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Bachelor's thesis

The effects of elevated levels of CO_2 on sulfur metabolism in *Arabidopsis thaliana*

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Abstract

Global atmospheric CO_2 levels are rising fast and expected to be doubled by the year 2100. C_3 plants increase their photosynthesis capacity under elevated CO_2 levels. Previous studies have already described the effects of the increase in photosynthesis on the nitrogen (N) assimilation pathway. However, how this increase in photosynthesis impacts the assimilation pathway of the essential macronutrient sulfur (S) is unknown. Plants acquire most sulfur through the uptake and reduction of sulfate. The sulfur metabolism is co-regulated with the pathways of photosynthesis and N-assimilation since products of all three pathways are needed for cysteine synthesis. In this thesis it is predicted how the S metabolism responds to elevated CO_2 levels by looking at the rate-limiting steps of the S metabolism. Predicted is that elevated CO_2 levels upregulate the reduction of sulfate by inducing APS reductase (APR) genes via several signaling metabolites. Glucose and fructose act as direct signals whereas amino acid levels, trehalose-6-phosphate (T6P) and O-acetylserine (OAS) act as indirect signals of elevated CO_2 levels on the sulfate reduction. The increase in sulfate reduction leads to an increase of sulfate uptake by the roots. This is regulated by inducing sulfur deficiency genes by OAS and dropping sulfate levels in the shoots.

Introduction

Sulfur (S) is one of the six macronutrients found in plants. Most sulfur in plants is found assimilated in molecules with an -SH group, so called thiols (Kopriva & Rennenberg, 2004). The most abundant thiols are the amino acids cysteine and methionine (Maathuis, 2009). Cysteine and methionine are important as building blocks for proteins, because of their ability to form disulfide bonds which stabilize the tertiary and quaternary structures of proteins (Gotor et al., 2015). The main source of sulfur is sulfate (SO_4^{2-}) which is actively taken up by the roots (Hawkesford & de Kok, 2006). Sulfate is then reduced to sulfide, which subsequently is used as a substrate in the synthesis of cysteine. This cysteine can be further used in the synthesis of methionine. Besides S, to synthesize cysteine, also nitrogen (N) and carbon (C) are needed. Because of this, there is a need for co-regulation between the pathways of S-, N- and C-assimilation as products of all three need to be present for the synthesis of cysteine. The pathways of sulfate assimilation and nitrate assimilation are especially well coordinated (Kopriva & Rennenberg, 2004). Since the pathways of photosynthesis, N-assimilation and sulfate assimilation are co-regulated, changes in one of the pathways have regulatory effects on the others.

Global atmospheric carbon dioxide (CO_2) levels have risen from 280 parts per million (ppm) at 1800 up to 400 ppm in 2013 (Pandey et al., 2015). Current levels are still rising fast and are expected to reach 1000 ppm by the year 2100 (Prentice et al., 2001). Elevated levels of CO_2 have been described to stimulate biomass production by an increased photosynthesis capacity especially in C_3 plants (Drake et al., 1997). Photosynthesis is the fixation of CO_2 into organic compounds (carbohydrates) using energy of light. The first step of this fixation is the carboxylation of Ribulose-1,5-biphosphate (RuBP) catalyzed by Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), which happens when a CO_2 molecule binds to RuBP (Stitt & Krapp, 1999). However, RuBP can also undergo oxygenation; this happens if RuBP binds to a O_2 molecule. Binding of O_2 to RuBP is also catalyzed by Rubisco and does not yield carbohydrates.

Oxygenation of RuBP leads to wasteful products which need to be detoxified in the so-called photorespiration pathway. Rubisco has a low affinity for CO_2 and at current CO_2 levels it does not solely catalyzes the carboxylation of RuBP but also the oxygenation (Izumi et al., 2012). Increasing levels of CO_2 result in a bigger proportion of Rubisco that catalyzes carboxylation compared to oxygenation (Drake et al., 1997). This increase in carboxylation leads to an upregulation of the photosynthesis pathway, resulting in the production of more carbohydrates. Since the pathways of photosynthesis, N-assimilation and sulfate uptake and reduction are co-regulated, changes in the photosynthesis pathway are expected to have effects on the other two pathways (Kopriva & Rennenberg, 2004). Upregulation of photosynthesis results in enhanced synthesis of proteins and amino acids, which subsequently increases the demand of other macronutrients like nitrogen (Drake et al., 1997). Thus, resulting in an upregulation of the N-assimilation pathway.

The effects upregulated photosynthesis on the N-assimilation have been described in literature, however the effects on the sulfur metabolism remain unknown. As global CO_2 levels are rising and it has been shown that other macronutrient pathways are influenced by this, changes in the S metabolism are expected as well. It is important to know what these effects are, as sulfur currently is a limiting factor in the agriculture and changes in its metabolism could lead to a need for other ways of fertilization (Schnug & Haneklaus, 1998).

The main goal of this thesis is to predict the effects of elevated CO_2 levels on the sulfur metabolism in a C_3 plant, specifically *Arabidopsis thaliana*.
An upregulation of the sulfate uptake and reduction is expected at elevated CO_2 levels. This predicted upregulation is an effect of the increased protein synthesis at elevated CO_2, resulting in more demand for the sulfur containing amino acids cysteine and methionine.

In order to predict the effects, a literature study has been done. Firstly, the steps that limit the flux through the sulfur metabolism and the signaling molecules of these steps are identified. Then the effects of elevated CO_2 levels on these flux controlling steps are described, following the predicted effects on the sulfur metabolism. The focus is on *Arabidopsis thaliana* as many studies of the pathways of the sulfur, nitrogen and carbon metabolisms have been done with this plant, whereas for other species knowledge for several steps of these pathways is lacking.

Sulfur (S) metabolism

Sulfate uptake

Plants get most of their sulfur from the uptake and reduction of sulfate by the roots. Members of the sulfate transporters family (Sultr) regulate the transport of sulfate into the roots and to other areas in the plant (Hawkesford & de Kok, 2006). The Sultr-family consists of five groups, of which group 1 (Sultr1) regulates the uptake of sulfate into the root (Kimura et al., 2019). Sultr1 transporters are predominantly present in the root epidermis (Hawkesford & de Kok, 2006; Smith et al., 1997).

Group 1 of the Sultr-family consists of three different transporters with two of them, Sultr1;1 and Sultr1;2, responsible for the sulfate uptake into the root (Maruyama-Nakashita et al., 2004; Takahashi et al., 2002). Sultr1;1 and Sultr1;2 are both high affinity sulfate transporters with K_m values of 3,6 ± 0,6 μM and 6,9 ± 1,0 μM respectively (Feldman-Salit et al., 2019; Takahashi et al., 2000). Sultr1;3, the third member of group 1 of the Sultr-family, is suspected to play a role in the transport of sulfate into the xylem (Hawkesford & de Kok, 2006).

From the roots, sulfate is transported through the xylem to the shoots via Sultr1;3 and transporters of group 2, Sultr2 (Hawkesford & de Kok, 2006). Most sulfate

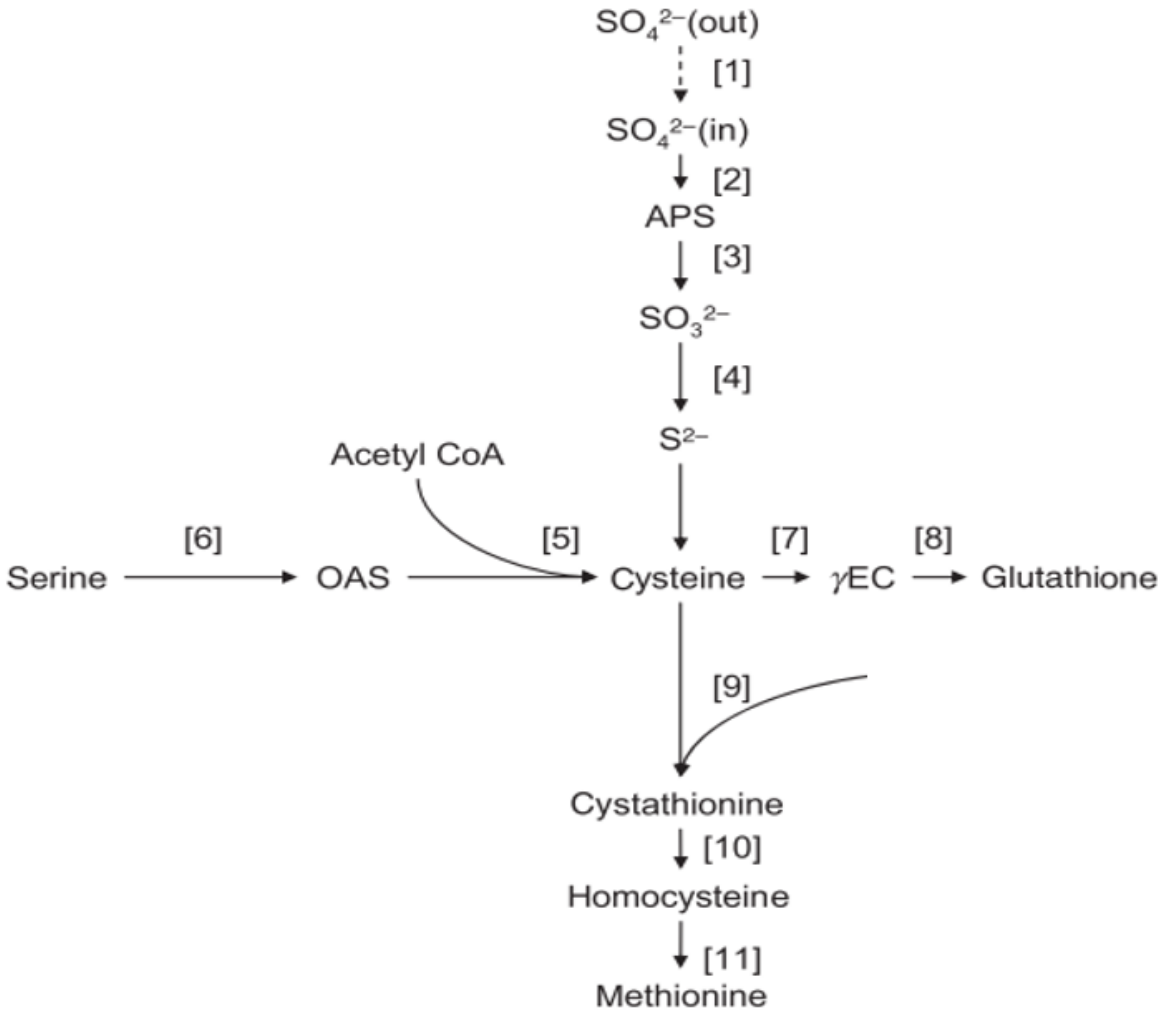

Figure 1. Steps of sulfate uptake/reduction and the synthesis of cysteine, methionine and glutathione. Involved enzymes: [1] Sultr1 transporters [2] ATP sulfurylase (ATPS) [3] APS reductase (APR) [4] sulfite reductase (Sir) [5] OAS thiolyase (OAS-TL) [6] serine acetyltransferase (SAT) [7] γ-glutamylcysteine synthetase (γ-GCS) [8] glutathione synthetase (GS) [9] cystathionine γ-synthase (CGS) [10] cystathionine β-lyase (CBL) [11] methionine synthase (MS). Figure modified after (Hawkesford & de Kok, 2006).

is reduced in the leaf's chloroplasts; however, a part is reduced in the plastids of the roots (Haneklaus et al., 2016). Sulfate transporters of group 3, Sultr3, subsequently transport the sulfate into plastids or chloroplasts. Notably, not all sulfate is reduced in chloroplasts and plastids: surplus sulfate is transported into the vacuoles via group 4 sulfate transporters, Sultr4, where it is stored (Takahashi et al., 2011).

Sulfate reduction

Sulfate reduction is a 4-step process (figure 1). First, sulfate (SO_4^{2-}) is activated to adenosine 5'-phosphosulfate (APS) by the enzyme ATP sulfurylase (ATPS) (Hawkesford & de Kok, 2006). APS reductase (APR) reduces APS to sulfite (SO_3^{-}), which subsequently is reduced to sulfide (S^{2-}) by sulfite reductase (SiR). This is the last step of the sulfate reduction; the formed sulfide is used as a substrate in the biosynthesis of cysteine (Takahashi et al., 2011).

Cysteine synthesis

Biosynthesis of cysteine consists of incorporating sulfide in a carbon- and nitrogen-containing backbone (Takahashi et al., 2011). This backbone is O-acetylserine (OAS), which has serine as a

precursor (figure 1). Serine is converted to OAS by the enzyme serine acetyltransferase (SAT). This is done by replacing the hydroxy group of serine with an acetate group, creating the ester OAS. Subsequently, cysteine is formed by the substitution of that acetate group by sulfide. This substitution is catalyzed by the enzyme OAS thiolyase (OAS-TL) and has acetate as a byproduct of the reaction.

Serine synthesis

As described before, OAS is the backbone for the synthesis of cysteine. OAS is synthesized out of its precursor serine by SAT. Serine is a product of both the C and N assimilation pathways. In C_3 plants serine can be synthesized by 2 different pathways; the phosphorylated serine pathway and the photorespiratory serine pathway (Igamberdiev & Kleczkowski, 2018). The phosphorylated serine pathway starts with the carboxylation of RuBP by Rubisco whereas the photorespiratory serine pathway stats with the oxygenation of RuBP, also catalyzed by Rubisco (Igamberdiev & Kleczkowski, 2018; Maurino & Peterhansel, 2010).

Under current levels of CO_2 the bulk of the serine in a plant is synthesized by the photorespiratory pathway (Igamberdiev & Kleczkowski, 2018). However, as stated before increasing CO_2 levels result in a lower proportion of oxygenation compared to carboxylation by Rubisco. Therefore, under elevated levels of CO_2 it would be expected that a bigger proportion of the serine would be synthesized by the phosphorylated pathway compared to current CO_2 levels.

Methionine synthesis

In addition to cysteine's important role as a building block for proteins, it can also be used as a precursor for the biosynthesis of methionine. Methionine is an amino acid, which just like cysteine is important in the protein synthesis (Maathuis, 2009). Moreover, it plays a role in the initiation of mRNA translation and is a precursor of S-adenosylmethionine (SAM). SAM regulates pivotal cellular processes like cell wall biosynthesis and cell division (Hacham et al., 2013). Furthermore, SAM is important for a plant because it is a precursor for ethylene (Hacham et al., 2013). Ethylene is an important hormone that regulates the plant's life cycle and has effects on the levels of other hormones. However, the precise mechanisms are still unclear (Guo & Ecker, 2004).

In order to synthesize methionine, cysteine is first converted into cystathionine by the enzyme cystathionine γ-synthase (CGS) (figure 1). Cystathionine consists of 2 serine groups bound together by a single sulfur atom (Hawkesford & de Kok, 2006). Subsequently, cystathionine is metabolized into homocysteine via the enzyme cystathionine β-lyase (CBL). Homocysteine resembles cysteine; however, it has an additional $-CH_2$ group in the backbone of the molecule. In order to create methionine out of homocysteine an extra methyl group is added by methionine synthase (MS). The enzyme SAM synthase (SAMS) catalyzes the reaction of methionine to SAM.

Glutathione (GSH) is a metabolite that has cysteine as a precursor as well (figure 1). GSH plays an important role in the plant's response to stress as it is capable of preventing damage of reactive oxygen species (ROS) (Maathuis, 2009). The first step of glutathione synthesis is the reduction of cysteine to γ-glutamylcysteine (γEC) by the enzyme γ-glutamylcysteine synthetase (γGSH). Glutathione synthetase (GS) adds a glycine group to γEC, which yields GSH.

Flux controlling steps

Multiple studies have found that the reduction of APS to sulfite by APR and the uptake of sulfate by Sultr1;1 and Sultr1;2 are rate-limiting steps for the flux through the sulfur metabolism (Feldman-Salit et al., 2019; Kopriva & Rennenberg, 2004). A flux control coefficient for APR was found at 0,92, but realistically lies lower (Vauclare et al., 2002). Furthermore, Vauclare et al.

(2002) suggested a coupled activation/inhibition of APR and sulfate uptake, however no clear evidence for this was found. Additionally, the reduction of sulfite to sulfide catalyzed by the enzyme SiR has been described as another rate-limiting step in this pathway (Khan et al., 2010). Therefore, up- and downregulation of these 3 steps can control the flux through the sulfur pathway. In order to look at the effects of an elevated CO_2 level on the sulfur metabolism, these three steps are being looked at.

Regulation of sulfate uptake

Sulfate demand

As described before, Sultr1;1 and Sultr1;2 are responsible for the uptake of sulfate from the soil into the roots. Sultr1;1 and Sultr1;2 are both high affinity sulfate transporters and both have a different regulating mechanism (Hawkesford & de Kok, 2006). Both transporters are regulated at transcriptional levels and regulation happens by in- or decreasing the mRNA levels (Rouached et al., 2008). Under normal conditions, with sufficient sulfate in the soil, Sultr1;2 is the main transporter for sulfate uptake. However, Sultr1;1 is the main sulfate uptake transporter under stressed conditions (Kimura et al., 2019).

Sulfate levels in the root environment acts as a local signal for the regulation of sulfate uptake. Takahashi et al. (2000) described that sulfate levels regulate the abundance of several transporters of the Sultr-family. If the sulfur concentration in the soil drops, an increase in mRNA levels and abundance of Sultr1;1 is found (Hawkesford & de Kok, 2006). This regulation of the sulfate uptake is independent of factors in the plant; it depends solely on the sulfate concentration in the soil (Hubberten et al., 2012a). Even though mostly Sultr1;1 mRNA is upregulated under low sulfate concentrations in the soil, Sultr1;2 mRNA also was upregulated albeit much less than Sultr1;1 mRNA (Rouached et al., 2008; Takahashi et al., 2002).

Not all signals for the regulation of sulfate uptake come from factors outside the plant. The demand for sulfur in the shoots is also determining sulfate uptake and distribution through the roots (Hawkesford & de Kok, 2006). Plants with low concentrations of sulfate in the shoots have been shown to increase the Sultr1;2 mRNA and abundance in the roots to increase the sulfate uptake (Hubberten et al., 2012a). This increase in Sultr1;2 mRNA is suspected to be signaled by sulfur limitation1 (SLIM1). SLIM1 is a transcriptional factor that upregulates the expression of Sultr1;2 (Maruyama-Nakashita et al., 2006). It is induced by sulfur deficiency and an ethylene-insensitive3-like (EIL)-family transcription factor and is the only transcription factor of this family that is only impacting the sulfur metabolism (Wawrzyńska & Sirko, 2014). Furthermore, if the shoot from plants is excised, then sulfate uptake strongly decreases (Ausma & De Kok, 2019).

O-acetylserine (OAS)

It is suspected that OAS plays a role as a positive regulator of the sulfate uptake and reduction and mediates the demand-driven regulation of the S-metabolism (Hubberten et al., 2012a). However, there is an ongoing debate about the mechanism of OAS that increases the uptake and reduction of sulfate and OAS as a signaling role in the sulfur metabolism (Hawkesford & de Kok, 2006; Hubberten et al., 2012b; Maruyama-Nakashita et al., 2004; Rouached et al., 2008; Vauclare et al., 2002). Rouached et al. (2008) found no correlation between the root OAS-levels and the mRNA levels of both Sultr1;1 and Sultr1;2, suggesting that OAS has no major role as a regulator of these genes. However, plants with exogenous application of OAS do show an increase in Sultr1;1 and Sultr1;2 protein abundance in the roots (Maruyama-Nakashita et al., 2004). A possible explanation is that only unmetabolized free OAS acts as a signal to increase mRNA of Sultr1;1 and Sultr1;2. Unmetabolized OAS becomes present if there is more OAS

compared to sulfide in the cysteine synthesis. Not all OAS is metabolized and leftover unmetabolized OAS can act as a signal to upregulate the sulfate uptake in order to increase sulfide abundance (Rouached et al., 2008).

Thiols

Whereas OAS is a positive regulator of the sulfate uptake, thiols are expected to be negative regulators of the sulfate uptake (Hawkesford & de Kok, 2006). Exogenous addition of cysteine and GSH, both thiols, showed decreases in the activity of Sultr1;1 and/or Sultr1;2 transporters suggesting that thiols are able to regulate the sulfate uptake and reduction (Vauclare et al., 2002). Accumulation of cysteine led to a decrease of OAS through reduced activity of SAT, the enzyme that catalyzes the synthesis of OAS (Hawkesford & de Kok, 2006). Accumulation of GSH, another thiol, in a plant triggered a decrease of Sultr1;1 activity, suggesting a direct regulation of sulfate uptake by GSH (Maruyama-Nakashita et al., 2004).

Regulation of APS reductase (APR)

APR is the enzyme that catalyzes the reduction of APS into sulfite. It is regulated at the transcriptional level with multiple regulating factors described in literature. Three genes are responsible for the expression of APR in *Arabidopsis thaliana*, these genes being APR1, APR2 and APR3 (Vauclare et al., 2002).

Sulfate demand

The sulfate deficiency reaction in the shoots, which is also activated by increased sulfate demand, is one of the most described upregulators of the activity of APR (Hawkesford & de Kok, 2006). An increase in APR activity results in an upregulation of the sulfate reduction and subsequently of the synthesis of cysteine (Lee et al., 2011) This results in less available sulfate in the shoots for reduction, which activates the sulfate deficiency response. As said before, increased demand of sulfate in the shoots leads to upregulation of Sultr1;2. Via this pathway APR can indirectly regulate the uptake of sulfate.

Another regulating factor of APR activity is the N-metabolism (Kopriva & Rennenberg, 2004). An increase in amino acid levels led to an increase in the activity of APR (Lee et al., 2011). This increase in amino acid levels meant that there was an increase in demand of sulfate in the shoots, which as stated before upregulates APR activity. Adding more nitrate or ammonium to the growth medium of a plant also increased APR activity (Lee et al., 2011). This regulation of APR activity by the N-metabolism is predicted to be mediated by OAS (Hawkesford & de Kok, 2006). Multiple other studies have found increases in APR activity after a rise in OAS levels, suggesting an important role of OAS in regulating APR (Hawkesford & de Kok, 2006; Kopriva & Rennenberg, 2004).

O-acetylserine (OAS)

OAS has been found to induce the expression of genes that play part in the sulfur deficiency reaction. The sulfur deficiency reaction is also activated as demand of sulfate in the shoots increases (Ohkama-Ohtsu et al., 2004). Six genes that play a role in the sulfur metabolism show an increase in expression by OAS, namely *APS reductase 3* (APR3), *sulfur-deficiency-induced 1* (sdi1), *sulfur-deficiency-induced 2* (sdi2), *low-sulfur-induced 1* (LSU-1), *serine hydroxymethyltransferase 7* (SHM7) and *ChaC-like protein* (ChaC) (Hubberten et al., 2012b). Sdi1 and sdi2 have been found to repress the synthesis of glucosinalates (GSL), whereas SHM7 plays a role in the development of fruit (Aarabi et al., 2016; Zhang et al., 2017). The function of LSU-1 and ChaC remains unknown and needs to be further explored. However, both do not seem to play a regulating role in the expression of the Sultr-family (Hubberten et al., 2012b). OAS thus induces the expression of APR-genes, while as stated before thiols inhibit this expression.

Carbohydrates

Glucose is a monosaccharide and the main product of photosynthesis along with fructose. Firstly, CO_2 is fixated in a pathway known as the Calvin-cycle. The Calvin-cycle consists of light-independent reactions, however the energy used for the reductions in the cycle are formed by the light-dependent part of photosynthesis. The first step of the Calvin-cycle is the carboxylation of Rubisco, which adds another carbon atom to the 5-carbon backbone of RuBP. This forms an unstable intermediate 6-carbon molecule, which immediately splits into two 3PG molecules. Phosphorylation of 3PG by ATP is catalyzed by phosphoglycerate kinase (PGK) and results in ADP and 1,3-biphosphoglycerate (1,3BPG). 1,3BPG is then reduced to glyceraldehyde 3-phosphate (G3P) by glyceraldehyde 3-phosphate dehydrogenase. G3P can than either be used for the regeneration of RuBP or it is used to synthesize glucose or fructose (Stitt et al., 2010). Both fructose and glucose are monosaccharides. To store and transport carbohydrates these monosaccharides are assimilated to polysaccharides like starch and sucrose (Martins et al., 2013).

APR activity is upregulated by glucose. Glucose induces the expression of APR2 genes which results in increased APR activity (Hesse et al., 2003). This is a direct regulation of the S-metabolism by photosynthesis as glucose is the main product of photosynthesis. Indirect regulation is also possible; this happens when for example glucose upregulates a pathway and the product of this pathway upregulates the S-metabolism.

Indirect regulation by carbohydrates

Carbohydrates are able to regulate several pathways like photosynthesis and amino acid synthesis by inducing the expression of genes (Stitt et al., 2010). Nitrate uptake and reduction are upregulated by the presence of glucose and sucrose. This upregulation is an effect of increased expression of genes of the nitrate transport family (Nrt) and of the nitrate/nitrite reduction family (NIA/NiR) by glucose and/or sucrose (Thompson et al., 2017). As stated before, APR activity increased as amino acids and nitrate levels increase. Since glucose and/or sucrose upregulate the synthesis of amino acids and the uptake of nitrate, they enhance the activity of APR through this indirect path.

Glucose is also able to promote plant growth through activation of the enzyme hexokinase (Thompson et al., 2017). Furthermore, trehalose-6-phosphate (T6P) abundance is also increased if glucose concentrations are higher. T6P is suggested to play a role in upregulation of multiple other macronutrient pathways if the supply of carbon is high (Wingler et al., 2012). Starch degradation is inhibited by T6P, whereas the synthesis of starch is upregulated (Martins et al., 2013). Exogenous feeding with T6P resulted in an increase of the N assimilation through upregulation of nitrate uptake and reduction genes, suggesting that T6P is an important regulating factor in this pathway (Lin et al., 2017). This upregulation of the N-assimilation leads, as described before to an increase in APR activity.

The activity of SNF1-related protein kinase Snrk1 is also inhibited by T6P. Snrk1 increases the proportion of phosphorylated nitrate reductase (NR) over non-phosphorylated NR (Jossier et al., 2009). Phosphorylated NR is the in-active form of NR, thus inhibition of Snrk1 leads to an increase in NR activity. This increase in NR activity leads to an upregulation of the N-assimilation, which subsequently upregulates the amino acid synthesis (Jossier et al., 2009). So, both T6P and Snrk1 upregulate the N-assimilation pathway. As described before, upregulation in the N-assimilation pathway increases the activity of APR. Thus, another way glucose indirectly increases the activity of APR is through increases in T6P abundance and the subsequent inhibition of Snrk1 by T6P.

The glucose-target of rapamycin (TOR) signaling pathway is another way glucose is predicted to regulate plant growth, uptake and reduction of other macronutrients (Dong et al., 2019). TOR is a protein kinase and downregulation of TOR has been found to reduce growth and protein synthesis (Pu et al., 2017). Snrk1 has been found to inhibit TOR activity, whereas presence of glucose induces TOR activity. It still remains unsure what exactly is the mechanism of TOR activation and how it influences other macronutrients, although it is expected that TOR plays a role in the nutrient deficiency pathways (Dong et al., 2019; Guan, 2017; Pu et al., 2017). This could be another indirect way of upregulation of APR by glucose as the sulfur deficiency pathway increases the activity of APR and this pathway is possibly induced by TOR.

Regulation of sulfite reductase (SiR)

The third rate-limiting step of the flux through the S-metabolism is the reduction of sulfite to sulfide (S^{2-}) catalyzed by the enzyme SiR. Khan et al. (2010) have described this catalyzation by SiR as a rate-limiting step, however, much remains unknown about what signals regulate this step. Changes in sulfur status, which can be done by varying the levels of sulfate or H_2S in the plant environment, do not affect SiR activity. This raises questions whether or not SiR can be regulated at all (Ausma & De Kok, 2019).

Elevated levels of CO_2

An increased CO_2 concentration in the air has multiple effects on plants. At current CO_2 levels (±350 ppm) Rubisco catalyzes the carboxylation as well as the oxygenation of RuBP. An increase of CO_2 concentration results in an increased photosynthesis capacity as the proportion of RuBP that undergoes carboxylation instead of oxygenation increases (Drake et al., 1997). This increase in photosynthesis capacity has the effects of increased plant growth and biomass. Increased carboxylation of RuBP results in more 3PG molecules entering the Calvin-cycle. This upregulation of the Calvin-cycle leads to an increase in glucose and sucrose synthesis as they are the main products of photosynthesis. Signaling by glucose and sucrose leads to an upregulation protein and amino acid synthesis (Lin et al., 2017; Stitt et al., 2010). Improved growth and biomass are an effect of this upregulation and results in a larger demand for other macronutrients, since they need to be present for protein and amino acid synthesis (Stitt & Krapp, 1999). The signaling for the increased demand is done in previously described mechanisms using either glucose or fructose as direct signals or indirect via metabolites like T6P and hexokinase (Stitt et al., 2010; Thompson et al., 2017; Wingler et al., 2012). Signaling through the glucose-TOR pathway also seems like a potential regulating mechanism of plant growth; however, many things are still unknown in this pathway (Dong et al., 2019; Guan, 2017).

Conclusion

The goal of this thesis was predicting how an elevated CO_2 concentration influences the sulfur metabolism in *Arabidopsis*. In order to make this prediction the flux controlling steps and the signal molecules affecting these steps have described. Subsequently, the effects of an elevated level of CO_2 on these signal molecules has been looked at.

The three flux controlling steps of the S-metabolism are the uptake of sulfate by Sultr1;1 and Sultr1;2, the reduction of APS to sulfite by APR and the reduction of sulfite to sulfide by SiR. SiR activity does not seem to change with changing sulfur status, suggesting that even though it is a flux controlling step it cannot be regulated (Ausma & De Kok, 2019). On the other hand, the uptake of sulfate and APR activity are regulated by several factors.

Sulfate uptake by Sultr1;1 and Sultr1;2 is upregulated at increased sulfate demand in

the shoots, dropping sulfate levels in the shoots and OAS levels. On the contrary, thiol levels downregulate the uptake of sulfate. Upregulation of APR results in increased demand of sulfate in the shoots, resulting in upregulation of sulfate uptake by Sultr1;1 and Sultr1;2.

The activity of APR is upregulated through induced gene expression by OAS (APR3) and glucose (APR2) (Hesse et al., 2003; Hubberten et al., 2012b). Increasing amino acid and nitrate levels also upregulate the activity of APR. Both glucose and sucrose induce the expression of nitrate uptake (Nrt) and reduction (NIA, NiR) genes, resulting in an increase of N-assimilation (Thompson et al., 2017). Furthermore, T6P, which is induced by glucose, upregulates the biosynthesis of amino acids which increases the demand for nitrogen(Lin et al., 2017). This also causes an upregulation of the N-assimilation pathway, which subsequently results in upregulation of APR activity. Thus, glucose and sucrose increase the activity of APR by inducing the expression of APR genes and the upregulation of the N-assimilation. Glucose-TOR signaling could also play a role in the regulation of plant growth and increased N-assimilation, however, many things about this pathway remain unknown(Dong et al., 2019; Guan, 2017). Moreover, glucose presence upregulates the expression of hexokinase, which subsequently enhances plant growth and biomass (Thompson et al., 2017). Enhanced plant growth means that the amino acid and protein synthesis are upregulated. Upregulation of the protein synthesis leads to a decrease of cysteine concentrations, resulting in downregulation of GSH synthesis. GSH and cysteine are both thiols and able to negatively regulate activity of sulfate uptake and reduction (Hawkesford & de Kok, 2006).

Elevated CO_2 levels leads to an increase in photosynthesis capacity in plants resulting in increased glucose and sucrose levels in a plant as they are the main products of photosynthesis (Drake et al., 1997). Plant growth and biomass are upregulated by glucose and fructose, subsequently upregulating amino acid and protein levels (Stitt & Krapp, 1999). This upregulation results in an increase in sulfate demand and thus upregulated sulfate uptake and reduction. Furthermore, glucose and sucrose can regulate the sulfate reduction pathway through affecting APR activity. Both induce expression of APR-genes, resulting in an increase in APR activity. The activity of APR also is increased by the level of amino acids and OAS. Increased sulfate reduction results in more demand of sulfate, which leads to an increase in OAS, which subsequently upregulates the APR activity. Furthermore, this increase in OAS also increases the uptake of sulfate in the roots. The increase in APR activity also creates more demand for sulfate in the shoots, this subsequently increases the activity of Sultr1;1 and Sultr1;2 in the roots.

Increased CO_2 levels thus increase APR activity directly with glucose as a signal and indirectly through T6P, increased amino acid and protein synthesis. This results in an increase in sulfate demand in the shoots, which subsequently upregulates the uptake of sulfate.

Thus, in *Arabidopsis* an upregulation of the sulfate uptake and reduction is predicted at elevated levels of CO_2.

Discussion

According to the prediction, it has been found that enzymes of the methionine synthesis were upregulated under elevated levels of CO_2 (Zinta et al., 2018). However, data for the sulfate uptake and reduction is not present for *Arabidopsis*. Nevertheless, sulfate uptake and reduction activity under elevated CO_2 levels have been sketchily studied in other plants. A study in *Brassica oleracea* showed increases of sulfate and sulfur levels in the roots and shoots (Rodríguez-Hernández et al., 2014). Furthermore, all measured enzymes and products of the S-metabolism showed an increase of abundance at elevated levels of

CO_2 (Rodríguez-Hernández et al., 2014). However, activity and abundance of enzymes of the rate-limiting steps of the sulfate uptake and reduction were not measure. Therefore, conclusions about up- or downregulation of the S-metabolism cannot be reliably made.

Moreover, in *Brassica pekinensis* sulfate levels in neither the roots or the shoots differed significantly at elevated levels of CO_2 (Reich et al., 2016). Total plant sulfur levels also did not show significant changes at different concentrations of CO_2 (Reich et al., 2016). This may suggest that, in contrast to the prediction, there is no upregulation of the uptake of sulfate. However, metabolite levels don not tell anything about the flux through a pathway. Metabolite levels could stay the same, even if the flux is increased. In the study of Reich et al. (2016) biomass production, free amino acids and nitrate levels did show an increase under elevated levels of CO_2. Since the metabolisms of S and N are co-regulated, this increase in N-assimilation would be expected to upregulate the sulfate uptake and reduction as well.

Nevertheless, it is interesting that S-metabolism in different species responds differently to changes in CO_2 levels. Experimental setups were similar, with plants in both experiments growing on Hoagland solutions. Levels of CO_2 were about the same range with the control/elevated concentrations for Reich et al. (2016) at 420/800 ppm and for Rodríguez-Hernández et al. (2014) at 380/800 ppm. The age of the plants at harvesting was different for both researches; 20 days old (Reich et al., 2016) and 36 days old (Rodríguez-Hernández et al., 2014). Furthermore, even though both used a species of the *Brassica* genus, they were different species.

These results thus suggest that even within one genus of plants, *Brassica*, there is variation at how plants adjust their sulfur metabolism to an increases CO_2 concentration.

Currently, the effects of elevated CO_2 levels on the sulfur metabolism are unknown. In this thesis a prediction about these effects has been made. However, further research on the precise metabolic changes in reaction to elevated CO_2 levels needs to be done in order to test the prediction made in this thesis. Especially the activity of enzymes of the rate-limiting steps at different CO_2 levels must, since they control the flux of the sulfur metabolism. Research in *Brassica* has shown that it is necessary to study this in different plant species, since under relatively similar conditions plants can show differences in sulfate and sulfur content at elevated CO_2 levels. This suggests the possibility of different reactions to elevated CO_2 levels for different species of plants.

References

Aarabi, F., Kusajima, M., Tohge, T., Konishi, T., Gigolashvili, T., Takamune, M., Sasazaki, Y., Watanabe, M., Nakashita, H., Fernie, A.R., Saito, K., Takahashi, H., Hubberten, H.-M., Hoefgen, R., & Maruyama-Nakashita, A. (2016) Sulfur Deficiency-Induced Repressor Proteins Optimize Glucosinolate Biosynthesis in Plants. *Science advances* 2: e1601087.

Ausma, T., & De Kok, L.J. (2019) Atmospheric H2S: Impact on Plant Functioning. *Frontiers in Plant Science* 10: 743.

Dong, Y., Silbermann, M., Speiser, A., Forieri, I., Linster, E., Poschet, G., Samami, A.A., Wanatabe, M., Sticht, C., Teleman, A.A., Deragon, J.-M., Saito, K., Hell, R., & Wirtz, M. (2019) Sulfur Availability Regulates Plant Growth via Glucose-TOR Signaling. *Nature Communications* 8: 1174.

Drake, B.G., Gonzàlez-Meler, M.A., & Long, S.P. (1997) MORE EFFICIENT PLANTS: A Consequence of Rising Atmospheric CO 2 ? *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 609–39.

Feldman-Salit, A., Veith, N., Wirtz, M., Hell, R., & Kummer, U. (2019) Distribution of Control in the Sulfur Assimilation in *Arabidopsis Thaliana* Depends on Environmental Conditions. *New Phytologist* 222: 1392–1404.

Gotor, C., Laureano-Marín, A.M., Moreno, I., Aroca, Á., García, I., Luis, ·, & Romero, C. (2015) Signaling in the Plant Cytosol: Cysteine or Sulfide? *Amino Acids* 47: 2155–64.

Guan, P. (2017) Dancing with Hormones: A Current Perspective of Nitrate Signaling and Regulation in Arabidopsis. *Frontiers in Plant Science* 8: 1697.

Guo, H., & Ecker, J.R. (2004) The Ethylene Signaling Pathway: New Insights. *Current Opinion in Plant Biology* 7: 40–49.

Hacham, Y., Matityahu, I., & Amir, R. (2013) Light and Sucrose Up-Regulate the Expression Level of Arabidopsis Cystathionine γ-Synthase, the Key Enzyme of Methionine Biosynthesis Pathway. *Amino Acids* 45: 1179–90.

Haneklaus, S., Bloem, E., Schnug, E., de Kok, L.J., & Stulen, I. (2016) Sulfur. In *Handbook of Plant Nutrition*, eds. Allen V. Barker and David J. Pilbeam. Boca Raton: CRC Press, 199–256.

Hawkesford, M.J., & de Kok, L.J. (2006) Managing Sulphur Metabolism in Plants. *Plant, Cell and Environment* 29: 382–95.

Hesse, H., Trachsel, N., Suter, M., Kopriva, S., von Ballmoos, P., Rennenberg, H., & Brunold, C. (2003) Effect of Glucose on Assimilatory Sulphate Reduction in Arabidopsis Thaliana Roots. *Journal of Experimental Botany* 54: 1701–9.

Hubberten, H.M., Drozd, A., Tran, B. V., Hesse, H., & Hoefgen, R. (2012) Local and Systemic Regulation of Sulfur Homeostasis in Roots of Arabidopsis Thaliana. *Plant Journal* 72: 625–35.

Hubberten, H.M., Klie, S., Caldana, C., Degenkolbe, T., Willmitzer, L., & Hoefgen, R. (2012) Additional Role of O-Acetylserine as a Sulfur Status-Independent Regulator during Plant Growth. *Plant Journal* 70: 666–77.

Igamberdiev, A.U., & Kleczkowski, L.A. (2018) The Glycerate and Phosphorylated Pathways of Serine Synthesis in Plants: The Branches of Plant Glycolysis Linking Carbon and Nitrogen Metabolism. *Frontiers in Plant Science* 9: 318.

Izumi, M., Tsunoda, H., Suzuki, Y., Makino, A., & Ishida, H. (2012) RBCS1A and RBCS3B, Two Major Members within the Arabidopsis RBCS Multigene Family, Function to Yield Sufficient Rubisco Content for Leaf Photosynthetic Capacity. *Journal of Experimental Botany* 63: 2159–70.

Jossier, M., Bouly, J.P., Meimoun, P., Arjmand, A., Lessard, P., Hawley, S., Grahame Hardie, D., & Thomas, M. (2009) SnRK1 (SNF1-Related Kinase 1) Has a Central Role in Sugar and ABA

Signalling in Arabidopsis Thaliana. *Plant Journal* 59: 316–28.

Khan, M.S., Haas, F.H., Samami, A.A., Gholami, A.M., Bauer, A., Fellenberg, K., Reichelt, M., Hänsch, R., Mendel, R.R., Meyer, A.J., Wirtz, M., & Hell, R. (2010) Sulfite Reductase Defines a Newly Discovered Bottleneck for Assimilatory Sulfate Reduction and Is Essential for Growth and Development in Arabidopsis Thaliana. *Source: The Plant Cell* 22: 1216–31.

Kimura, Y., Ushiwatari, T., Suyama, A., Tominaga-Wada, R., Wada, T., & Maruyama-Nakashita, A. (2019) Contribution of Root Hair Development to Sulfate Uptake in Arabidopsis. *Plants* 8: 106.

Kopriva, S., & Rennenberg, H. (2004) Control of Sulphate Assimilation and Glutathione Synthesis: Interaction with N and C Metabolism. *Journal of Experimental Botany* 55: 1831–42.

Lee, B.-R., Koprivova, A., & Kopriva, S. (2011) The Key Enzyme of Sulfate Assimilation, Adenosine 5′-Phosphosulfate Reductase, Is Regulated by HY5 in Arabidopsis. *The Plant Journal* 67: 1042–54.

Lin, Y., Zhang, J., Gao, W., Chen, Y., Li, H., Lawlor, D.W., Paul, M.J., & Pan, W. (2017) Exogenous Trehalose Improves Growth under Limiting Nitrogen through Upregulation of Nitrogen Metabolism. *BMC Plant Biology* 17: 1–13.

Maathuis, F.J. (2009) Physiological Functions of Mineral Macronutrients. *Current Opinion in Plant Biology* 12: 250–58.

Martins, M.C.M., Hejazi, M., Fettke, J., Steup, M., Feil, R., Krause, U., Arrivault, S., Vosloh, D., Figueroa, C.M., Ivakov, A., Yadav, P., Piques, M., Metzner, D., Stitt, M., & Lunn, J.E. (2013) Feedback Inhibition of Starch Degradation in Arabidopsis Leaves Mediated by Trehalose 6. *Physiology* 163: 1142–63.

Maruyama-Nakashita, A., Nakamura, Y., Tohge, T., Saito, K., & Takahashi, H. (2006) Arabidopsis Slim1 Is a Central Transcriptional Regulator of Plant Sulfur Response And. *Source: The Plant Cell* 18: 3235–51.

Maruyama-Nakashita, A., Nakamura, Y., Yamaya, T., & Takahashi, H. (2004) Regulation of High-Affinity Sulphate Transporters in Plants: Towards Systematic Analysis of Sulphur Signalling and Regulation. *Journal of Experimental Botany* 55: 1843–49.

Maurino, V.G., & Peterhansel, C. (2010) Photorespiration: Current Status and Approaches for Metabolic Engineering. *Current Opinion in Plant Biology* 13: 248–55.

Ohkama-Ohtsu, N., Kasajima, I., Fujiwara, T., & Naito, S. (2004) Isolation and Characterization of an Arabidopsis Mutant That Overaccumulates O-Acetyl-LSer. *Plant Physiology* 136: 3209–22.

Pandey, R., Zinta, G., Abdelgawad, H., Ahmad, A., Jain, V., & Janssens, I.A. (2015) Physiological and Molecular Alterations in Plants Exposed to High [CO 2] under Phosphorus Stress. *Biotechnology Advances* 33: 303–16.

Prentice, I.C., Farquhar, G.D., Fasham, M.J.R., Goulden, M.L., Heimann, M., Jaramillo, V.J., Kheshgi, H.S., Quéré, C. Le, Scholes, R.J., & Wallace, D.W.R. (2001) The Carbon Cycle and Atmospheric Carbon Dioxide. In *Climate Change 2001: The Scientific Basis*, eds. J T Houghton, Y Ding, D J Griggs, M Noguer, P J van der Linden, X Dai, K Maskell, and C A Johnson. Cambridge: Cambridge University Press, 183–237.

Pu, Y., Luo, X., & Bassham, D.C. (2017) TOR-Dependent and -Independent Pathways Regulate Autophagy in Arabidopsis Thaliana. *Frontiers in Plant Science* 8: 1204.

Reich, M., van den Meerakker, A.N., Parmar, S., Hawkesford, M.J., & De Kok, L.J. (2016) Temperature Determines Size and Direction of Effects of Elevated CO 2 and Nitrogen Form on Yield Quantity and Quality of Chinese Cabbage ed. D. Grantz. *Plant Biology* 18: 63–75.

Rodríguez-Hernández, M. del C., Moreno, D.A., Carvajal, M., & Martínez-Ballesta, M. del C. (2014) Genotype Influences Sulfur Metabolism in Broccoli (Brassica Oleracea L.) Under Elevated CO2 and NaCl Stress. *Plant and Cell Physiology* 55: 2047–59.

Rouached, H., Wirtz, M., Alary, R., Hell, R., Arpat, A.B., Davidian, J.-C., Fourcroy, P., & Berthomieu, P. (2008) Differential Regulation of the Expression of Two High-Affinity Sulfate

Transporters, SULTR1.1 and SULTR1.2, in Arabidopsis. *Plant Physiology* 147: 897–911.

Schnug, E., & Haneklaus, S. (1998) Diagnosis of Sulphur Nutrition. In *Sulphur in Agroecosystems*, ed. Ewald Schnug. Dordrecht: Springer, 1–38.

Smith, F.W., Hawkesford, M.J., Ealing, P.M., Clarkson, D.T., Vanden Berg, P.J., Belcher, A.R., & Warrilow, A.G.S. (1997) Regulation of Expression of a CDNA from Barley Roots Encoding a High Affinity Sulphate Transporter. *The Plant Journal* 12: 875–84.

Stitt, M., & Krapp, A. (1999) The Interaction between Elevated Carbon Dioxide and Nitrogen Nutrition: The Physiological and Molecular Background. *Plant, Cell and Environment* 22: 583–621.

Stitt, M., Lunn, J., & Usadel, B. (2010) Arabidopsis and Primary Photosynthetic Metabolism - More than the Icing on the Cake. *Plant Journal* 61: 1067–91.

Takahashi, H., Kopriva, S., Giordano, M., Saito, K., & Udiger Hell, R.¨. (2011) Sulfur Assimilation in Photosynthetic Organisms: Molecular Functions and Regulations of Transporters and Assimilatory Enzymes. *Annual Review of Plant Biology* 62: 157–84.

Takahashi, H., Smith, F.W., Yamaya, T., & Saito, K. (2002) Two Distinct High-Affinity Sulfate Transporters with Different Inducibilities Mediate Uptake of Sulfate in Arabidopsis Roots. *The Plant Journal* 29: 465–73.

Takahashi, H., Watanabe-Takahashi, A., Smith, F.W., Blake-Kalff, M., Hawkesford, M.J., & Saito, K. (2000) The Roles of Three Functional Sulphate Transporters Involved in Uptake and Translocation of Sulphate in Arabidopsis Thaliana. *The Plant Journal* 23: 171–82.

Thompson, M., Gamage, D., Hirotsu, N., Martin, A., & Seneweera, S. (2017) Effects of Elevated Carbon Dioxide on Photosynthesis and Carbon Partitioning: A Perspective on Root Sugar Sensing and Hormonal Crosstalk. *Frontiers in Physiology* 8: 578.

Vauclare, P., Kopriva, S., Fell, D., Suter, M., Sticher, L., Von Ballmoos, P., Krähenbühl, U., Op Den Camp, R., & Brunold, C. (2002) Flux Control of Sulphate Assimilation in Arabidopsis Thaliana: Adenosine 5¢-Phosphosulphate Reductase Is More Susceptible than ATP Sulphurylase to Negative Control by Thiols. *The Plant Journal* 31: 729–40.

Wawrzyńska, A., & Sirko, A. (2014) To Control and to Be Controlled: Understanding the Arabidopsis SLIM1 Function in Sulfur Deficiency through Comprehensive Investigation of the EIL Protein Family. *Frontiers in plant science* 5: 575.

Wingler, A., Delatte, T.L., O'hara, L.E., Primavesi, L.F., Jhurreea, D., Paul, M.J., & Schluepmann, H. (2012) Trehalose 6-Phosphate Is Required for the Onset of Leaf Senescence Associated with High Carbon Availability 1[W][OA]. *Plant Physiology* 158: 1241–51.

Zhang, Y., Dai, L., Liu, Y., Zhang, Y., & Wang, S. (2017) Identifying Novel Fruit-Related Genes in Arabidopsis Thaliana Based on the Random Walk with Restart Algorithm ed. Quan Zou. *PLOS ONE* 12: 1–15.

Zinta, G., Abdelgawad, H., Peshev, D., Weedon, J.T., Van Den Ende, W., Nijs, I., Janssens, I.A., Beemster, G.T.S., & Asard, H. (2018) Dynamics of Metabolic Responses to Periods of Combined Heat and Drought in Arabidopsis Thaliana under Ambient and Elevated Atmospheric CO 2. *Journal of Experimental Botany* 69: 2159–70.