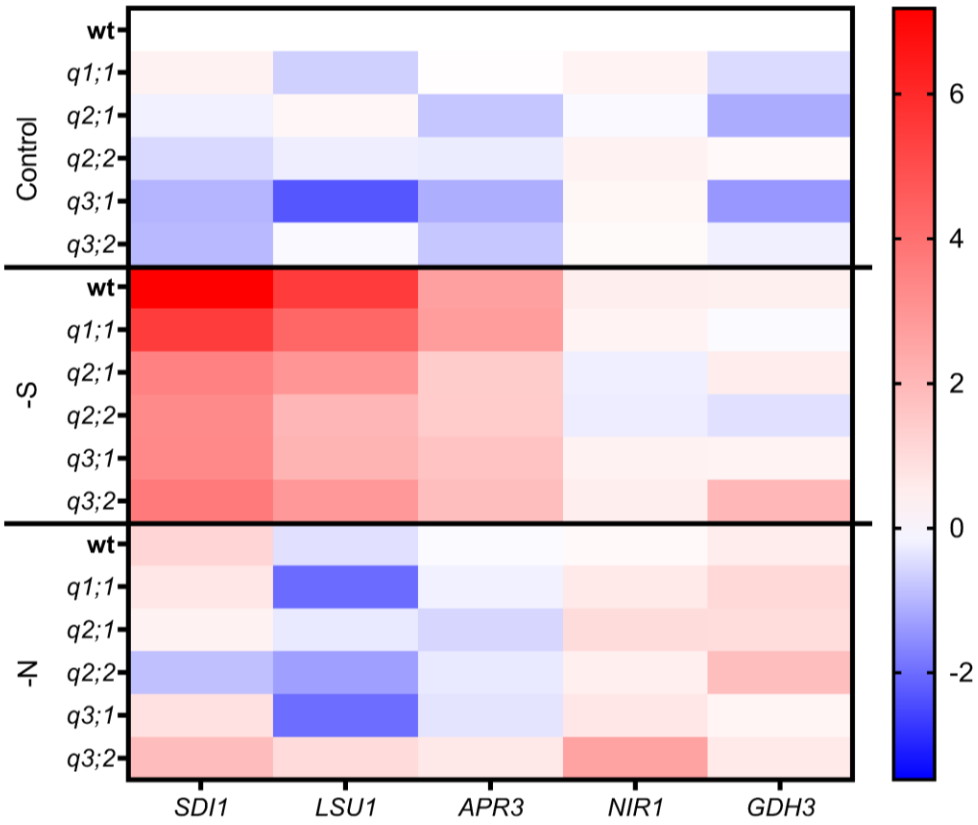

Figure 10: Relative expression of sulfur and nitrogen marker genes in roots. This heatmap shows the log2 Fold Change of the relative gene expression of wild type and all quadruple SERAT mutants in control condition (control), sulfur deficiency (-S), and nitrogen deficiency (-N) for shoots and roots. Fold Change was calculated against wild type under control conditions and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

The expression pattern in roots did not match completely with what was observed in shoots. Some similarities could be found, with the mutants *q2;2*, *q3;1* and *q3;2* showing lower transcript levels of some sulfur marker genes and the *q3;1* again with the lowest expression level of *GHD3*. In roots, this gene was also downregulated in *q1;1* and *q2;1* while *NIR1* showed no changes. As seen in shoots, sulfur starvation led to a strong response of the sulfur marker genes and once again we find the highest induction in WT and *q1;1*. Unlike in shoots, the nitrogen marker genes remained unaltered in this condition. Under nitrogen deficiency, the differences between roots and shoots were more remarkable. Among the sulfur marker genes, only *LSU1* showed lower expression

levels, *APR3* was close to control levels and *SDI1* was even slightly induced in some cases. Both nitrogen marker genes were induced among all the lines, even though *NIR1* is expected to be downregulated under nitrogen deficiency. Moreover, the mutant *q3;2* showed upregulation of all 5 genes, having the strongest induction for *SDI1* and *NIR1* which is the opposite of what we could observe in shoots.

Additionally, the expression levels of all *SERAT* isoforms were analyzed (Supplemental Table S4) and to our surprise, the only substantial changes were found for *SERAT3;2*, whose transcripts accumulated under sulfur deficiency. However, none of the isoforms had an increased expression in the mutants to compensate for the lack of other isoforms.

Taken together, these results suggest that SERAT isoforms might have further functions than just OAS production. Despite having relatively similar levels of OAS, the concentration of other metabolites were different and the metabolic and transcriptional profiles of the mutants did not match. Remarkably, the level of induction of sulfur deficiency markers by low S was significantly reduced in several mutants, most prominently in *q3;1*. Out of all the mutants, *q3;2* was the most outstanding one, we could observe that it could not perceive the starvation signals, as it usually mimicked WT levels or sometimes even surpassed it. It was expected to find higher levels of OAS in plants grown under sulfur deficiency (Nikiforova et al. 2003; Hirai et al., 2003), but it was surprising to find it as well in the shoots of plants grown under nitrogen deficiency. It would be logical to think that the OAS cluster genes expression would be induced after the accumulation, however we found those genes to be downregulated. This could be the consequence of a signalling system more robust than OAS signalling, which makes the plant downregulate the sulfur assimilation pathway to compensate for the lack of nitrogen. It is a great example of how complex metabolite and gene regulation is in plants.

3.2 OAS concentration remains stable after nitrogen depletion and resupply

Previous studies have explored the connection between sulfur and nitrogen metabolism. Koprivova and colleagues (2000) demonstrated how OAS restored sulfate assimilation in nitrogen starved plants more rapidly than any other nitrogen sources, making it a good candidate for a signalling molecule connecting nitrogen and sulfur metabolism. In this study we have also seen

the interplay between sulfur and nitrogen pathways, so we decided to investigate the connection in more detail. To evaluate whether the endogenous levels of OAS change after nitrogen depletion and further resupply, WT plants were grown hydroponically under full nutrient supply for 2 weeks, transferred to media without any N source for 24 hours and then resupplied with either ammonium succinate or potassium nitrate (Figure 11).

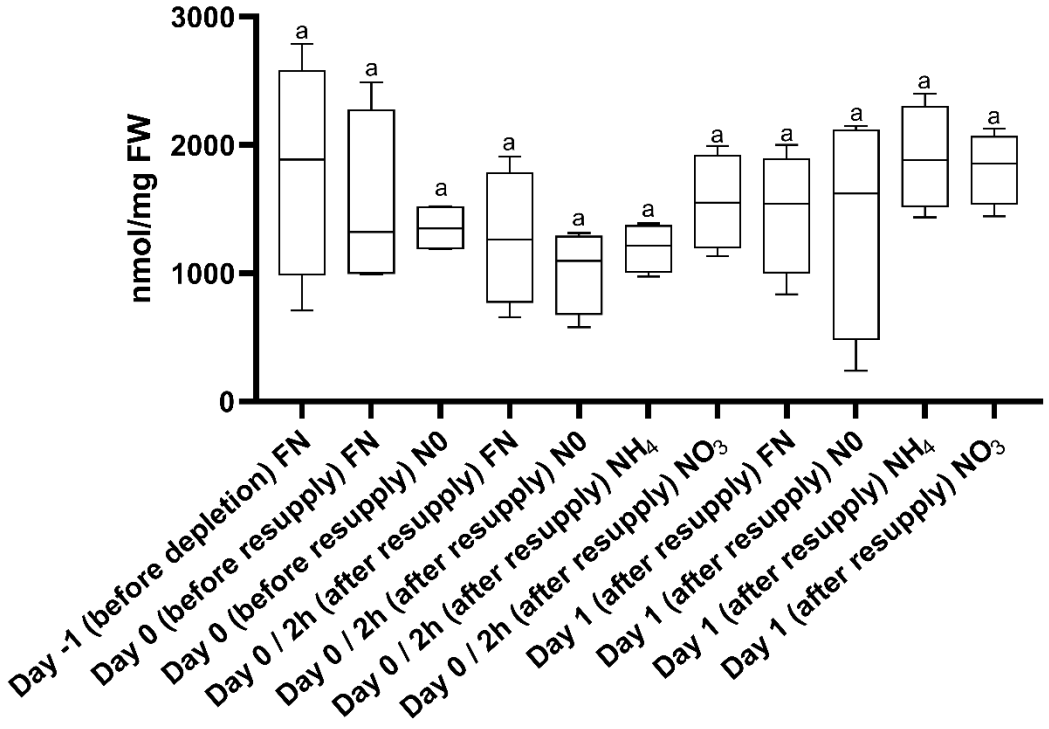

Figure 11: OAS content in roots of WT plants after nitrogen depletion and resupply. Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a one-way ANOVA followed by TukeyHSD and letters are displayed. FN: full nutrient media; N0: media without Nitrogen; NH4: plants supplemented with ammonium succinate; NO3: plants supplemented with potassium nitrate.

Interestingly, the concentration of OAS in roots of plants grown hydroponically is about 2-fold higher than the one seen in plants grown on agar plates. This supports the findings of Watanabe et and collaborators (2008b), proving how OAS production is dependent on the growth condition. Apart from that, no significant differences were found across the different time points, independently of the treatment applied, independently of whether the plants were resupplied with Nitrogen or the starvation was maintained. We can conclude that OAS accumulation in roots does not change after a short term nitrogen depletion, nor after resupply and, therefore, is not acting as

a signal, but we cannot dismiss the possibility that it might play a role in a long term nitrogen starvation, as our previous results suggested (Figures 6 and 9).

3.3 SERAT mutants contribution to light-darkness transition

Using the data produced by Caldana et al. (2011), Hubberten and colleagues (2012) proved the signalling function of OAS based on metabolite-gene correlation studies. They found that after transferring plants from light to darkness OAS displayed a transient increase after 5-10 minutes followed by an increase in the transcript levels of the OAS cluster genes. We replicated the experimental set-up with the single and quadruple SERAT KO mutants with the goal of identifying the isoform(s) responsible for this increase to better understand the OAS signalling function. We expected that, if a single SAT isoform is responsible for the transient increase, the corresponding single mutant should not show OAS increase nor transcriptional activation of the OAS cluster genes, but that should be restored in the quadruple mutant with that remaining isoform. While the OAS results were inconclusive, the expression levels of the OAS cluster genes in the single (Figure 12) and quadruple (Figure 13) mutants showed significantly altered patterns.

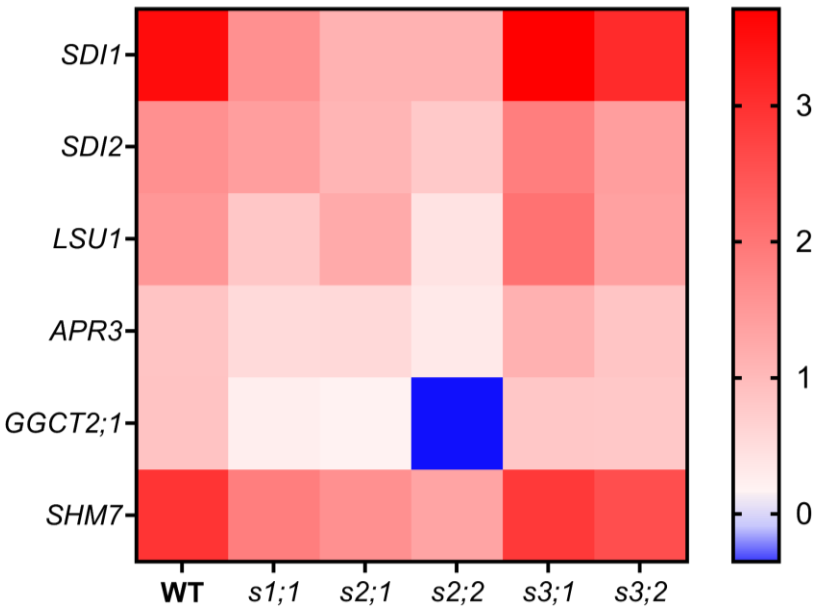

Figure 12: Relative expression of the OAS cluster genes after light-darkness transition is single *serat* mutants. These heatmaps show the log2 Fold Change of the relative gene expression of wild type and single SERAT mutants 40 minutes after transition from light to darkness. Fold Change was calculated per genotype vs time 0 and the

housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

We could observe the induction of the OAS cluster genes 40 minutes after the transition in all the lines. Interestingly, knocking out the isoforms SERAT3;1 or SERAT3;2 had no impact on the transcriptional activation of the OAS cluster genes, since both mutants reached the same levels of induction as WT. This could mean that the cytosolic SERAT isoforms are not necessary for the light-darkness transition. On the other hand, the *s1;1*, *s2;1*, and *s2;2* showed lower increase in expression levels than WT in most of the genes, suggesting they have an active role in this regulatory network. Among them, the single mutant *s2;2* (mitochondrial isoform) seems to be the most affected by the loss of a single isoform, which agrees with the previously described importance of mitochondrial OAS synthesis for cysteine synthesis (Watanabe et al., 2018).

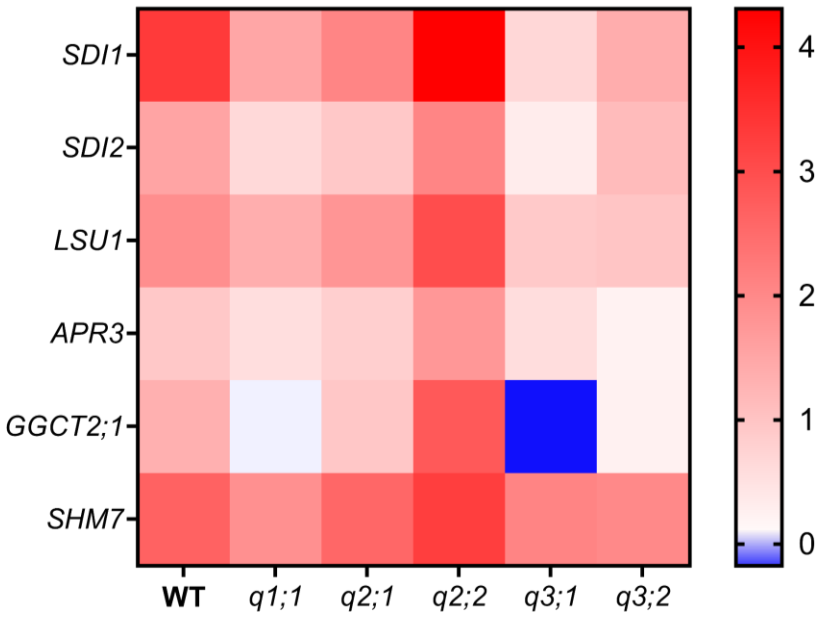

Figure 13: Relative expression of the OAS cluster genes after light-darkness transition in quadruple *serat* mutants. These heatmaps show the log2 Fold Change of the relative gene expression of wild type and quadruple SERAT mutants 40 minutes after transitioning from light to darkness. Fold Change was calculated per genotype vs time 0 and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

The analysis of light-darkness transition in the quadruple mutants revealed that all the lines were generally able to induce the expression of the OAS cluster genes 40 minutes after the shift. Corresponding to the previous data with single mutants, the cytosolic isoforms (SERAT3;1 and

SERAT3;2), while not necessary when the other isoforms are present, are able to activate the transcription of the OAS cluster genes albeit to much lower extent. Remarkably, the *q2;2* mutant, with only the mitochondrial isoform remaining, is able to generate the same response (or even higher) than WT. This points towards this isoform being the main contributor to this regulation, which again corresponds to the single mutant data, and is logical considering that it is the isoform that accounts for 90% of the SERAT activity in leaves (Watanabe et al., 2018). Taken together, our results suggest that there is not a single isoform responsible for the response of OAS cluster genes to light-darkness transition and, while their contribution might differ, all the isoforms are able to activate the OAS cluster genes after transitioning from light to darkness, with SERAT2;2 playing the largest role.

3.4 Sucrose in the media influences OAS accumulation and OAS cluster genes induction

As stated before, OAS acts as a convergence point for carbon, nitrogen and sulfur pathways. In this study, we observed how perturbations in sulfur and nitrogen metabolism have an impact on OAS accumulation and OAS cluster genes expression. Therefore, we also decided to study whether changes in carbon metabolism affect OAS and OAS cluster genes. To do so, the transition from light to darkness was performed in plants grown under 3 different conditions: no sucrose in the media, sucrose in the media, and germinated in sucrose-containing media and later grown in no sucrose media. OAS accumulation was determined via HPLC (Figure 15) and the expression of the OAS cluster genes was evaluated 20, 40 and 60 minutes after the shift (Figure 14).

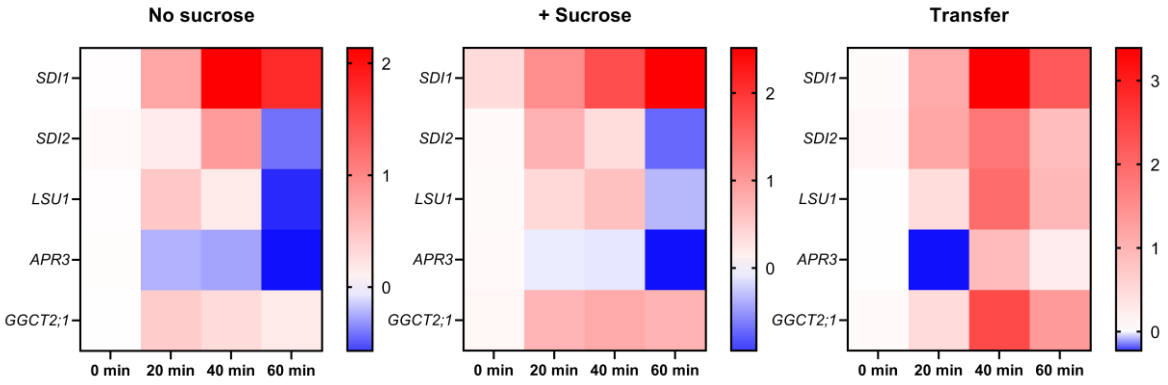

Figure 14: Relative expression of the OAS cluster genes after light-darkness transition in different sucrose conditions. These heatmaps show the log2 Fold Change of the relative gene expression of wild type plants 20, 40 and

60 minutes after transitioning from light to darkness. Plants were either grown on no sucrose in the media, sucrose containing media and germinated in sucrose containing media and then transferred to no sucrose media. Fold Change was calculated per treatment vs time 0 and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

The presence of sucrose in the media had an effect in the induction of the OAS cluster genes. Transferred plants showed the clearest induction after 40 minutes, not only in absolute values for each gene but also the induction was uniform and all the genes showed the same pattern. Under no sucrose or when sucrose was present during the whole developmental process, the induction of the cluster genes was not only lower than in transferred plants, but also the cluster did not behave uniformly. *APR3* was downregulated in both cases. Besides, transferred plants only showed a slight reduction in the transcript levels after 60 minutes compared to the 40-minute peak, while the other 2 conditions had a clear downregulation in some genes, reaching lower levels than at time 0.

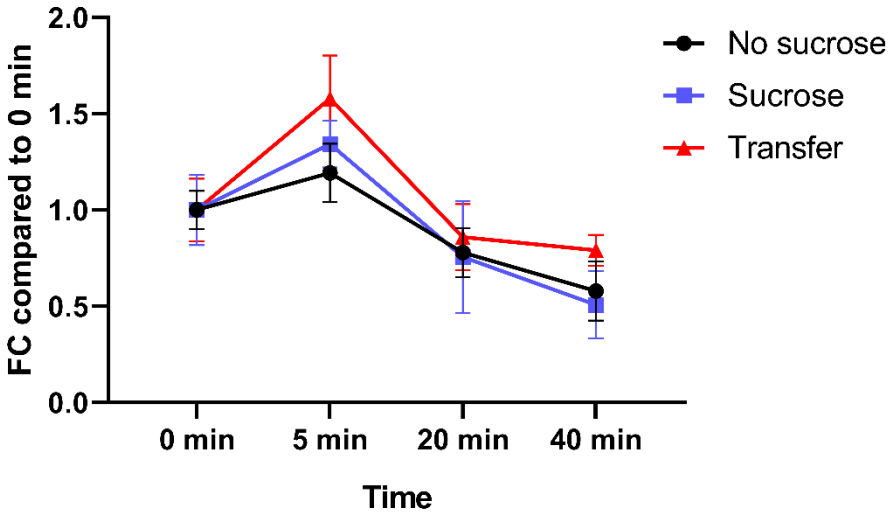

Figure 15: OAS concentration after light-darkness transition in different sucrose conditions. Fold Change of OAS concentration compared to time 0 for every condition is represented. Plants were either grown on no sucrose in the media, sucrose containing media and germinated in sucrose containing media and then transferred to no sucrose media. Data was collected from 4 biological replicates.

OAS concentration data matched with what was observed for the relative expression. While all 3 conditions had some sort of increase of OAS 5 minutes after the transition followed by a decrease in concentration, transferred plants showed the highest increase.

The fact that both OAS accumulation and OAS cluster genes expression match and show the same differences under the different sucrose conditions provides some insight on how carbon

metabolism perturbations can affect OAS signalling, once again showing how this metabolite could be acting as a mediator between different pathways.

4 RESULTS – CHAPTER 2: Mechanisms of regulation of the OAS cluster genes

4.1 Transcription factors SLIM1, RVE1 and RVE8 regulate the OAS cluster genes

The OAS cluster genes are a tightly co-regulated group of genes. As a first approach to shed some light into their regulation, the online platform Plant Regulomics (Ran et al., 2020) was used to find transcription factors predicted to bind to the promoter of all the genes. Seven transcription factors were identified (see Supplemental Table S8) and we initially focussed on three candidates: SLIM1, RVE1 and RVE8. SLIM1 is a central regulator of Sulfur metabolism (Maruyama-Nakashita et al., 2006), RVE8 is part of the circadian clock core (Rawat et al., 2011) and RVE1, while being connected to the circadian clock, acts as an output and has also been connected to auxin metabolism (Rawat et al., 2009). Homozygous mutant lines *slim1-1*, *rve1* and *rve8* were obtained and used for analyses of the involvement of these transcription factors in regulation of OAS cluster genes. First, we tested the condition which led to identification of the OAS cluster, the light-darkness transition. The mutants were subjected to light-darkness transition and transcript levels of the OAS cluster genes were determined after 40 min (Figure 16).

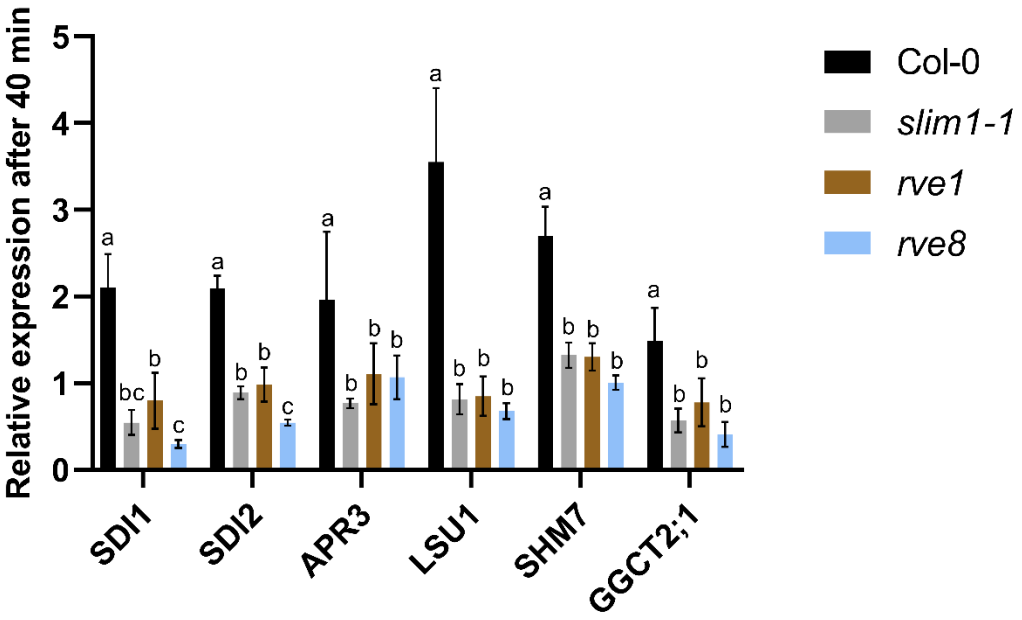

Figure 16: Relative expression of the OAS cluster genes after light-darkness transition in WT, *slim1-1*, *rve1* and *rve8*. The plants were subjected to light-darkness transition, shoots were harvested after 40 minutes, and expression levels of the OAS cluster genes were determined by qRT-PCR. Shown is mean (2-ΔΔCT method) and standard deviation. Relative expression changes were calculated per genotype vs time 0 and the housekeeping gene *TIP41* served as a reference. Significances were calculated using a one-way ANOVA followed by TukeyHSD for every gene individually and letters are displayed

While WT plants showed an induction in the expression of all genes 40 minutes after the transition, none of the mutants did. Those differences were statistically significant for all the mutants and all the genes. In the mutant lines, none of the genes were upregulated, in fact, in some cases they were downregulated. These results show that SLIM1, RVE1 and RVE8 are necessary for the induction of the OAS cluster genes after transitioning from light to darkness and, therefore, participate in their regulation.

OAS levels were also measured via HPLC after the transition (Figure 17), to test whether OAS accumulation might also be affected in the mutants.

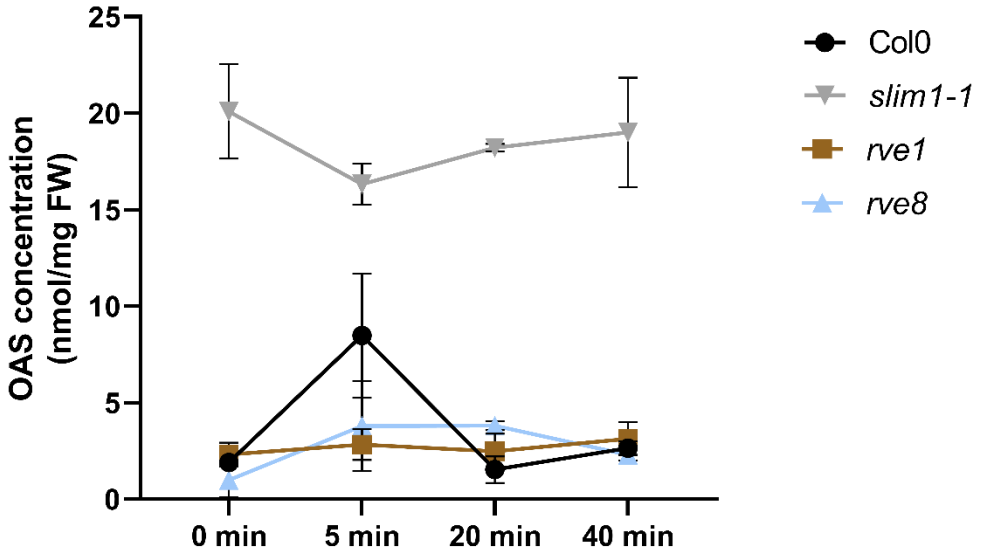

Figure 17: OAS concentration after light-darkness transition in WT *slim1-1*, *rve1* and *rve8*. Data was collected from 4 biological replicates and average is shown, with standard deviation as error bars. Samples were collected right before the transition (0min) and then after 5, 20 and 40 minutes.

Surprisingly, the transient OAS peak after 5 minutes was only observed in WT. The OAS concentration remained stable over time without significant changes in the mutant lines. OAS levels were similar in WT and the *rve1* as well as *rve8* mutants, however, *slim1-1* showed

significantly higher OAS concentration, around 4-fold higher. These results suggest that SLIM1, RVE1 and RVE8 not only regulate the OAS cluster genes directly, but they also participate in the OAS signalling cascade upstream of the OAS accumulation. However, the question of whether or how they are involved in translating the OAS accumulation into a transcriptional signal remains unanswered.

To further understand how these transcription factors participate in the regulation of the OAS cluster genes, we analyzed their expression levels after transitioning from light to darkness (Figure 18).

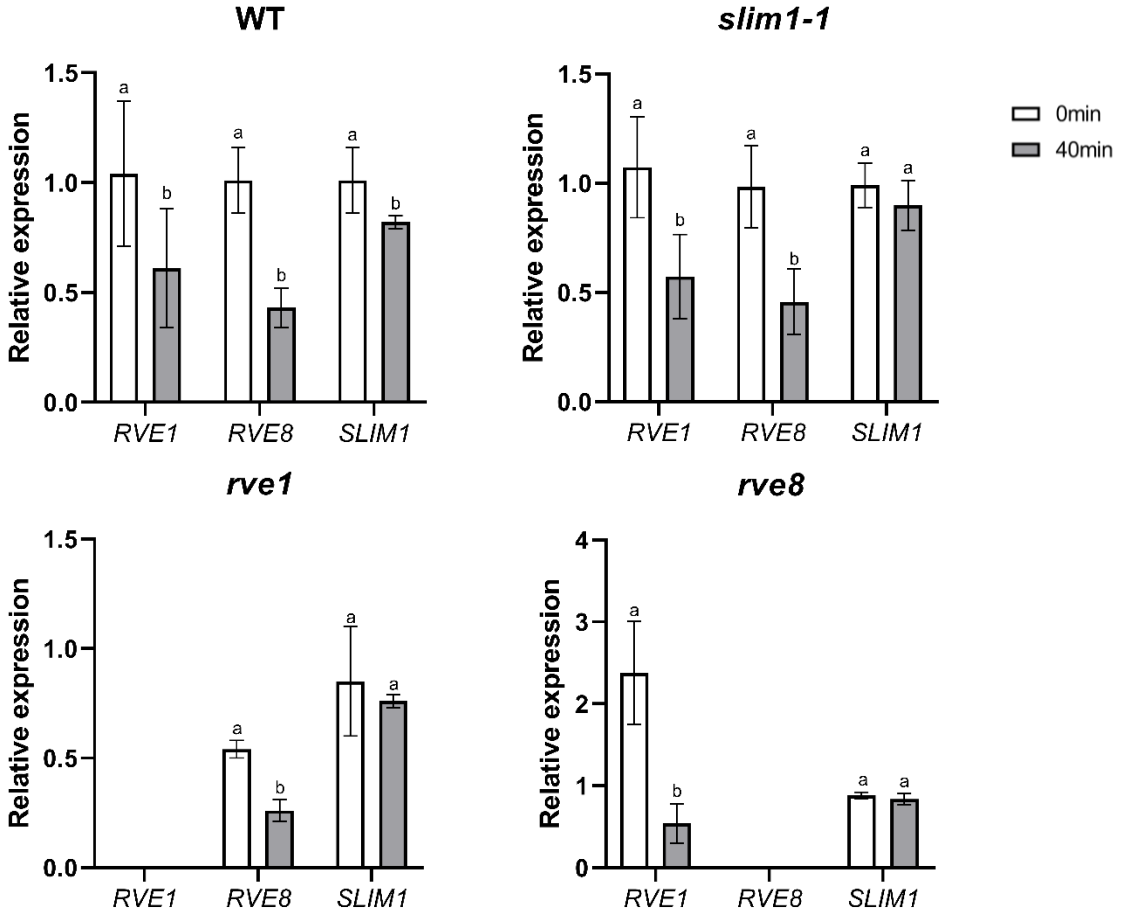

Figure 18: Relative expression of *RVE1, RVE8* and *SLIM1* after light-darkness transition in WT, *slim1-1, rve1* and *rve8*. Relative gene expression 40 minutes after transitioning from light to darkness, absolute mean (2-ΔΔCT method) and standard deviation represented. Expression was normalized vs WT time 0 and the housekeeping gene *TIP41* served as a reference. Significances were calculated using a one-way ANOVA followed by TukeyHSD for every gene individually and letters are displayed.

Interestingly, both *RVEs* showed a similar behaviour in the WT, and are both downregulated 40 min after light-darkness transition. The loss of function of *RVE1* resulted in an upregulation of *RVE8* (around 2-fold higher), but on the other hand, the knock-out of *RVE8* led to the downregulation of *RVE1* (around 2-fold lower). The expression levels of *SLIM1* also decreased slightly but significantly in WT plants, however, it was not altered in the mutants. The mutation of *SLIM1* had no impact in the expression levels of both *RVE* genes and the significant downregulation was also present. These results hint that the control of the OAS cluster genes is quite complex, and there might be more layers than simply transcriptional regulation.

4.2 Circadian oscillations of the OAS cluster genes

4.2.1 Expression pattern of the OAS cluster genes during the night

Due to their sessile nature, plants need a robust endogenous regulatory network to cope with environmental changes and anticipate diurnal changes. The clock is also tightly connected to metabolism at many levels, allowing plants to allocate resources during the most beneficial time of the day and thus improving fitness, defense and performance in general (Venkat & Muneer, 2022).

Previous data (Caldana et al., 2011; Hubberten et atl., 2012) showed how OAS accumulates in the middle of the night and the transcript levels of the OAS cluster genes correlate with that increase. The cause of this OAS peak or its significance for the downstream processes are completely unknown, so in order to shed some light, we replicated the original experiments with the three mutants and evaluated the expression of the OAS cluster genes during the night as well as OAS concentration (Figures 19 and 20). Samples were taken at the beginning of the subjective night and then after every 2 hours.

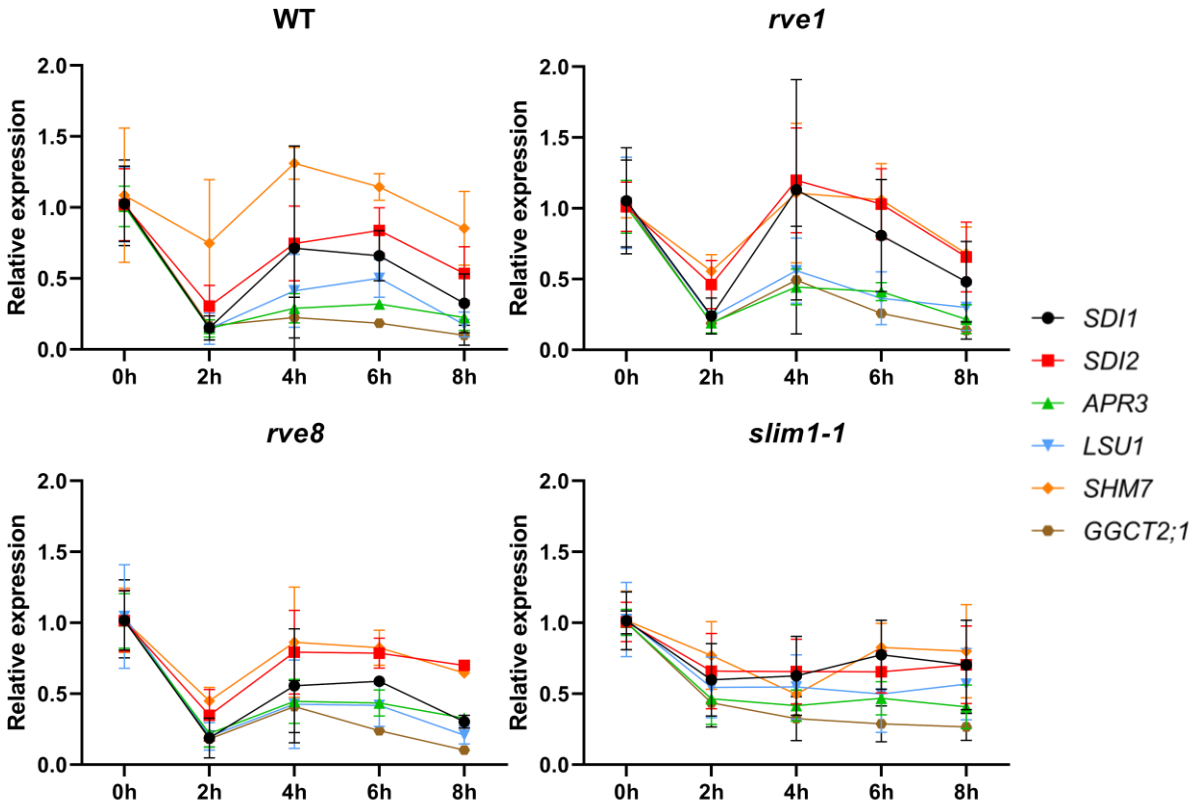

Figure 19: Relative expression of the OAS cluster genes during the night in shoots of WT, *slim1-1*, *rve1* and *rve8*. Relative gene expression at the beginning of the night (0h) and after 2, 4, 6 and 8 hours, absolute mean (2-ΔΔCT method) and standard deviation represented. Relative expression changes were calculated per genotype vs time 0 and the housekeeping gene *TIP41* served as a reference.

While the original experiment showed an expression peak of the OAS cluster genes 4 hours after the beginning of the night, this was not seen in our data, possibly due to different growth conditions. Nevertheless, our experimental setup was able to provide a more detailed picture of how these genes behave during the night, since we collected data after every 2 hours. The expression pattern of OAS cluster genes was similar in WT and *rve1* and *rve8* mutants. The transcript levels of all the genes were strongly diminished 2 hours after the beginning of the subjective night, then for most of them the levels increased again after 4 h to starting point levels and then decreased again. *SHM7* and *SDI2* showed higher expression at 4 hours and afterwards, while *LSU1*, *APR3* and *GGCT2*;1 were always lower than at the beginning of the night. *SDI1* showed an intermediate expression pattern. The *slim1-1* mutant showed a different pattern. While the expression levels of the OAS cluster genes also decreased after 2 hours, they did not increase

after 4 hours, and remained mostly stable for the rest of the night. These results suggest that RVE1 and RVE8 are not involved in the regulation of the cluster genes during the night, while SLIM1 is.

Mutations in the 3 transcription factors proved to have a negative impact in the transcriptional activation of these genes after transitioning from light to darkness, however only SLIM1 seemed to participate in controlling these genes during the night, showing how complex and context-dependent regulation can be.

To complement the transcriptional data, OAS concentration was also measured via HPLC and the results can be seen in Figure 20.

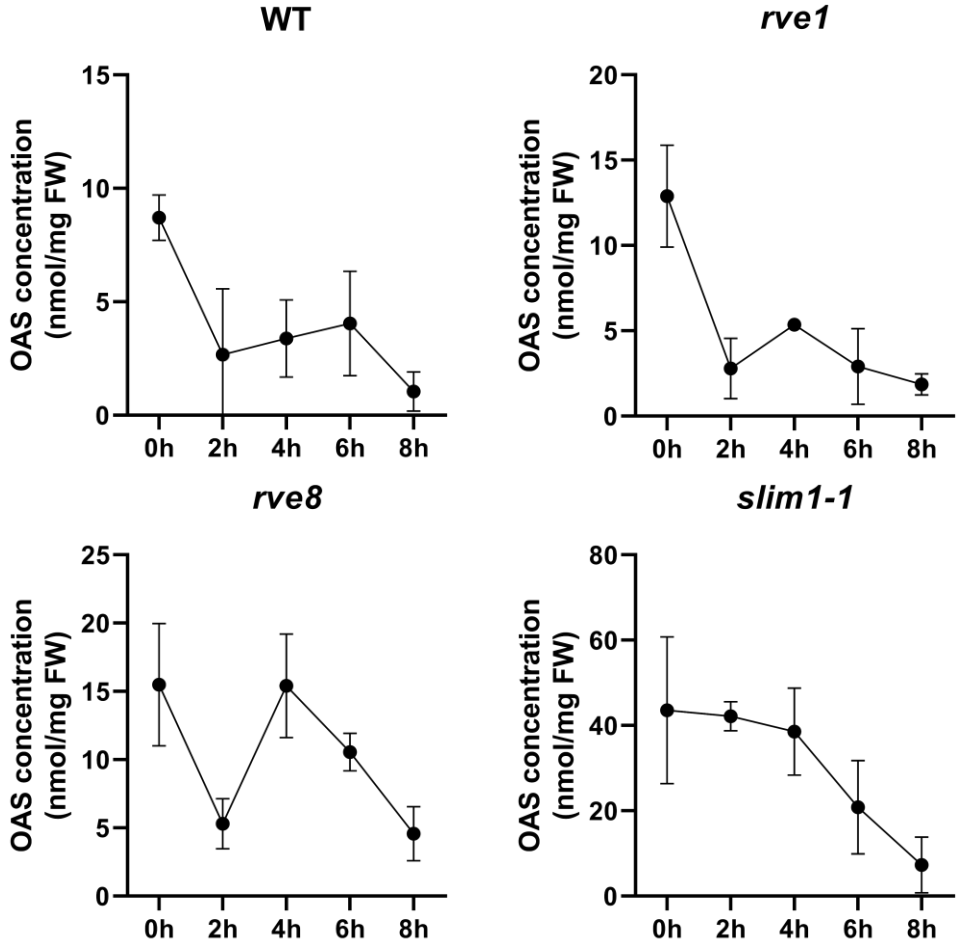

Figure 20: OAS concentration during the night in WT *slim1-1*, *rve1* and *rve8*. Data was collected from 4 biological replicates and average was represented, with standard deviation as error bars. Samples were collected at the beginning of the night (0h) and after 2, 4, 6 and 8 hours.

The original data from Caldana and colleagues in 2011 showed an OAS peak 4 hours after the beginning of the night that correlated with the expression peak of the OAS cluster genes. We did not observe said expression peak and OAS concentration did not increase either. However, the changes in OAS levels matched perfectly with the expression pattern of the OAS cluster genes. Again, WT, *rve1* and *rve8* showed a similar profile, with OAS concentration decreasing after 2 hours, then going up again after 4 h and later slowly decreasing as the night goes on. While the dynamics are the same, the absolute values were slightly different, with *rve1* and *rve8* having a slightly higher amount of OAS than WT. In the *slim1-1* mutant we found a different pattern. The levels were around 4-fold higher than in WT, and the changes over time were also different. OAS remained stable during the first 4 hours of the night and then its concentration was diminished after 6 and 8 hours. The changes in OAS levels, thus, correlated quite well with the changes in expression levels of OAS cluster genes and again *slim1-1* showed a different pattern than WT and the other mutants, supporting the idea of the transcription factors working upstream of the OAS production and not only controlling the expression of the OAS cluster genes by OAS.

Similarly to above, the expression levels of the 3 transcription factors were checked in WT plants during the night (Figure 21).

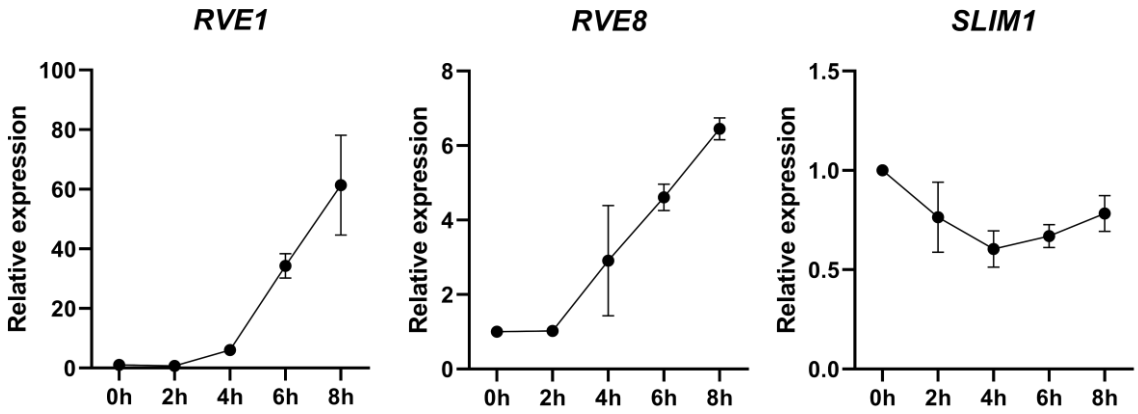

Figure 21: Relative expression of the *RVE1*, *RVE8* and *SLIM1* during the night. Relative gene expression at the beginning of the night in WT plants and after 2, 4, 6 and 8 hours, absolute mean (2-ΔΔCT method) and standard deviation represented. Relative expression changes were calculated vs the beginning of the night (0h) and the housekeeping gene *TIP41* served as a reference.

The expression profiles of both *RVEs* were very similar, their transcripts started to accumulate 4 hours after the beginning of the night and then increased until the end albeit to different extent.

These results might explain why these transcription factors are not involved in the regulation of the OAS cluster genes during the night, since their expression levels are not matching the OAS accumulation nor the transcripts of the OAS cluster. Regarding *SLIM1*, the expression changes were very small during the night, with only a slight decrease after 4 hours and then return to initial levels. Despite not being regulated transcriptionally in the context of sulfur metabolism, we can still observe that the expression of *SLIM1* changes overnight and follows a circadian pattern.

4.2.2 OAS cluster genes expression is also induced after the transition from end of the day to beginning of the night

As described before, the circadian clock in plants contributes to the adaptation of predicted changes in the environment, such as the succession of day and night. Contrary to the light-darkness transition studied before, the natural shift from the end of the day to the beginning of the night is an expected and repeating event, therefore plants have developed regulatory networks that tune gene expression and metabolism to adapt to those changes. Hence, it would be very interesting to check the expression values of the OAS cluster genes and compare both events to gain some more insight into the mechanism behind. We checked the expression of the OAS cluster genes 30 minutes after the beginning of the night and also evaluated the effect of the mutations in *SLIM1*, *RVE1* and *RVE8* in this context (Figure 22)

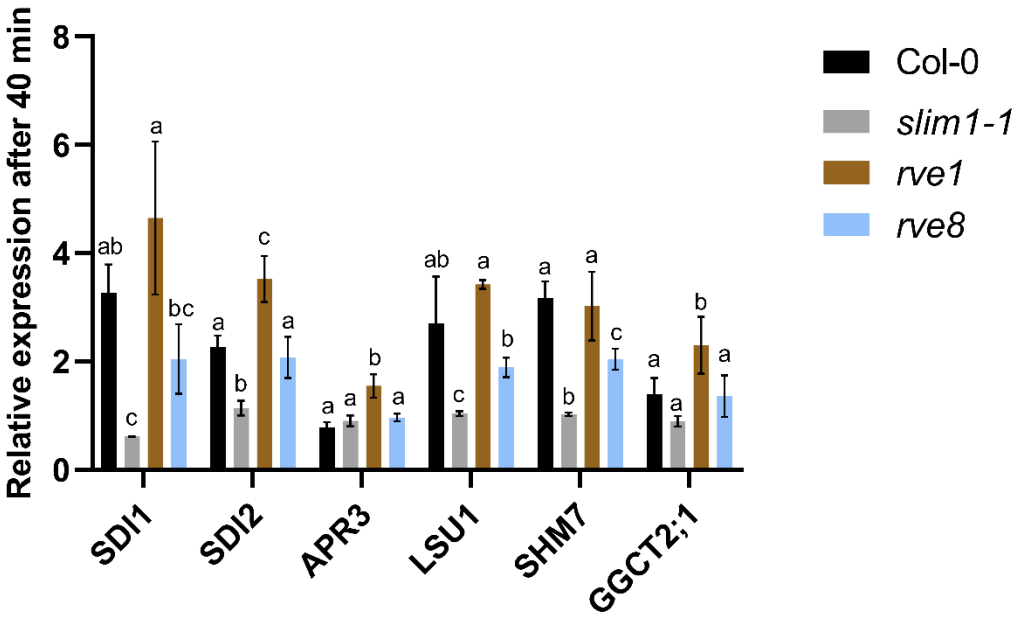

Figure 22: Relative expression of the OAS cluster genes after transitioning from day to night in WT *slim1-1*, *rve1* and *rve8*. Relative gene expression 30 minutes after the beginning of the night, absolute mean (2-ΔΔCT method) and standard deviation represented. Relative expression changes were calculated per genotype vs the beginning of the night and the housekeeping gene *TIP41* served as a reference. Significances were calculated using a one-way ANOVA followed by TukeyHSD for every gene individually and letters are displayed.

Interestingly, in WT plants all the genes but *APR3* were induced 30 minutes after the natural shift from day to night. Once again, *rve1* and *rve8*, despite having some significant but not substantial differences, exhibited a pattern comparable to WT, whereas the *slim1-1* mutant showed no expression changes at all. The increase in the transcript levels after 30 min is unexpected given the general decrease in the expression after 2 h in WT, *rve1*, and *rve8*, pointing to a large dynamics of gene expression in the first phase of the night. Comparing the mutants, these results support the conclusions that SLIM1 controls OAS cluster genes during the course of the night, while RVE1 and RVE8 are not involved. Taken together, our findings point towards SLIM1 acting as the main regulator of the OAS cluster genes, with RVE1 and RVE8 taking a more context-dependent role in the regulation. It is also very interesting that in both situations, after a sudden transition from light to darkness and after the shift from day to night, the OAS cluster genes are induced. Even though the downstream processes are yet unknown, it shows that it is a conserved mechanism and the consequences of the activation of these genes might be necessary for the adaptation from light to darkness.

Additionally, levels of OAS were measured in the same way as described before in order to check whether OAS content correlates with the expression levels of the OAS cluster genes (Figure 23)

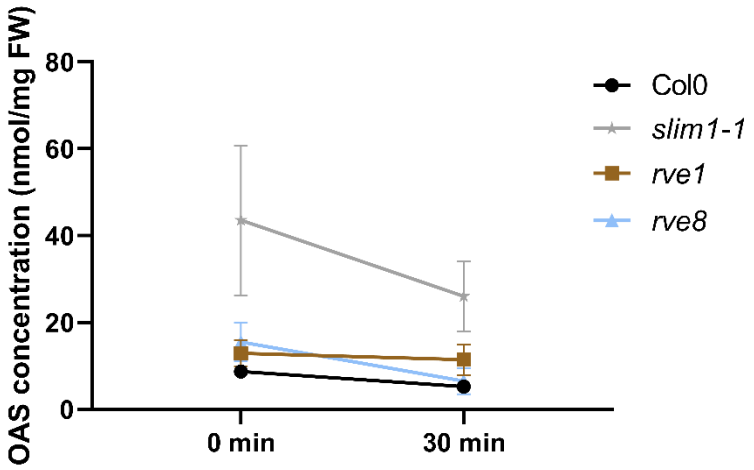

Figure 23: OAS concentration after transitioning from day to night in WT, *slim1-1*, *rve1* and *rve8*. Data was

collected from 4 biological replicates and average was represented, with standard deviation as error bars. Samples were collected at the beginning of the night and after 30 minutes.

As previously observed, WT, *rve1* and *rve8* show similar OAS concentration and the levels did not change 30 minutes after the beginning of the night. The *slim1-1* mutant showed again higher absolute values than WT and the other mutants but the changes after 30 minutes were not significant. However, due to the similarities between the sudden light-darkness transition and the shift at the end of the day and the beginning of the night, it could be possible that, if there is a change in OAS levels, the increase is transient and it happens very fast after the day ends.

4.3 SLIM1 acts as a repressor of the OAS signal

For a long time, OAS has been discussed as a potential signalling molecule in sulfur metabolism in plants, one of the reasons being the fact that it is accumulated during sulfur deficiency (Nikiforova et al., 2003). In 2003, Hirai and colleagues also showed how exogenous OAS produces a transcriptional response that overlaps substantially with the sulfur deficiency response. However, it was discussed controversially, since it has also been shown that the transcriptional changes can precede the accumulation of OAS (Hopkins et al., 2005).

Regardless of its role in sulfur deficiency, it is yet unknown how the accumulation of OAS is translated into a transcriptional signal. In our study, we have observed how the transcription factors SLIM1, RVE1 and RVE8 regulate the OAS cluster genes and also potentially work upstream of OAS accumulation, therefore they are good candidates to be tested. WT, *rve1*, *rve8* and *slim1-1* plants were grown hydroponically for 2 weeks and then fed with 1 mM OAS for 4 hours. After the treatment, roots were collected and the expression of the OAS cluster genes, as well as the sulfate transporter *SULTR1;2*, was evaluated (Figure 24).

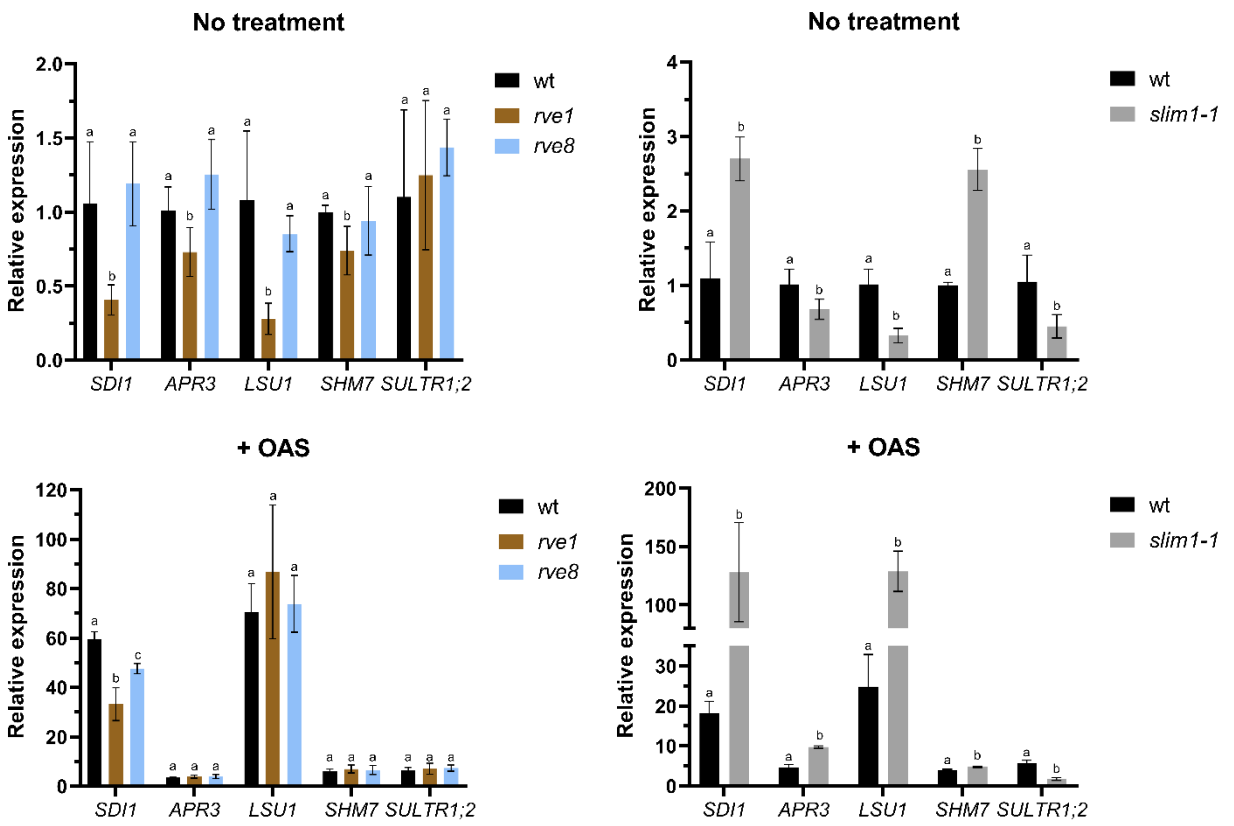

Figure 24: Relative expression of the OAS cluster genes and *SULTR1;2* after OAS feeding in WT, *slim1-1*, *rve1* and *rve8*. Relative gene expression before (No treatment) and 4 hours after OAS (+OAS) treatment, absolute mean (2-ΔΔCT method) and standard deviation represented. Relative expression changes were calculated vs WT no treatment and the housekeeping gene *TIP41* served as a reference. Data for *slim1-1* and *rve*s mutants was collected in independent experiments. Significances were calculated using a one-way ANOVA followed by TukeyHSD for every gene individually and letters are displayed.

The RT-qPCR data revealed some differences between the mutants and WT already under control conditions. The transcript levels of the *SDI1*, *APR3*, *SHM7* and *LSU1* were significantly lower in the *rve1* mutant than in WT, whereas there were no differences for *SULTR1;2*. The *rve8* mutant showed no differences to WT, and *slim1-1* did not have a regular expression pattern. When compared to WT, *SDI1* and *SHM7* were upregulated, and *APR3*, *LSU1* and *SULTR1;2* were downregulated. These differences already under control conditions are further evidence that these transcription factors directly regulate the OAS cluster genes.

After the OAS treatment, as expected, all the genes were upregulated. Interestingly, the *rve1* and *rve8* mutants exhibited the same response to OAS than WT (except for *SDI1*, which had a slightly

lower induction in both mutants). Conversely, *slim1-1* showed substantially higher transcriptional response to OAS in all the OAS cluster genes compared to WT, but lower one for *SULTR1;2*. This suggests that SLIM1 acts as a repressor for the OAS signal, since its mutation results in higher levels of transcriptional activation. The fact that *SULTR1;2* does not show a response to OAS in the *slim1-1* background is interesting and possibly consistent with this gene not being part of the cluster and therefore undergoing different regulation.

In order to further understand the connection between OAS and the transcription factors, their expression was also studied after the treatment (Figure 25).

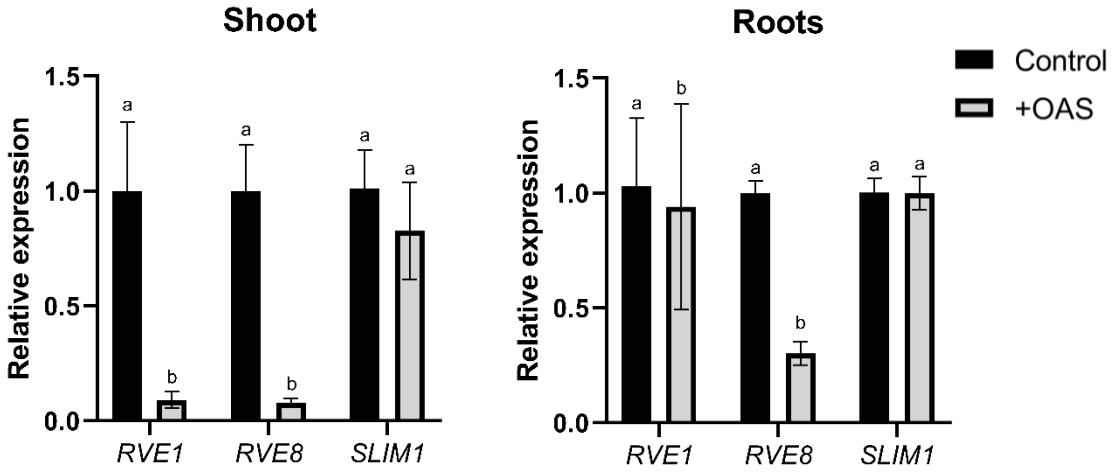

Figure 25: Relative expression of *RVE1*, *RVE8* and *SLIM1* after OAS feeding in WT. Relative gene expression in shoots and roots before (Control) and 4 hours after OAS feeding (+OAS), absolute mean (2-ΔΔCT method) and standard deviation represented. Relative expression changes were calculated vs no treatment in every organ and the housekeeping gene *TIP41* served as a reference. Significances were calculated using a one-way ANOVA followed by TukeyHSD for every gene individually and letters are displayed.

Despite the mutants *rve1* and *rve8* having almost no differences in their response to OAS compared to WT, the expression of these transcription factors was strongly affected. In shoots, both were severely downregulated 4 hours after the OAS feeding, while in roots only *RVE8* showed that pattern. *SLIM1*, on the other hand, remained unaltered in both organs. The response of *RVE1* and *RVE8* to OAS accumulation might indicate the presence of a feedback regulatory mechanism, further supporting the hypothesis of these transcription factors working upstream of the OAS accumulation in the signalling cascade. Besides, the fact that the effect can be observed in shoots and roots suggests that either OAS or the OAS signal (or both) are transmitted from the root to the shoot.

Our results provided evidence not only about RVE1 and RVE8 not being involved in the OAS signal translation, but also established a new connection between SLIM1 and OAS, two very important components of sulfur regulatory networks.

4.4 Loss of function of RVE1 and RVE8 does not impact sulfur deficiency response at a metabolic level

The connection between OAS and sulfur metabolism, together with SLIM1 being a key transcription factor in sulfur deficiency response, is widely known. Our results showed how the transcription factors RVE1 and RVE8 regulate the OAS cluster genes and, in some contexts, they show a similar effect as SLIM1. Therefore, we hypothesized that they may participate in the sulfur deficiency response working together with SLIM1, similarly as EIL1 (Dietzen et al., 2020). To test our hypothesis, homozygous lines for *rve1 slim1-1*, *rve8 slim1-1* and *rve1 rve8* were obtained and, together with the respective single mutants, were grown on agar plates under control and sulfur deficiency conditions. Anions, low-molecular-weight thiols and expression levels of OAS cluster genes (here also acting as sulfur marker genes) were analyzed (Figures 26, 27, 28 and 29)

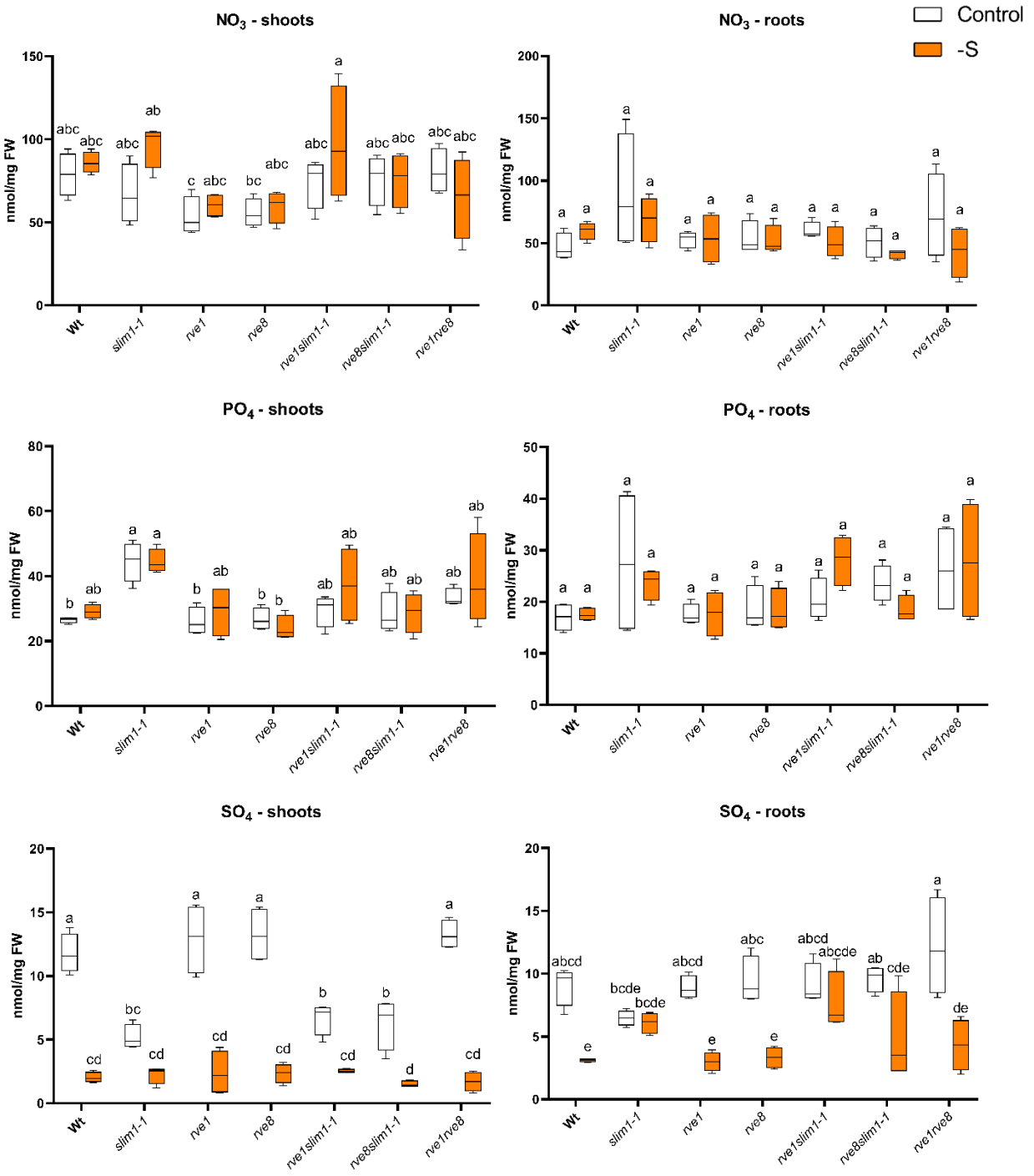

Figure 26: Anion content in shoot and roots of WT, *rve1*, *rve8*, *slim1-1* and the respective double mutants. Control conditions (white) and sulfur deficiency (-S; orange). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and letters are displayed.

The nitrate content in WT and the mutants did not show any differences among the genotypes in both organs. Phosphate was also very similar among the lines, the only significant difference was found in *slim1-1* having higher phosphate than WT in the shoots under control conditions. Sulfur deficiency did not have a significant impact on the concentration of phosphate and nitrate. Sulfate concentration, as expected because of the treatment, was reduced in plants grown on sulfur-deficient media. Under control conditions, *slim1-1* showed significantly lower foliar sulfate than WT, *rve1*, *rve8* and *rve1 rve8*. Interestingly, the double mutants with *slim1-1* background showed the same behaviour, so the knock-out of *RVE1* or *RVE8* had no impact in sulfate accumulation in both WT and *slim1-1* backgrounds. Sulfate levels under sulfur deficiency conditions in shoots were comparable in all the lines. Due to its lower levels under control conditions, the differences in *slim1-1* mutant between the two conditions were not statistically significant. In roots, the differences are not that remarkable. When having a look at each condition, all the lines have comparable sulfate levels. However, there are some interesting differences when the changes from control to sulfur deficiency conditions are compared. Again, WT, *rve1*, *rve8* and *rve1 rve8* had a significantly lower amount of sulfate when grown on sulfur deficiency media. The *slim1-1* mutant, together with *rve1 slim1-1*, had no significant differences. In roots, the double mutant *rve8 slim1-1* exhibited a WT-like pattern. Taken together, our results show how, as previously described (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020), a mutation in *SLIM1* has a negative impact in sulfate accumulation but it seems that RVE1 and RVE8 are not involved in this regulation.

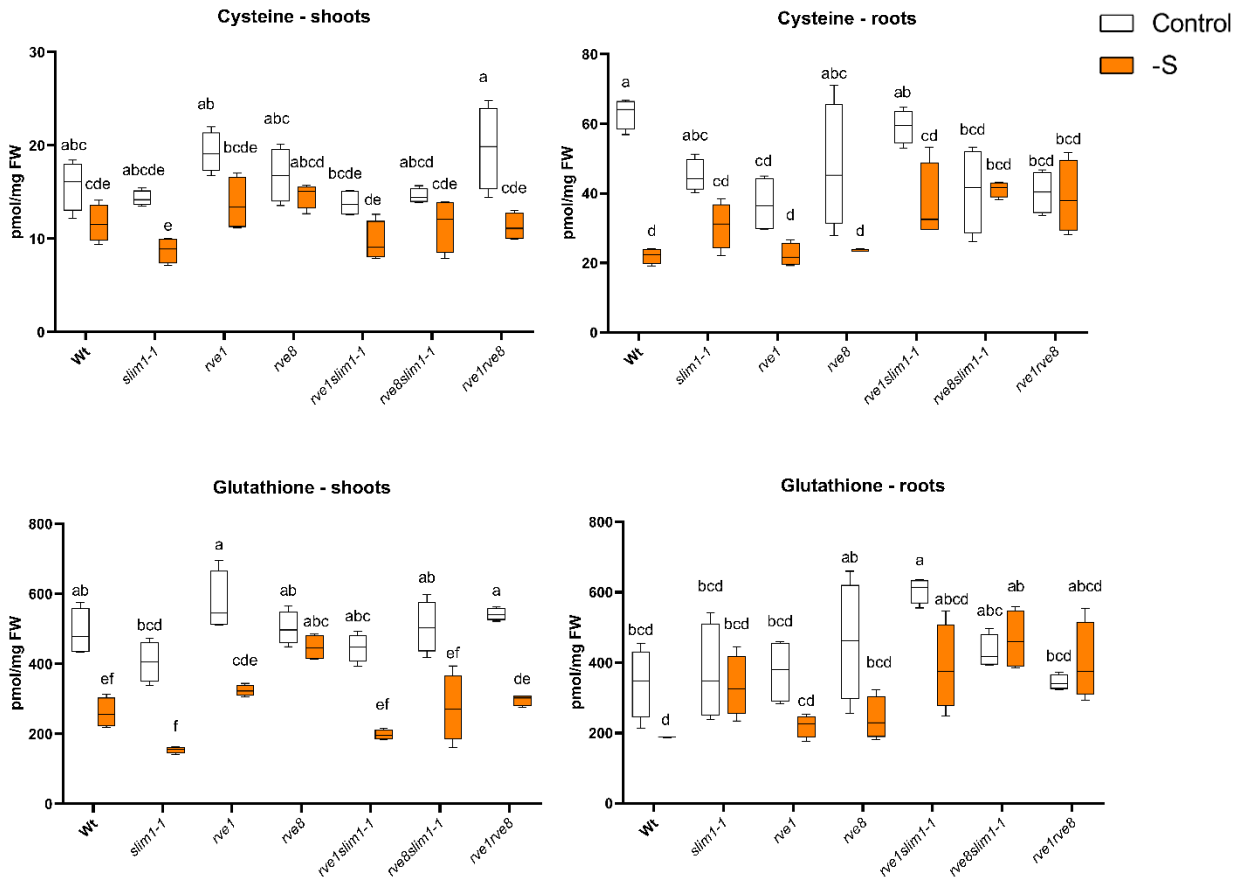

Figure 27: Low-molecular-weight content in shoot roots of WT, *rve1*, *rve8*, *slim1-1* and the respective double mutants. Control conditions (white) and sulfur deficiency (-S; orange). Data was collected from 4 biological replicates and presented as box plots showing lower (Q1) and upper (Q3) quartiles, with the line representing the mean and the whiskers 5 and 95 percentiles. Significances were calculated using a two-way ANOVA followed by TukeyHSD and letters are displayed.

Foliar cysteine levels were similar in all lines under control conditions, and the same pattern could be observed under sulfur deficiency. In plants grown on low-sulfur media, cysteine accumulated to a lesser extent, even though the changes were not statistically significant. In roots, cysteine was significantly reduced under sulfur deficiency conditions in WT, *rve8* and *rve1 rve8*. The rest of the lines had comparable levels among the two conditions. Interestingly, *rve1* and *rve8 slim1-1* showed significantly lower cysteine than WT under control conditions.

Glutathione exhibited a relatively similar pattern to cysteine, but the differences between the conditions in shoots were more substantial. It was significantly reduced in all lines but *rve8* under

sulfur deficiency. In roots, no remarkable changes were observed, so overall the low-molecular-weight thiols data did not lead to any unexpected findings.

To complement the metabolic profile, the expression levels of 5 OAS cluster genes (which, as stated before also work as sulfur marker genes) was studied under control (Figure 28) and long term sulfur deficiency conditions (Figure 29). RNA was extracted from shoots and RT-qPCR was performed.

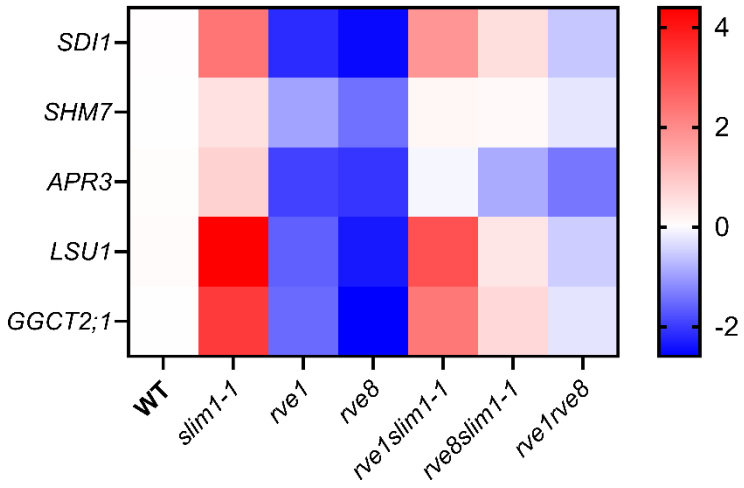

Figure 28: Relative expression of OAS cluster/sulfur marker genes in shoots of WT, *rve1*, *rve8*, *slim1-1* and the respective double mutants. This heatmap shows the log2 Fold Change of the relative gene expression of wild type and all mutants under control condition for shoots. Fold Change was calculated vs wild type under control conditions and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

While the loss of function of RVE1 and RVE8 did not affect sulfur metabolism at a metabolic level, we found interesting changes in the mutants under control conditions. The *slim1-1* mutant had higher transcript levels of *SDI*, *LSU1* and *GGCT2;1*. Both *rve1* and *rve8* showed a strong downregulation of all 5 genes. The profile of these mutants under control conditions did not match the one previously seen before the OAS treatment (Figure 24), but since the growth conditions and the organs are different, it is not entirely surprising. The study of the double mutants led to interesting findings about how they interact with each other. While *rve1* and *rve8* showed a severe downregulation of the OAS cluster genes, when combined the effect was mitigated and the transcript levels were less reduced, therefore not having an additive effect. When combining *rve1* and *rve8* mutations with *slim1-1*, the profiles are closer to the *slim1-1* mutant but again the effects

are less substantial. Also, the results suggest that the loss of function of RVE8 had a bigger impact than that of RVE1: not only the single mutant is more affected than *rve1*, but the double mutant *rve8 slim1-1* shows transcript levels similar to WT, unless the *rve1 slim1-1*.

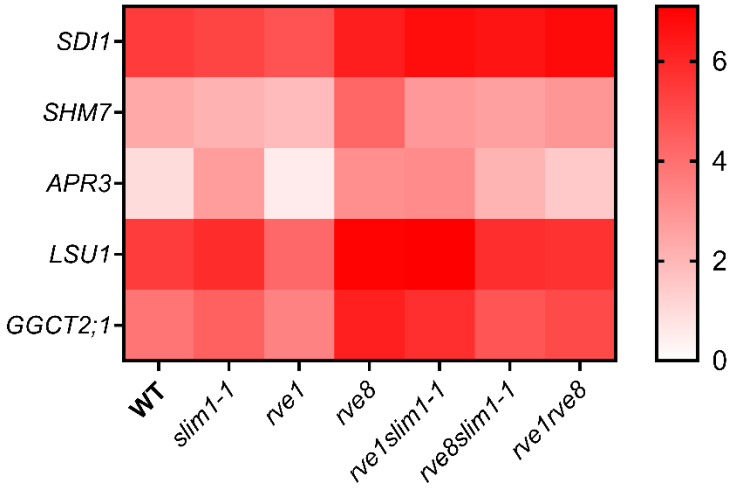

Figure 29: Relative expression of OAS cluster/sulfur marker genes in shoots of WT, *rve1, rve8, slim1-1* and the respective double mutants under sulfur deficiency conditions. This heatmap shows the log2 Fold Change of the relative gene expression of wild type and all mutants under sulfur deficiency condition for shoots. Fold Change was calculated vs wild type under control conditions and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

Despite their differences under control conditions, all the mutants had similar transcript levels of the OAS cluster genes under sulfur deficiency, which was unexpected for *slim1-1* since it is known to have an impaired response. The relative transcriptional sulfur deficiency response was different in every mutant, since they displayed different control levels of the genes, however they were all able to reach the same levels as WT.

Taken together, our results show how RVE1 and RVE8 are not directly connected to sulfur metabolism or sulfur deficiency response. Their role as regulators of the OAS cluster genes might be important in other contexts, perhaps signalling as described before, but they are not necessary to develop an adequate sulfur starvation response.

4.5 Early sulfur deficiency response varies at different times of the day

In this study, we have revealed how RVE1 and RVE8, circadian clock-related transcription factors, regulate the OAS cluster genes as well as the effect of the time of the day in the expression levels of these genes. Therefore, it was of further interest to explore the connection between sulfur metabolism and the circadian clock. As a first approach, hydroponically grown WT and *slim1-1* plants were transferred to no-sulfur-containing media for 4 hours and the expression level of OAS cluster/sulfur marker genes was evaluated in roots at different times of the day (Figure 30).

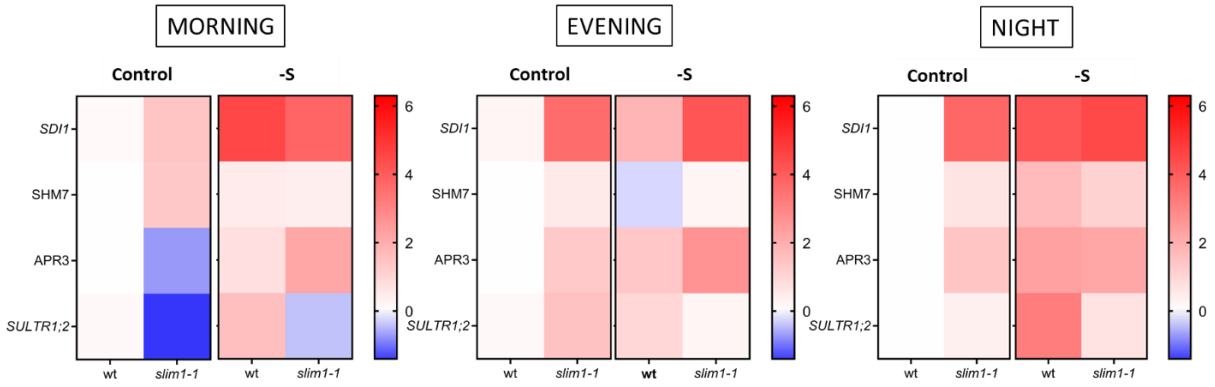

Figure 30: Relative expression of OAS cluster/sulfur marker genes in roots of WT and *slim1-1* and their sulfur deficiency response at different times of the day. These heatmaps shows the log2 Fold Change of the relative gene expression of wild type and *slim1-1* mutant under control condition and after short term (4 hours) sulfur starvation (-S) at different time points: Morning corresponds to 3 hours after subjective dawn, evening to 11 hours after subjective dawn and night to 3 hours after onset of subjective night. Fold Change was calculated vs wild type under control conditions for every time of the day and the housekeeping gene *TIP41* served as a reference. Absolute mean values were calculated using the 2-ΔΔCT method and can be found in supplemental data.

Before any treatment, the *slim1-1* mutant already showed differences compared to WT. *SDI1* was always upregulated independently of the time of the day and *SHM7* always showed a slight upregulation. On the other hand, *APR3* was slightly downregulated in the *slim1-1* mutant in the morning and slightly upregulated at the other time points, while *SULTR1;2* was severely downregulated in the morning, slightly upregulated in the evening and similar to WT in the night. These results suggest that, even though SLIM1 is a key transcription factor in sulfur metabolism, the circadian clock also plays a role in its action, since the expression of sulfur marker genes differed in the mutant. Interestingly, only 4 hours after being transferred to a media without sulfate plants already displayed a transcriptional sulfur deficiency response. However, the intensity of this

response was dependent on the time of the day, again pointing towards the important role of the circadian clock. In WT plants, *SDI1* transcript levels reached the highest levels in the morning and in the night, while the increase in the evening was very mild in comparison. *SHM7* early response was very mild in the morning, it was even slightly downregulated in the evening and showed a stronger increase in the night. *APR3* was also only slightly upregulated after the short sulfur deficiency treatment, with transcript levels increasing slightly in subsequent time points. *SULTR1;2* was mildly upregulated in the morning and evening, but in the night, surprisingly, the upregulation was substantially stronger. The *slim1-1* mutant did not show substantial differences in response to sulfur starvation when compared to WT. In general, the response of the genes was comparable to or higher than WT, with the exception of *SULTR1;2* which was always significantly lower than WT. The lack of differences in the *slim1-1* mutant provides evidence that the early events leading to the adaptation to a lower sulfur supply are SLIM1-independent.

4.6 SLIM1, RVE1 and RVE8 bind to the promoters of the OAS cluster genes

Based on published data, the transcription factors SLIM1, RVE1 and RVE8 were predicted to bind to the promoter of the OAS cluster genes. Our experiments revealed that mutations in these genes affect not only the regulation of the OAS cluster genes, but also have an impact at other levels of the OAS signalling. Therefore, it was necessary to prove experimentally that the transcription factors indeed bind the promoters of the corresponding genes. To do so, *A. thaliana* cells were co-transformed with *A. tumefaciens* containing either promoter::GUS constructs or overexpressing the transcription factors by 35S promoter, and the binding of the transcription factors to the promoters of OAS cluster genes was tested via a transactivation assay with GUS staining (Figure 31).

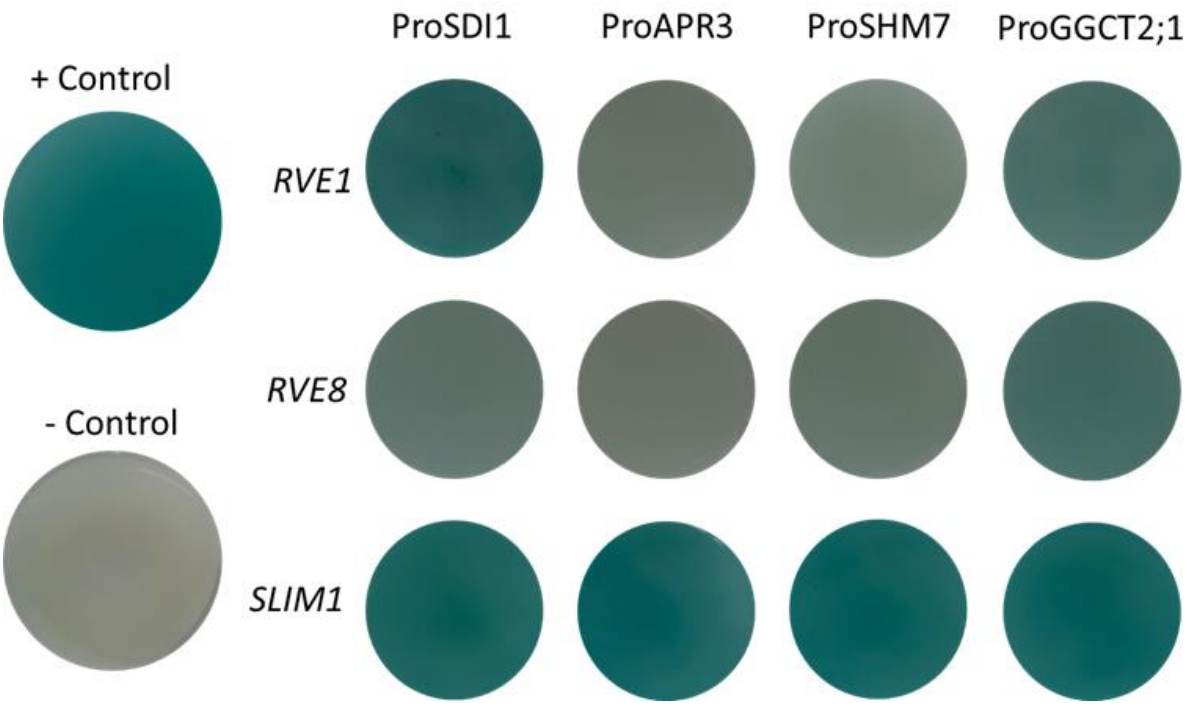

Figure 31: Histochemical GUS staining of the *Agrobacterium*-transformed cultured *Arabidopsis thaliana* cells. 35S::GUS was used as a positive control (+) and a promoter construct without transcription factors as effector was used as a negative control (-). Pictures of 1 well (out of 3 total replicates) for each experimental set-up are shown after the staining overnight. Original pictures can be found in supplemental data (Supplemental Figures S1, S2 and S3).

The transactivation assay confirmed the binding of the 3 transcription factors to the chosen representatives of the OAS cluster genes. The combination of the SLIM1 effector construct with all the promoters showed the strongest activation among the different setups (activation was visible already after 6 hours, data not shown) further supporting our previous results and the hypothesis of SLIM1 acting as the main regulator of the cluster. RVE1 and RVE8 binding to the promoter of these 4 OAS cluster genes was also confirmed, however the intensity differed. Both of them bound more strongly to the promoters of *SDI1* and *GGCT2;1* and only slightly activated the promoters of *APR3* and *SHM7*. They also showed individual differences, with RVE1 showing a stronger activation of the promoter of *SDI1* than RVE8.

Confirming the binding of these transcription factors to the promoter of the OAS cluster genes provided some valuable insight on the regulation of these genes and further validated our results, however questions such as differential activation during different times of the day (considering

that RVE1 and RVE8 are circadian-clock related transcription factors) or the effect of potential other factors remain to be explored.

5 DISCUSSION

Our study aimed to unravel the function of OAS and propose a model for its signalling (Figure 32). In an attempt to dissect it, we studied the mechanisms leading to OAS accumulation, such as sulfur starvation and transition to darkness, to reveal the importance of every SERAT isoform in these processes. Perturbations in carbon, nitrogen and sulfur metabolism also helped us understand the central and connecting role of OAS. The delay between OAS accumulation and the induction of the OAS cluster genes suggests the involvement of transcription factors in translating the accumulation into a transcriptional signal, therefore several candidates were identified and tested. The downstream processes affected by the OAS cluster genes were initially out of the scope of this study, however, preliminary data generated points towards a connection to ABA and potentially other hormones.

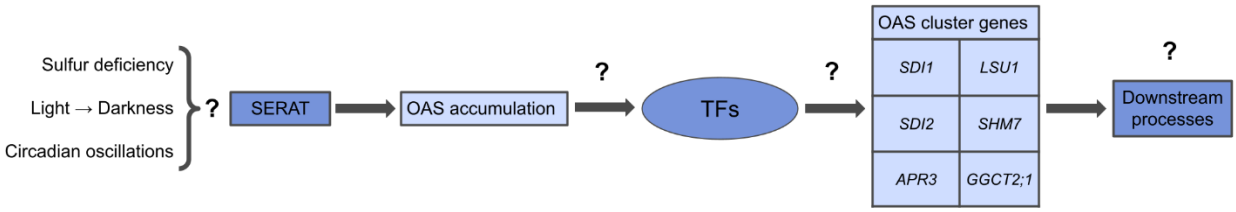

Figure 32: Open questions in the model for OAS signalling. Several conditions lead to OAS accumulation. After a delay, the OAS accumulation is followed by an induction of the OAS cluster genes. The (expected) transcription factor(s) involved in translating the OAS accumulation into a transcriptional signal is(are) unknown. The induction of the OAS cluster genes leads to an impact in downstream processes, yet uncharacterized.

5.1 OAS, a connection between carbon, nitrogen and sulfur metabolism

Unlike animals, which can actively search for food sources, plants are sessile organisms. Therefore, they have developed efficient nutrient acquisition and regulatory mechanisms to ensure their survival and growth. Adaptation to nutrient availability and integration between the different pathways are key elements for plant fitness, and the intricate connection between sulfur and nitrogen metabolism is a good example. It is known that lack of nitrogen decreases the activity of ATPS and APR, thus reducing the sulfur flux into both primary and secondary metabolism. Conversely, the addition of nitrogen in the form of nitrate or ammonium quickly restored the

activity of both enzymes (Reuveny et al., 1980; Brunold & Suter, 1984). Our results further validated this data in the model plant *A. thaliana*, since we could show how the expression of sulfur marker genes was downregulated in plants grown under nitrogen deficiency conditions (Figure 9). Apart from nitrate and ammonium, other nitrogen sources can also restore the sulfur pathway after nitrogen starvation. Interestingly, OAS was the most efficient among them (Koprivova et al., 2000). OAS provides the carbon and nitrogen backbone to incorporate sulfur into organic molecules, which makes it a good candidate to act as a signal connecting and adjusting the pathways to the current nitrogen status. We did not observe changes in OAS contents in roots in our depleting and restoration of nitrogen supply experiment (Figure 11). However, the fact that no changes were found in this particular set-up are not sufficient to rule out the potential role of this compound. In fact, during our long-term starvation studies, plants grown under nitrogen deficiency accumulated OAS in the shoots (Figure 6). Therefore, the pool of OAS could be acting as a long-term mediator between the pathways instead of as a quick signal to trigger changes. This idea is consistent with the current knowledge about how OAS accumulates in plants grown under long-term sulfur deficiency (Hirai et al., 2003; Nikiforova et al., 2003; Figure 6). Our results, together with previous literature, seem to support the hypothesis of OAS accumulating for reasons beyond just lack of sulfur and inability to form cysteine. However, the question of whether that accumulation is a consequence of an upstream signal and/or a trigger for further changes remains unknown.

Carbon and sulfur metabolism are also tightly connected in plants. Being the most important non-mineral element, carbon metabolism plays a central role in every living being, including plants. In photosynthetic organisms, its regulation seems to be even more complex. In fact, several sugars such as glucose or sucrose not only act as metabolites and energy sources, but they also have hormone-like functions and influence processes like germination, development or senescence (reviewed in Li et al., 2021). It has been long known that carbohydrates can influence sulfate assimilation. In 1999, Kopriva and colleagues demonstrated how APR activity and expression levels (of all three isoforms) were highly induced after adding sucrose to the media, suggesting that a higher sugar content results in an improved sulfur flux towards the primary pathway. Moreover, maintaining physiological levels of sulfite, an intermediate in the sulfur assimilation pathway that can be toxic if it accumulates, seems to be more important than just a simple detoxifying process since the activity of the sulfite oxidase is necessary to maintain a proper

balance between carbon and sulfur metabolism (Oshanova et al., 2021). In this study, we depicted how three different sucrose-containing media affected not only OAS accumulation, but also the magnitude of induction of the OAS cluster genes. Our data provides a new link between sugars and an important precursor in sulfur metabolism. It also shows how the changes in sugars content can dynamically impact OAS and the OAS cluster genes, as our set-up contained plants grown under no sucrose, grown under sucrose during the whole process and only germinated under sucrose and then transferred to no-sucrose media. Since we found differences in the three conditions, it suggests that the communication between sucrose and OAS is dynamic, which is logical considering the non-static nature of light and photosynthesis. Additionally, OAS can be directly connected to carbon metabolism via its precursor, serine. In plants, serine can be synthesized by three different pathways: photorespiration, phosphorylated pathway of serine biosynthesis (PPSB) and glycerate pathway. Serine produced by photorespiration is the dominant precursor for OAS in photosynthetic tissue (Samuilov et al., 2018a). However, perturbations in the PPSB pathway produce alterations in sulfate uptake and fluxes (Samuilov et al., 2018a, 2018b; Anoman et al., 2019). Due to the complex interconnection between carbon, nitrogen and sulfur metabolism, it is sensible to consider the existence of many layers connecting the different pathways, as we have observed in the literature and the results from this study. Therefore, despite the several hints towards OAS, it is most likely the action of many molecules and different levels of regulation what keeps an adequate balance between the major nutrient pathways.

5.2 The SERAT family confers an evolutionary advantage

In Arabidopsis, duplication of chromosomal segments has occurred several times, providing a source of evolutionary novelty. A comparative genomics study indicated that such was the origin of the SERAT family, strongly supported by the fact that they are conserved in many other plant species (Watanabe et al., 2008b). Furthermore, the presence of SERAT isoforms in all compartments (cytosol, mitochondria and plastids) brings the possibility of differential regulation and additional functions to individual isoforms. Previous studies have stated that the mitochondrial isoform (SERAT2;2) is the most efficient one and provides the majority of OAS in *A. thaliana* (Haas et al., 2008; Watanabe et al., 2008b). However, the plastidic isoform SERAT2;1 was proven to be a key element in stress acclimation. During oxidative stress, SERAT2;1 is (redox) activated

via CYP20-3 to produce OAS and ensure the availability of precursors for glutathione synthesis (Domínguez-Solis et al., 2018). Besides, the cytosolic isoform SERAT3;2, presumed to have little to no contribution to OAS formation *in vivo* (Watanabe et al., 2008b), was found to be induced under sulfur deficiency and cadmium stress (Kawashima et al., 2005), also confirmed in our study (Supplemental Table S4).

Despite individual functions being associated to single isoforms, the viability of every quadruple *serat* mutant implies that every isoform is efficient enough to produce the necessary OAS for survival (Watanabe et al., 2008b, 2018). Survival of a quintuple *serat* mutant was not possible, making evident that the only way to produce OAS is via SERAT (Watanabe et al., 2008b). Further supporting how robust this system is, our deficiency study with the quadruple mutants confirms that only one isoform of SERAT is enough for the plant to survive under stressful conditions such as sulfur or nitrogen starvation. However, the differences found in the different metabolites among the mutants and different conditions show how every isoform behaves distinctly, differentially sensing the nutrient status of the plant and also affecting the concentration of downstream metabolites. According to our data, the *q3;1* mutant is the most affected one, with the lowest levels of almost all metabolites and under all conditions. These results are consistent with similar studies (Watanabe et al., 2008b, 2018) and support the idea of SERAT3;1 not being very relevant for OAS production due to its low activity and tissue expression (Kawashima et al., 2005). On the other hand, the data obtained from the *q3;2* mutant appears to be contradictory. While previous studies reported that the SERAT3;2 isoform has little relevance in OAS production *in vivo* and the corresponding quadruple mutant is severely affected, our results show otherwise. With higher levels of OAS under control conditions and mimicking or even surpassing WT in almost all metabolite levels under the different conditions, the *q3;2* mutant seems to be rather insensitive to the nutrient status of the plant. Knowing how this isoform is upregulated under stress conditions (Kawashima et al., 2005), it could be expected that the lack of other SERAT isoforms might induce the expression of SERAT3;2. Surprisingly, we did not find that (Supplemental Table S4) but the differences could be explained based on different media and growing conditions. It has been shown before that differences in growth conditions (soil/agar, light intensity and duration, etc.) have a massive impact in the transcriptional and metabolic profiles of the mutants (Watanabe et al., 2018). Our data also shows substantial differences in OAS accumulation in roots when comparing plants grown in agar and hydroponic plates (Figures 6 and 11).

Examining the transcriptional regulation of the different isoforms in the mutants did not bring clear conclusions. Therefore, the action of other mechanisms could help understand how each individual isoform is regulated. When studying the SERAT enzyme, it is essential to consider how it works in the context of the CSC. Successful cysteine synthesis requires the sequential reactions catalyzed by SERAT and OAS-TL, working together to form the hetero-oligomeric CSC. Computational data suggest that the complex contains SERAT dimers or trimers and a homodimer of OAS-TL. To be functional, SERAT needs to be bound to OAS-TL and, conversely, OAS-TL needs to be free to be active (reviewed in Wirtz & Hell, 2006). The localization of both enzymes implies the formation of CSC in every compartment. Sulfide promotes the formation of CSC, while OAS disassociates the complex and allows cysteine formation. However, only two cytosolic SERAT isoforms (SERAT1;1 and SERAT3;2) display feedback inhibition by cysteine (Watanabe et al., 2008b). In principle, OAS and cysteine can be freely transported in and out of the different compartments to maneuver around the inhibitory mechanisms, providing each compound where necessary. Besides, post-translational modifications seem to affect feedback inhibition (Wirtz & Hell, 2006). Additionally, the presence of three different SERAT isoforms in the cytosol allows the formation of several combinations of CSC, unlike in plastids or mitochondria. The different possibilities could indicate that the CSC might have yet undiscovered regulatory functions, which could be interesting to explore. Therefore, the knock-out of 4 out of the 5 SERAT isoforms, as observed in our results, might have consequences beyond what could be logically expected. Last, but not least, OAS-TL is known to physically interact with SULTR1;2 (Shibagaki & Grossman, 2010) and, while the role of this complex as a sulfur sensor has been generally dismissed, further studying this interaction and the role of OAS in the stability could shed some light on a potential regulator of sulfur metabolism.

Taken together, the CSC has proven to be a sophisticated regulatory complex that allows the synthesis of both compounds to be finely tuned not only in response to the plant demands but also more specifically in response to every compartment requirement. However, while recent literature and this study have raised concerns about the vital function of OAS and cysteine transporters, it is a topic that has not been properly addressed in plants. Transport between compartments, as stated before, might be essential not only in the context of sulfur metabolism but also for many other potential regulatory functions. Therefore, future research should be carried out to characterize these transporters and establish their functions in plants.

5.3 OAS acts as a signalling molecule, not simply a metabolite

Nutrient signalling is one of the most fundamental mechanisms to modulate cellular activities and organismal development, integrating internal and environmental changes. Originally, it was thought that nutrients automatically feed into cellular metabolism and growth. Contrary to the initial belief, they are also part of complex signalling mechanisms, participating in the control of a plethora of cellular and physiological functions. Attributing a dual function to nutrients is a very elegant and efficient manner to allocate resources, preventing the use of energy and metabolites for the generation of molecules that would solely have a signalling purpose. Furthermore, plants have evolved and adapted in a way that they can use even toxic compounds as signalling molecules. ROS are highly reactive and can be detrimental, but they allow the plant to properly react to different stimuli and activate stress-response networks, thus contributing to the establishment of defense mechanisms (reviewed in Mittler et al., 2022). Hydrogen sulfide, an intermediate in the sulfur assimilation pathway (Figure 1), was always considered a toxic gas and environmental hazard but recent research has confirmed that it is part of important signaling pathways. The most known is persulfidation, a form of post-translational modification that impacts protein activities, structures, and subcellular localization that happens to one out of every twenty proteins in Arabidopsis, suggesting a significant role (reviewed in Aroca et al., 2018)

Regarding nutrients, sugars, as described before, are a perfect example of regulation of not only their own pathways but many more. Another interesting group is amino acids, well known for their ability to not only be metabolized but also influence gene expression in plants. For instance, glutamine rapidly induces the expression of genes involved in nitrogen metabolism and stress responses (Kan et al., 2015), while serine affects the transcript accumulation of photorespiration-associated genes (Timm et al., 2013). OAS has long been discussed in the literature and this study as a signalling molecule, both as sulfur status dependent and independent. Our work provides further evidence about OAS not being just a metabolite. We could observe that the metabolic profile of the quadruple SERAT mutants did not match their respective transcriptional profile. While the *q3;2* mutant had a similar metabolic profile to WT, the most similar transcriptomic profile was from *q1;1*. This, together with the fact that OAS concentration was not vastly different among the mutants, suggests that OAS is not just a metabolite acting as a precursor for cysteine. Our results support the idea of OAS as a signalling molecule and suggest that the compartment where it is produced is a key part of it. Relatively similar amounts of OAS (Figure 6) led to

significantly different transcriptional profiles (Figures 9 and 10). If OAS is, indeed, working as a signal, it is sensible to assume the existence of an OAS sensor. The differences found between OAS produced in plastids, mitochondria and cytosol might suggest the existence of different sensors in every compartment. Consequently, the CSC comes up as a potential candidate, due to the effect of OAS in its stability, and its presence along the different compartments (reviewed in Wirtz & Hell, 2006). The hypothesis of the CSC as the main sulfur sensor is not strongly supported, however, it could still act as an OAS sensor and contribute partially. Different combinations of CSC in every compartment could explain the differences found in the transcriptional profiles of the quadruple mutants. This idea would be further supported if we consider the importance of plastid-synthesized OAS to deal with stress acclimation (Domínguez-Solis et al., 2018), despite the hypothetical free transport of OAS and cysteine among the different organelles. The fact that these compounds can be transported in order to assure survival (Watanabe et al., 2008b, this study) while specific processes are heavily affected by the mutation of a single isoform reveals the existence of very complex multilayer regulatory networks. Nevertheless, the CSC hypothesis alone cannot answer the question of how OAS accumulation is translated into a transcriptional signal affecting genes in the sulfur pathway.

While the main question about OAS signalling remains unanswered, this study could provide new information. Increased OAS accumulation in plants grown under sulfur deficiency conditions is widely known (Hirai et al., 2003; Nikiforova et al., 2003), however, our study is the first one exploring its regulation under low nitrogen conditions. We could observe that OAS accumulated significantly in the shoots of plants grown under nitrogen deficiency. The same pattern could be observed in roots but the accumulation was, in general, lower than under sulfur deficiency. Contrary to expectations, the OAS accumulation in nitrogen-starved plants did not lead to an induction of the expression of any of the sulfur marker/OAS cluster genes. In fact, the transcript levels were significantly lower than under control conditions. While this finding is contradictory to what we know about OAS accumulation and the consequential sulfur marker genes induction, the fact that they are downregulated is consistent with the literature. Nitrogen is the most important mineral nutrient and a limiting factor in plant growth, therefore under low nitrogen supply plants reduce their sulfur assimilation (Reuveny et al., 1980; Koprivova et al., 2000). Our results are in line with those of previous studies and provide additional information about OAS signalling, indicating the action of a more robust signalling system capable of suppressing the effect of OAS

accumulation in order to adjust the sulfur pathway to the corresponding nitrogen supply. With our current knowledge, it is only possible to make assumptions, but a plausible candidate could be TOR, given its central role in nutrient sensing and regulation and the already-established connections with nitrogen and sulfur (Li et al., 2021; Yu et al., 2021).

5.4 Transcriptional regulation of the OAS cluster genes

The identification of the OAS cluster genes through a systems biology approach by Hubberten and colleagues (2012) unequivocally proved the role of OAS as a signal. It is important to notice the relevance of this work, since usually it is not easy to correlate gene expression with changes in a single metabolite. Through their approach, they found the expression of these 6 genes connected directly to OAS accumulation and no other sulfur-containing or related compound. The analysis of publicly available data revealed that these 6 genes are co-expressed (Obayashi et al., 2022), not only in response to endogenous and exogenous OAS (Hirai et al., 2003; Hubberten et al., 2012), but also in plants grown under sulfur deficiency (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020) and under oxidative stress (Ristova & Kopriva, 2022). The functions of the proteins encoded by these genes are directly connected to sulfur metabolism: SDI1 and SDI2 downregulate glucosinolates biosynthesis in response to sulfur limitation, APR3 is a key enzyme in sulfate assimilation, GGCT2;1 participates in glutathione recycling, SHM7 is necessary for epigenetic regulation in response to sulfur starvation and although the function of LSU1 is not fully elucidated, its expression is highly upregulated in response to sulfur starvation. However, the different contexts in which they are co-expressed together with previous studies point towards a role that likely goes beyond the sulfur status of the plant, perhaps connecting and adjusting the sulfur assimilation pathway in response to different stimuli.

Despite the lack of knowledge about the detailed functions of all the OAS cluster genes, our work aimed to provide new insights into the regulation. The original experimental set-up that led to the identification of the cluster procured an excellent system to study the regulation of the OAS cluster genes and their connection to OAS. When transferring plants from light to darkness, there is a transient accumulation of OAS and after a short delay the expression of the cluster genes is induced (Caldana et al., 2011; Hubberten et al., 2012). Since there is a time gap between the OAS accumulation and the gene induction, we hypothesized the action of transcription factors involved

in the sensing of the metabolite signal and further translation into a transcriptional signal. Due to their co-expression under different conditions, it is sensible to assume that the cluster genes are co-regulated. Therefore, the online tool Plant Regulomics (Ran et al., 2020) was used to find transcription factors predicted to bind to the promoters of all OAS cluster genes. Among the obtained candidates (Supplemental Table S8), we selected SLIM1, RVE1 and RVE8 for further analysis. Due to the sulfur-related nature of the OAS cluster genes, it is not surprising to find SLIM1 among the predicted transcription factors since it is considered the main transcription factor in sulfur deficiency (Maruyama-Nakashita et al., 2006). Interestingly, the expression of *SDI2* and *APR3* under control and sulfur deficiency conditions was not affected by its mutation (Dietzen et al., 2020). On the other hand, no previous connections have been established between RVE1 or RVE8 and sulfur metabolism or the OAS cluster genes, however, they offer a logical connection to the circadian clock considering how light and circadian regulation affect sulfur metabolism and these genes (Kopriva et al., 1999; Caldana et al., 2011; Hubberten et al., 2012; Hornbacher et al., 2019).

Remarkably, when the transition from light to darkness was conducted with mutants from these transcription factors, none of them showed an induction of the OAS cluster genes (Figure 16). Our results supported the initial hypothesis of transcription factors being involved in the regulation of these genes and we could directly find three positive candidates. Moreover, the binding of these three transcription factors was tested with the promoters of *SDI1*, *APR3*, *SHM7* and *GGCT2;1* and it was confirmed for all of them (Figure 31). SLIM1 showed the strongest activation among all the combinations, while RVE1 and RVE8 activated all of them but the intensity varied. The mutation of only one transcription factor prevented the transcriptional activation of the OAS cluster genes after transitioning to darkness, suggesting that they might work together. This claim is further supported by the expression changes of *RVE1* and *RVE8* after the transition, both being downregulated after 40 minutes (Figure 18), together with a compensation effect in the *rve1* and *rve8* mutants, with *rve1* having lower transcript levels for *RVE8* and *rve8* showing higher ones for *RVE1*. *SLIM1*, on the other hand, was only slightly downregulated in the WT background but not in any of the other lines, suggesting that transcriptional regulation is not how this gene is regulated in this context, in line with previous studies about SLIM1 regulation under sulfur deficiency (Maruyama-Nakashita et al., 2006).

Surprisingly, the analysis of the mutants unveiled that transcriptional activation was not the only element that was affected, but also the accumulation of OAS. None of the mutants displayed the transient OAS increase after 5 minutes (Figure 17), suggesting that they might also work upstream of OAS production in this signaling cascade. In the case of *slim1-1*, it could be argued that OAS production is generally affected by the mutation in the transcription factor since OAS accumulation is significantly higher than the rest of the lines (Figure 17) and also because the SERAT3;1 isoform is under SLIM1 control (Maruyama-Nakashita et al., 2006). Regarding RVE1 and RVE8, no previous connection has been established to OAS but a quick analysis of the first 1000 bp of the promoter region of all *SERAT* genes revealed binding sites for both transcription factors in the promoters of *SERAT2;1*, *SERAT2;2*, and *SERAT3;2* (Chow et al., 2019). While the binding needs to be confirmed, this prediction is in line with our results. General OAS levels were not affected in *rve1* or *rve8* (Figure 17), but the function of these transcription factors is likely to be context-dependent. Moreover, OAS accumulation led to the downregulation of both transcription factors (Figure 25), implying the action of a feedback mechanism and providing additional support to their potential implication upstream of the OAS accumulation.

Despite their essential role in the transcriptional activation of the OAS cluster genes after transitioning to darkness, we could not answer the question of whether SLIM1, RVE1 and/or RVE8 are involved in translating the OAS accumulation into a transcriptional signal, due to the absence of the transient OAS increase (Figure 17). Therefore, a different approach was taken and experiments with exogenous OAS were conducted with the mutants. As expected from the literature, the addition of exogenous OAS led to the induction of sulfur marker genes (Hirai et al., 2003) and, among them, the OAS cluster genes (Figure 24). Interestingly, the mutants already showed differences in the expression levels compared to WT prior to the treatment, providing additional evidence of the role of these transcription factors in their regulation and suggesting that they might be necessary to maintain basal levels as well (Figure 24). The knockout of *RVE1* or *RVE8* did not have any effect on how the plant responded to OAS, therefore they were ruled out. However, the response in the *slim1-1* mutant surpassed significantly that of WT, indicating that under physiological conditions SLIM1 might be acting as a repressor of the OAS signal. Exogenous OAS did not affect the expression levels of *SLIM1*, in accordance with the lack of changes in its mRNA levels under sulfur deficiency (Maruyama-Nakashita et al., 2006), which is logical considering the substantial overlap between the transcriptional profile of sulfur starved

plants and plants treated with exogenous OAS (Hirai et al., 2003). Interestingly, preliminary data generated by our collaborators suggests that OAS can stabilize SLIM1 protein (Sirko & Wawrzyńska, unpublished). Given the crucial role of SLIM1 in sulfur deficiency response (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020) and since OAS accumulates under sulfur starvation it is likely that one of its functions is to maintain a controlled transcriptional response of the sulfur marker genes in response to the OAS accumulation. Nevertheless, while a new connection has been established between two important elements in sulfur metabolism, the question of how the OAS accumulation is translated into expression changes remains unanswered but studying other transcription factors predicted to bind to the promoters of all the OAS cluster genes (Supplemental Table S8) might be a good starting point.

5.5 Circadian regulation of sulfur metabolism

To compensate for their sessile nature, plants have developed a robust circadian clock regulatory network in order to prevent and cope with environmental and diurnal changes. Proper coordination between daily and seasonal changes and cellular processes helps plants attain fitness, growth and development. In recent years, knowledge about the Arabidopsis circadian clock has grown significantly, with special interest in the connection to metabolism. Light is one of the main inputs for the endogenous clock and necessary for photosynthesis, therefore it is logical that carbon metabolism is regulated by the circadian clock. Many genes along the pathway display rhythmic expression patterns and/or are directly regulated by circadian clock transcription factors (reviewed in Venkat & Muneer, 2022). Simultaneously, sugars are able to, in turn, regulate many circadian clock related genes and transcription factors (Haydon et al., 2013). Another interesting example can be found in nitrogen metabolism, with nitrate reductase exhibiting diurnal expression and activity oscillations and glutamine or glutamate directly regulating *CCA1* expression, which in turn regulates many nitrogen assimilation genes (reviewed in Cervela-Cardona et al., 2021).

The circadian regulation of sulfur metabolism has been long established. Sulfate transporters and APR are controlled by light and circadian oscillations (Kopriva et al., 1999; Hornbacher et al., 2019) and the flux into thiols, proteins and glucosinolates is dependent on the time of the day (Huseby et al., 2013). Genetic perturbations in the glucosinolate pathway altered the length of the circadian period and the expression levels of core circadian clock genes such as *CCA1, PRR7* and

GI (Kerwin et al., 2001) and comparably, our results show how OAS can affect the expression of *RVE1* and *RVE8*. Additionally, binding sites for transcription factors like CCA1 and LHY are present in many genes along the sulfate assimilation pathway (Ran et al., 2020). In this study, we confirmed the binding of RVE1 and RVE8 to the promoters of *SDI1*, *APR3*, *SHM7* and *GGCT2;1* (Figure 31). Moreover, we proved how they are essential for the induction of the OAS cluster genes upon transitioning from light to darkness (Figure 16). Besides, the knock-out of *RVE1* or *RVE8* led to the downregulation of the OAS cluster genes under control conditions (Figure 28). This effect seems to be more prominent in shoots (Figure 28) than in roots (Figure 24), consistent with the expression pattern of both genes in the two organs (Supplemental Figure S4). Under control conditions, these transcription factors might be necessary to maintain an appropriate expression level of these genes. However, sulfur deficiency response or sulfur-containing metabolites were not affected in the *rve* mutants, revealing a dissociation between transcriptional regulation and metabolic impact (Figures 26,27,28 and 29). Perhaps subjecting the plants to a constant light regime would result in a greater effect at a metabolite level in the mutants, but that remains to be tested.

In 2012, Hubberten and colleagues (using the data from Espinoza et al., 2011) showed how the expression of the OAS cluster genes follows a circadian pattern. OAS concentration also followed this rhythm and it correlated very well with the expression levels of the cluster genes. In our study, we focused mainly on the night events, since the original study showed a peak in expression and OAS content in the middle of the night. While our results did not match with the original, perhaps due to a different experimental set-up, the observed expression pattern consistently matched the OAS concentration (Figures 19 and 20). In this context, RVE1 and RVE8 are not involved in controlling the expression levels, potentially due to their significantly lower expression levels during the night (Figure 21). SLIM1, again, appears to be involved since the expression pattern of the *slim1-1* mutant is different from the rest of the lines (Figure 19). Further evidence of the strong connection between the OAS cluster genes and the circadian clock could be found through the analysis of their expression levels at the beginning of the night. The transition from day to night led to the induction of all the OAS cluster genes but *APR3*. Similar to what was previously observed, the *rve* mutants exhibited a comparable pattern to WT while *slim1-1* did not show any induction (Figure 22). Altogether, SLIM1 emerges as the potential main regulator of the OAS cluster genes.

Not only the OAS cluster genes showed a circadian expression pattern, but also OAS accumulation (Espinoza et al., 2011; Hubberten et al., 2012). While our work only focuses on the changes during the night, we could observe the oscillations. Perhaps even more interesting is the fact that OAS accumulates after a sudden transition from light to darkness, a mechanism very likely to be regulated, at least partially, by the circadian clock. This claim is supported by the influence of light and the essential role of the circadian-clock-related transcription factors RVE1 and RVE8. In addition, OAS is known to accumulate under high light as well, although that could also happen in response to ROS accumulation (Speiser et al., 2015). Our initial hypothesis was that only one SERAT isoform was responsible for the synthesis of OAS in this particular event. However, the transcriptional data points towards the possibility of all isoforms being able to achieve it, with SERAT2;2 (mitochondrial isoform) as the main contributor (Figures 12 and 13). These results, despite contradicting the initial hypothesis, provide further evidence about the relevance of OAS transporters. Due to technical difficulties and the quick nature of the changes, no clear data could be obtained about OAS concentration, so this should be a priority task in the near future. Nevertheless, the fact that all the quadruple *serat* mutants had induced expression of the OAS cluster genes after the transition could have two explanations: (I) every SERAT isoform is able to synthesize OAS in response to a transition to darkness or (II) OAS accumulation is correlated with the OAS cluster genes expression in this condition, but it is not the cause of it. The lack of reliable OAS data for the quadruple *serat* mutants does not allow the distinction between correlation and causality between these two events. Therefore, as stated before, generating robust data is indispensable. Regardless, the connection between OAS production and circadian clock has collected strong evidence and one of the next steps to further validate it could be the confirmation of the binding of circadian-clock-related transcription factors to the promoters of the *SERAT* family. This, of course, could be done as well for many other genes along the sulfate assimilation pathway. Our study also provided new insights on the regulation of SLIM1. The transcript levels of this transcription factor are not affected by sulfur deficiency (Maruyama-Nakashita et al., 2006; Dietzen et al., 2020) and post-translational modifications are suggested as the regulation mechanism in this context, however, we could observe how *SLIM1* expression changed during the night (Figure 21), adding an extra layer in the already complex regulation mechanisms of SLIM1.

Due to the evidence connecting the circadian clock and sulfur metabolism, we hypothesized that the response to sulfur deficiency could also be dependent on the time of the day. Since metabolites,

redox state and expression levels oscillate following circadian patterns, it is sensible to assume that the sensing of the sulfur status as well as the signalling and response to sulfur limitation might work differently according to the time point. The massive reprogramming that the plant suffers when coping with long-term sulfur deficiency (Nikiforova et al., 2003; Nikiforova et al., 2005; Dietzen et al., 2020) could dilute the impact of the circadian clock in the response, therefore we decided to study the early transcriptional response to sulfur deficiency in the subjective morning, evening and night (Figure 30). Our results corroborated our initial hypothesis, as we found that the intensity of the gene induction was significantly different at the three selected time points. Perhaps even more interesting, we also found that SLIM1 did not play any role in this early response. While essential for the adaptation to long-term sulfur deficiency, the early events reprogramming the plant transcriptome to cope with sulfur limitation are SLIM1-independent.

The complex regulation mechanisms of SLIM1, together with the context-dependent function of this transcription factor as well as RVE1 and RVE8, might suggest the action of protein-protein interaction. While the study of the double mutants containing mutations in *SLIM1*, *RVE1* and *RVE8* in the context of long-term sulfur deficiency did not bring any clear conclusions, the possibility that these transcription factors are working together cannot be dismissed. It would be interesting to explore this possible connection using techniques like yeast two-hybrid or split luciferase to help elucidate the concrete functions of these regulators.

5.6 Conclusions and outlook

Our study was able to shed some light on the open questions in sulfur signaling and more specifically in OAS signaling (Figure 33). We were able to show how all the SERAT isoforms are able to contribute to the transition from light to darkness while the mitochondrial one, SERAT2;2, seems to be the major enzyme. However, this claim needs further validation through reliable OAS data. Additionally, studying mutants impaired in the different serine biosynthesis pathways would procure valuable knowledge, revealing which pathway is important for the provision of serine to fuel the increase of OAS. With our deficiency experiments, we could provide new knowledge on the connection of sulfur and nitrogen metabolism, with OAS potentially adjusting them but likely not as the main regulator. OAS accumulation under long-term nitrogen deficiency but not under short-term depletion and resupply points towards a role in long-term adaptation rather than a signal

triggering quick changes. Moreover, despite the theoretically free transport of OAS and cysteine, local production in every organelle proved to be biologically relevant. Therefore, further research on the different combinations of CSC as potential sensors of OAS should be conducted together with the characterization (especially functionally) of the OAS/cysteine transporters in plants.

We identified RVE1, RVE8 and SLIM1 as transcription factors which directly regulate the OAS cluster genes, both their induction by light-dark transition and under control conditions and their binding to the candidate genes selected was confirmed. Furthermore, we found that they were also involved in the signalling upstream of the OAS accumulation, a claim further supported by the fact that *RVE1* and *RVE8* expression levels are feedback inhibited by OAS. Confirming the binding of RVE1 and RVE8 to the predicted *SERAT* promoters would provide additional evidence. Unfortunately, the question about the OAS sensor and how the accumulation is translated into a transcriptional signal could not be fully answered. RVE1 and RVE8 were not involved in OAS signalling, but, remarkably, SLIM1 appears to work as a repressor of the transcriptional signal, regulating the intensity of the response. While the original question needs to still be addressed, our results contributed new knowledge on the interaction between SLIM1 and OAS, two key elements in sulfur metabolism. Several other transcription factors predicted to bind to all the OAS cluster genes remain to be studied, and their future analysis might provide new and useful insights on the cluster regulation as well as OAS signalling.

The physiological role of the induction of the OAS cluster genes remains a mystery, but our data and the literature imply a potential role as a central regulator of nutrient pathways (mainly sulfur) in response to different biotic and abiotic stresses. The similarities found between the sudden transition to darkness and the natural day to night shift, together with the multiple conditions in which these genes are co-expressed, point towards a conserved and important adaptive function of the OAS cluster genes. The dissection of the individual functions of the cluster components will help elucidate the biological role of this group of genes, since the connection to different nutrient and hormone pathways provides a plethora of downstream processes to be affected. In fact, preliminary data suggest that ABA plays an important role in OAS signalling, since mutants in the ABA pathway show impaired induction of the OAS cluster genes. Additionally, the study of different combinations of mutants in the OAS cluster genes would allow the identification of processes controlled by them.

This study gave us the opportunity to explore the already known connection between the circadian clock and sulfur metabolism. While RVE1 and RVE8 binding to the tested OAS cluster genes was confirmed, they only appear to have an impact at a genetic level that is not translated into metabolite changes. OAS, as many other metabolites, is capable of directly influencing the circadian clock affecting the expression of *RVE1* and *RVE8*, therefore it would be interesting to evaluate the impact at other levels, for example the length of the circadian period. Besides, the early transcriptional response to sulfur deficiency was dependent on the time of the day and, perhaps even more interesting, this event was SLIM1-independent. Of course, this is only one layer of the response, and the analysis of metabolite and flux changes will give a better idea and understanding of the whole picture, as well as provide more insights on the biological implication of these findings.

In summary, our study tackled crucial questions in sulfur metabolism. Our work provided new knowledge and understanding and our contribution also laid the foundation for future research on the different connections to sulfur metabolism, with special interest in the circadian clock.

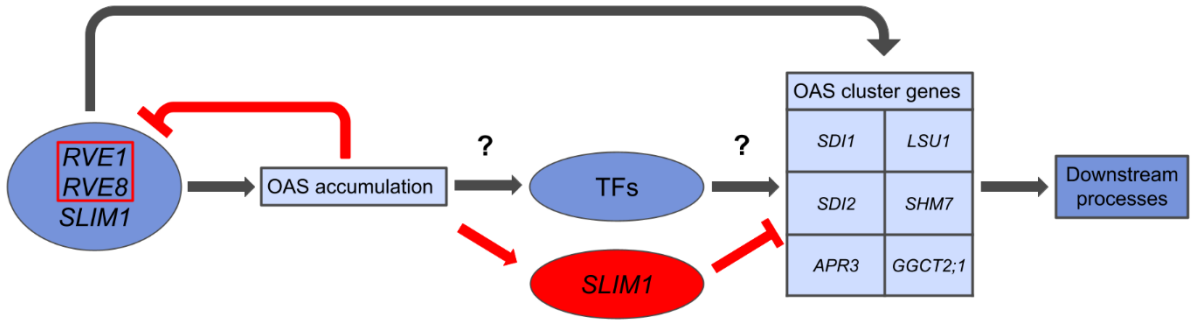

Figure 33: Proposed model for OAS signalling. RVE1, RVE8 and SLIM1 directly regulate the OAS cluster genes and also OAS accumulation. The expression of RVE1 and RVE8 is feedback inhibited by OAS. SLIM1 acts as a repressor of OAS but the transcription factor(s) involved in translating the OAS accumulation into a transcriptional signal remain(s) unknown. The induction of the OAS cluster genes leads to an impact in downstream processes, yet uncharacterized.

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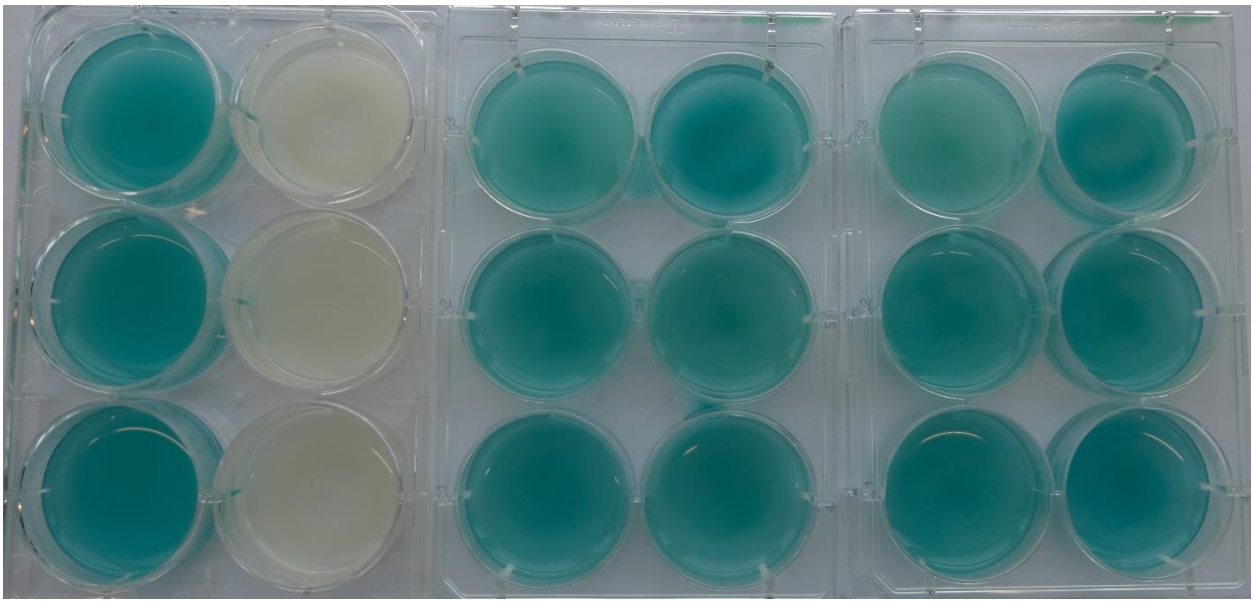
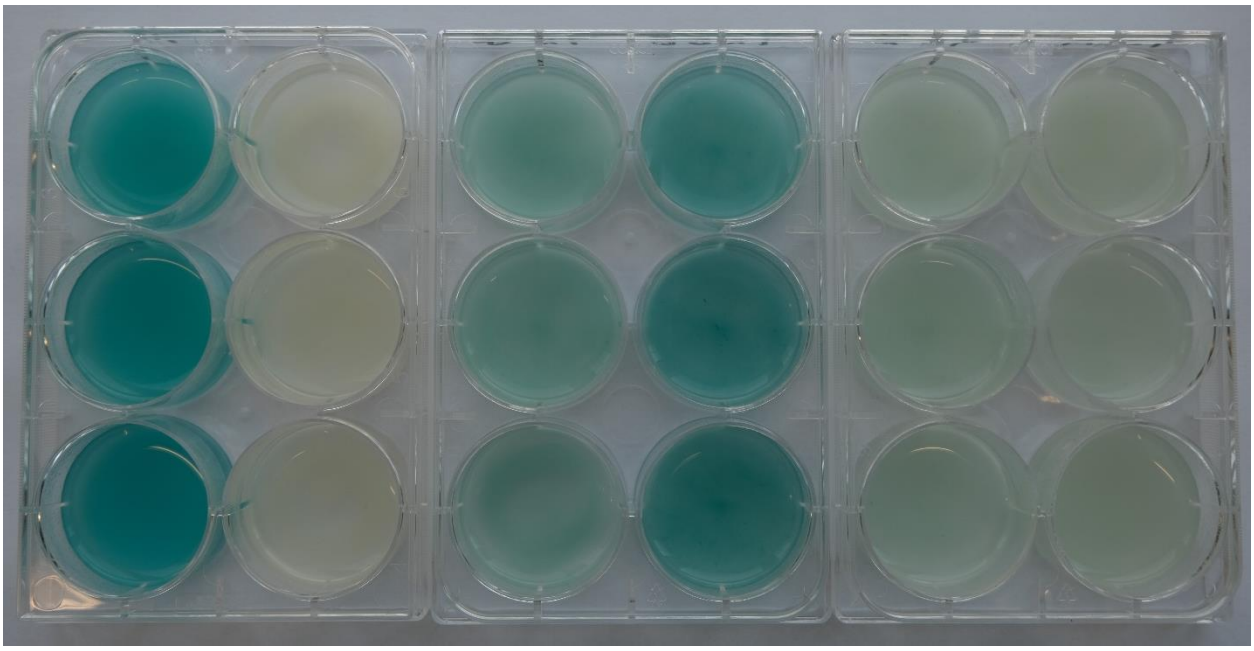
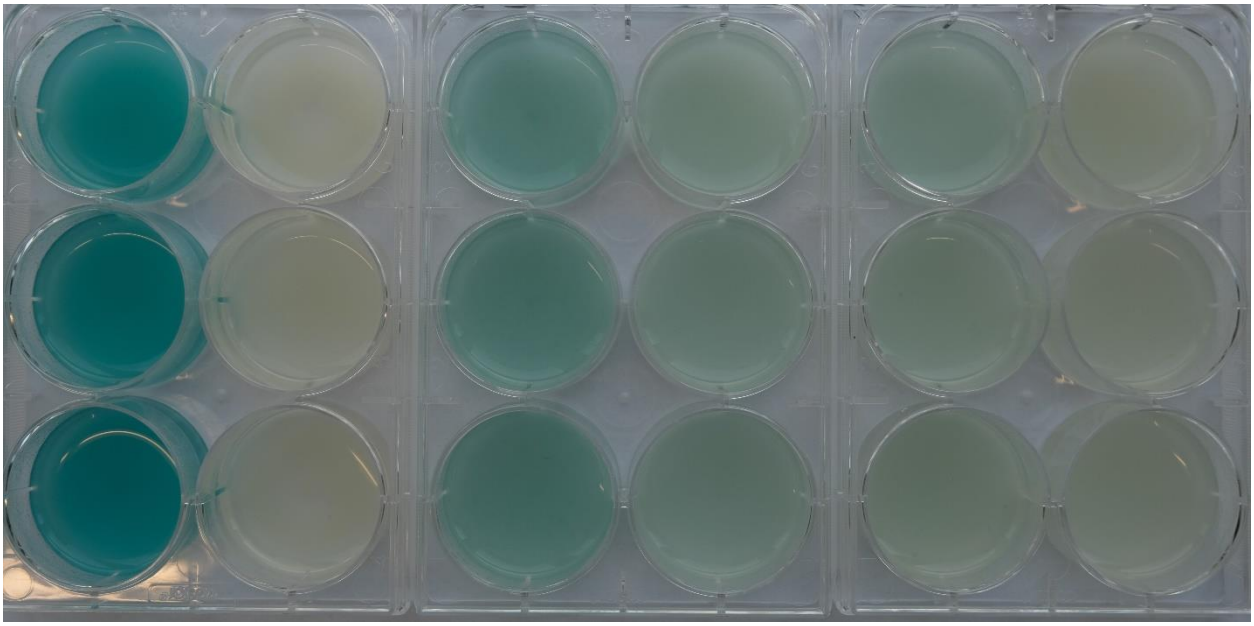
SUPPLEMENTAL DATA



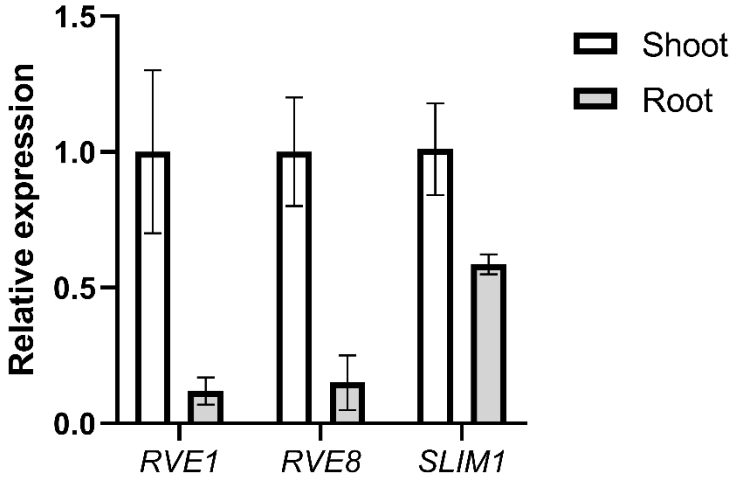
Supplemental Figure S1: Histochemical GUS staining of the *Agrobacterium*-transformed cultured *Arabidopsis thaliana* cells – EFECTOR SLIM1. Every column corresponds to 3 biological replicates. From left to right: positive control, negative control, promoter of GGCT2;1, promoter of SDI1, promoter of SHM7 and promoter of APR3.



Supplemental Figure S2: Histochemical GUS staining of the *Agrobacterium*-transformed cultured *Arabidopsis thaliana* cells – EFECTOR RVE1. Every column corresponds to 3 biological replicates. From left to right: positive control, negative control, promoter of GGCT2;1, promoter of SDI1, promoter of SHM7 and promoter of APR3.



Supplemental Figure S3: Histochemical GUS staining of the *Agrobacterium*-transformed cultured *Arabidopsis thaliana* cells – EFECTOR RVE8. Every column corresponds to 3 biological replicates. From left to right: positive control, negative control, promoter of GGCT2;1, promoter of SDI1, promoter of SHM7 and promoter of APR3.



Supplemental Figure S4: Relative expression of *SLIM1, RVE1* and *RVE8* in shoots and root. Mean (2-ΔΔCT method) and standard deviation shown, fold-change was calculated vs shoot and the housekeeping gene *TIP41* served as a reference.

Supplemental Table S1: Compact letter display from metabolite analysis in section 3.1. Letters were generated using Two-Way-Anova followed by a Tukey HSD in RStudio.

		SO4 shoots	SO4 roots	PO4 shoots	PO4 roots	NO3 shoot	NO3 roots	OAS shoots
ctrl	wt	a	ef	ab	abcd	a	fgh	hi
	q1;1	a	ef	a	abcd	ab	efg	hi
	q2;1	bcde	def	ab	abcd	bcde	cdef	ghi
	q2;2	abc	f	ab	abcd	abc	cdef	i
	q3;1	de	f	ab	abcd	de	bc	fghi
	q3;2	a	cd	ab	abcd	a	bcdef	ab
-S	wt	f	ab	ab	abcd	f	bcd	efghi
	q1;1	f	bc	ab	bcd	f	bcd	defghi
	q2;1	f	cd	abc	abc	f	ab	ghi
	q2;2	f	de	ab	abcd	f	bcd	efghi
	q3;1	f	def	bcd	ab	f	bcde	cdefg
	q3;2	f	a	abcde	a	f	cdef	cdefgh
-N	wt	abcd	d	f	d	abcd	ij	a
	q1;1	bcde	def	def	c	cde	ghi	bcde
	q2;1	de	cd	cdef	cd	de	j	a
	q2;2	cde	d	ef	cd	cde	hij	abcd
	q3;1	e	def	ef	cd	e	ij	abc
	q3;2	cde	cd	f	d	de	defg	bcdef
		OAS roots	Cys shoots	Cys roots	GSH shoots	GSH roots	Prot shoot	Prot root
ctrl	wt	cd	cde	ab	ab	a	efgh	bc
	q1;1	bcd	efg	cdef	defg	abcde	efgh	b
	q2;1	bcd	fgh	cdef	defg	abc	defgh	bc
	q2;2	d	def	cde	bcde	cdefg	bcd	a
	q3;1	bcd	h	ef	gh	cdefg	cdefg	c
	q3;2	abc	bcde	cde	ab	abcde	bcdef	bc
-S	wt	a	def	cde	fgh	cdefg	h	bc
	q1;1	ab	ef	def	h	efg	gh	bc
	q2;1	abcd	def	def	efgh	fg	fgh	bc
	q2;2	bcd	def	cde	fgh	cdefg	efgh	bc
	q3;1	abcd	gh	f	i	h	bcdef	bc
	q3;2	a	efg	def	gh	defg	bc	bc
-N	wt	bcd	a	abc	a	bcde	efgh	bc
	q1;1	bcd	abcd	abcd	bcd	abcd	bcd	bc
	q2;1	abcd	bcde	bcde	cdef	bcdef	bcd	bc
	q2;2	abcd	abc	a	abc	ab	bcdef	bc

	q3;1	abcd	efg	ef	fgh	gh	b	bc
	q3;2	abcd	ab	cde	abc	cdefg	a	a

Supplemental Table S2: Mean values of relative gene expression from Figure 9.

Condition	Line	*SDI1*	*LSU1*	*APR3*	*NIR1*	*GDH3*
ctrl	WT	1,065	1,102	1,014	1,016	1,013
	q1;1	1,518	0,922	1,202	0,996	1,215
	q2;1	2,338	1,417	0,952	0,871	1,279
	q2;2	0,533	0,453	0,617	0,869	1,250
	q3;1	1,248	0,680	0,722	0,782	0,545
	q3;2	0,505	0,524	0,661	1,009	1,221
-S	WT	39,943	64,273	3,940	0,567	0,337
	q1;1	32,002	47,386	3,097	0,464	0,604
	q2;1	14,214	19,562	1,570	0,439	0,550
	q2;2	11,938	18,034	1,295	0,659	0,529
	q3;1	6,721	13,646	1,707	0,486	0,583
	q3;2	13,831	20,940	1,686	0,598	0,726
-N	WT	0,159	0,271	0,424	0,407	1,327
	q1;1	0,162	0,131	0,426	0,445	1,793
	q2;1	0,308	0,330	0,327	0,350	1,167
	q2;2	0,223	0,220	0,291	0,458	1,693
	q3;1	0,307	0,355	0,477	0,368	1,242
	q3;2	0,096	0,282	0,267	0,312	1,371

Supplemental Table S3: Mean values of relative gene expression from Figure 10.

Condition	Line	*SDI1*	*LSU1*	*APR3*	*NIR1*	*GDH3*
ctrl	WT	1,034	1,002	1,022	1,021	1,207
	q1;1	1,319	0,637	1,051	1,274	0,853
	q2;1	0,901	1,159	0,587	0,959	0,546
	q2;2	0,720	0,847	0,849	1,309	1,356
	q3;1	0,513	0,201	0,472	1,211	0,448
	q3;2	0,531	0,944	0,598	1,118	1,042
-S	WT	149,151	45,492	6,439	1,398	1,601
	q1;1	45,847	19,667	6,834	1,278	1,161
	q2;1	12,011	7,806	2,759	0,876	1,693
	q2;2	10,092	4,107	2,793	0,861	0,906
	q3;1	10,198	4,285	3,292	1,295	1,519
	q3;2	13,708	7,403	3,598	1,417	4,843
-N	WT	2,343	0,754	0,976	1,138	1,729

	q1;1	1,627	0,245	0,888	1,548	2,538
	q2;1	1,309	0,813	0,687	1,984	2,301
	q2;2	0,566	0,401	0,828	1,381	4,264
	q3;1	1,843	0,251	0,787	1,612	1,450
	q3;2	3,803	2,008	1,597	6,242	1,844

Supplemental Table S4: Mean values of relative gene expression of all 5 *SERAT* genes in WT and quadruple mutants.

SHOOTS						
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
Ctrl	wt	1,020	1,011	1,013	1,056	1,002
	q1;1	1,468				
	q2;1		0,737			
	q2;2			1,653		
	q3;1				1,112	
	q3;2					0,634
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
-S	wt	1,318	1,010	1,018	1,699	5,041
	q1;1	1,268				
	q2;1		0,695			
	q2;2			1,195		
	q3;1				1,150	
	q3;2					1,296
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
-N	wt	0,629	0,682	0,462	0,984	0,223
	q1;1	0,829				
	q2;1		0,664			
	q2;2			0,566		
	q3;1				0,833	
	q3;2					0,161
ROOTS						
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
Ctrl	wt	1,036	1,018	1,040	1,025	1,130
	q1;1	0,645				
	q2;1		1,002			
	q2;2			0,995		
	q3;1				0,656	
	q3;2					0,564
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
-S	wt	0,673	0,673	0,951	2,950	5,774

	q1;1	0,557				
	q2;1		0,765			
	q2;2			0,719		
	q3;1				0,879	
	q3;2					3,146
		SERAT1;1	*SERAT2;1*	*SERAT2;2*	*SERAT3;1*	*SERAT3;2*
-N	wt	1,318	1,518	1,205	1,895	4,761
	q1;1	1,200				
	q2;1		1,172			
	q2;2			1,362		
	q3;1				1,208	
	q3;2					0,304

Supplemental Table S5: Mean values of relative gene expression from Figure 12.

	SDI1	*SDI2*	*LSU1*	*APR3*	*GGCT2;1*	*SHM7*
WT	11,513	3,056	2,830	1,799	1,820	7,720
s1;1	3,042	2,636	1,745	1,447	1,185	3,647
s2;1	2,160	2,093	2,343	1,458	1,135	3,046
s2;2	2,155	1,722	1,322	1,245	0,784	2,477
s3;1	13,057	3,657	4,127	2,179	1,743	7,294
s3;2	8,401	2,649	2,556	1,783	1,737	5,904

Supplemental Table S6: Mean values of relative gene expression from Figure 13.

	SDI1	*SDI2*	*LSU1*	*APR3*	*GGCT2;1*	*SHM7*
WT	9,995	2,899	3,725	1,898	2,505	6,275
q1;1	2,817	1,554	2,569	1,467	0,993	3,661
q2;1	4,150	1,890	3,444	1,750	1,919	5,888
q2;2	19,801	4,166	7,979	3,366	6,979	9,479
q3;1	1,582	1,243	1,875	1,473	0,886	4,204
q3;2	2,588	2,197	1,963	1,156	1,168	3,975

Supplemental Table S7: Mean values of relative gene expression from Figure 14.

No sucrose					
	SDI1	*SDI2*	*LSU1*	*APR3*	*GGCT2;1*
0 min	1,008	1,030	1,011	1,017	1,004
20 min	1,661	1,123	1,375	0,889	1,341
40 min	4,393	1,786	1,129	0,869	1,225
60 min	3,415	0,801	0,708	0,673	1,128

+ Sucrose					
	SDI1	*SDI2*	*LSU1*	*APR3*	*GGCT2;1*
0 min	1,264	1,035	1,026	1,028	1,061
20 min	2,157	1,685	1,305	0,952	1,647
40 min	3,344	1,257	1,534	0,938	1,776
60 min	5,710	0,674	0,835	0,520	1,669
Transfer					
	SDI1	*SDI2*	*LSU1*	*APR3*	*GGCT2;1*
0 min	1,034	1,072	1,008	1,004	1,039
20 min	2,158	2,230	1,363	0,854	1,385
40 min	10,468	3,514	3,856	1,858	5,267
60 min	4,585	1,830	1,920	1,187	2,526

Supplemental Table S8: Transcription factors predicted to bind to the promoters of the OAS cluster genes. According to Plant Regulomics (Ran et al., 2020)

Name	**AGI code**	**Description**
SLIM1	AT1G73730	Key transcription factor in sulfur deficiency response
RVE1	AT5G17300	Circadian clock related transcription factors
RVE8	AT3G09600	
PRR5	AT5G24470	
LFY	AT5G61850	Promotes the transition to flowering
SOL1	AT3G22760	Regulates fate transition and cell division in stomata lineage
WRKY33	AT2G38470	Involved in defense pathways and response to abiotic stress

Supplemental Table S9: Mean values of relative gene expression from Figure 28.

	SDI1	*SHM7*	*APR3*	*LSU1*	*GGCT2;1*
wt	1,018	1,002	1,029	1,038	1,003
slim1-1	5,177	1,418	1,708	8,217	7,792
rve1	0,225	0,519	0,261	0,325	0,350
rve8	0,175	0,367	0,241	0,197	0,166
rve1slim1-1	3,542	1,112	0,936	7,957	5,005
rve8slim1-1	1,467	1,072	0,549	1,343	1,569
rve1rve8	0,674	0,835	0,379	0,702	0,824

Supplemental Table S10: Mean values of relative gene expression from Figure 29.

	SDI1	*SHM7*	*APR3*	*LSU1*	*GGCT2;1*
wt	43,56201	5,172794	1,964793	42,7875	14,62699
slim1-1	36,30621	4,413322	5,441448	49,07274	21,17439
rve1	27,44353	3,68322	1,458504	18,11815	11,14114
rve8	76,81336	18,88641	8,562776	128,5705	74,817
rve1slim1-1	106,7619	7,162242	9,229143	137,4258	55,11997
rve8slim1-1	91,76378	6,164556	4,218258	55,83321	26,24274
rve1rve8	113,0058	7,552686	2,796309	52,32736	32,41874

Supplemental Table S11: Mean values of relative gene expression from Figure 30.

morning ctrl		morning -S	
wt	slim1-1	wt	slim1-1
1,09570923	2,70152681	23,0858508	13,8474555
1,00057756	2,55637414	1,38776953	1,33364714
1,01561809	0,67865488	1,70708986	4,5404365
1,11214598	0,45117843	2,97913603	0,80804077
evening ctrl		evening -S	
wt	slim1-1	wt	slim1-1
1,17051554	12,3675674	3,51491807	18,33613
1,00450804	1,41940384	0,85965791	1,17674524
1,01062653	2,49648625	2,58482672	6,25226159
1,11214598	2,80947916	1,96942275	1,17979471
night ctrl		night -S	
wt	slim1-1	wt	slim1-1
1,00908185	13,4682137	17,5648723	22,5666334
1,00748597	1,56897197	3,19644309	2,15110564
1,00200045	2,68100829	4,92206592	4,50558101
1,00740138	1,27701316	9,22606202	1,62891278

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

ABA	Abscisic acid
APK	APS kinase
APR	Adenosine 5′-phosphosulfate reductase
APS	Adenosine 5′-phosphosulfate
AT	*Arabidopsis thaliana*
bp	Base pair
cDNA	Complementary DNA
Chl	Chloramphenicol
Col-0	*Arabidopsis* ecotype Columbia-0
Ctrl	Control
Cys	Cysteine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
e.g.	*exempli gratia* (for example)
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii*/*ae* (and others)
FC	Fold-Change
FW	Fresh weight
gDNA	Genomic DNA
GUS	β-glucoronidase
GSH	Glutathione
H_2O	Water
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
Hyg	Hygromycin

Kan	Kanamycin
K_2SO_4	Potassium sulfate
KH_2PO_4	Potassium biphosphate
KNO_3	Potassium nitrate
LB	Lysogenic broth
Ler	Landsberg erecta
LiCl	Lithium chloride
LD	Light-Darkness transition
mRNA	Messenger RNA
µg	microgram
µl	microliter
µM	micromolar
miR395	microRNA-395
min	Minute
mL	milliliter
MS	Murashige and Skoog
N	Nitrogen
NO_3	Nitrate
$NaCO_3$	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
ng	nanogram
OAS	O-acetylserine
OAS-TL	O-acetylserine (thiol)lyase
PCR	Polymerase chain reaction
PO_4	Phosphate
qRT-PCR	Reverse transcription–quantitative PCR

Rif	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Sulfur
SERAT	Serine acetyltransferase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SO_4	Sulfate
SULTR	Sulfate transporter
TF	Transcription factor
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
WT	Wild type

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ERKLÄRUNG

Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

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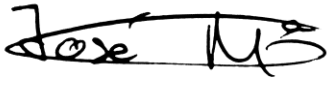

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PUBLICATIONS

Pavlů, J.,Kerchev, P., Černý, M., Novák, J., Berka, M., Jobe, T.O., López Ramos, J.M., Saiz-Fernández, I., Rashotte, A.M., Kopriva, S. and Brzobohatý, B., 2022. Cytokinin modulates the metabolic network of sulfur and glutathione. Journal of Experimental Botany, 73(22), pp.7417-7433.

CONFERENCES

July 2022	Oral communication: Transcripition factors *RVE1*, *RVE8* and *SLIM1* regulate the OAS cluster genes International Sulfur Workshop, Western University, London, Ontario, Canada.
Oct 2021	Poster: Role of transcription factors *RVE1* and *RVE8* in the control of OAS cluster genes and sulfur metabolism. Joint meeting for Plant and Human Sulfur Biology and Glucosinolates, CicCartuja, Sevilla.
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