Identification and characterization of *gcc8*, a glucosinolate-related mutation of *Arabidopsis thaliana*

Dissertation

To obtain the Doctoral Degree in the Natural Sciences
(Dr.rer.nat.)

Submitted to

The Faculty of Natural Sciences I (Biological Science)

Martin Luther University, Halle-Wittenberg, Germany

by

Kalidoss Ramamoorthy

Born on 15.02.1984 in Sivakasi, India

Halle (Saale) August-2015

Reviewers:

Prof. Dr. Steffen Abel

Prof. Dr. Marcel Quint

Dr. Tamara Gigolashvili

Date of Defense: 18-03-2016

DECLARATION BY THE CANDIDATE

I Kalidoss Ramamoorthy, hereby declare that the work presented in this dissertation, entitled "Identification and characterization of gcc8, a glucosinolate-related mutation of Arabidopsis thaliana" is an authentic record of by my own research conducted under the supervision of Professor Dr. Steffen Abel at the Department of Molecular Signal Processing, Leibniz Institute of Plant Biochemistry, Halle (Saale). I have not submitted the results embodied in this dissertation in this dissertation for the award of any other degree or diploma.

Place and Date:

Halle (Saale), 06-08-2015 (Kalidoss Ramamoorthy)

DECLARATION BY THE SUPERVISOR

This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

Place and Date:

Halle (Saale), 06-08-2015 Professor Dr. Steffen Abel

Leibniz Institute of Plant Biochemistry (IPB)

TABLE OF CONTENTS

Table of contents

SUMMARY

Glucosinolates (GS) are sulfur- and nitrogen-containing plant secondary metabolites involved in plant defense against insect herbivores. Their hydrolysis products have many different biological functions, acting as cancer-preventing agents, biopesticides and flavor compounds. GS-derived isothiocyanates are involved in carcinogen metabolism in mammalian liver cells. Quinone reductase (QR) is one such detoxification enzyme induced by 4-methylsulfinylalkyl isothiocyanates in mouse liver cells. Assaying this activity in cultured cells faithfully reports the relative GS content of leaf extracts of the plant model system *Arabidopsis thaliana*. The QR induction bioassay was used in a forward genetic screen of Arabidopsis to identify a number of *glucosinolate content and composition* (*gcc*) mutants, including *gcc8,* with altered levels of GS (Grubb, et al., 2002). The GS content of *gcc8* is only about 10% of the wild-type (Col-0 accession) level, suggesting that it could be one of the major regulatory loci of the GS biosynthetic pathway. To identify the causative mutation of the *gcc8* line, we followed a mapping by next generation sequencing (NGS) approach. We identified the causative mutation as a premature stop codon in the first exon of *SULTR1;2*, a gene encoding one of the major high-affinity sulfate transporters in Arabidopsis. Earlier, *AtSULTR1;2* mutants were identified by their tolerance to selenate (*sel* alleles), a toxic structural analogue of sulfate (Shibagaki, et al., 2002; Kassis, et al., 2007). As the *gcc8* line proved allelic to *sel* mutants, it shows resistance to selenate. The previously reported *sel* mutants exhibit low GS content which was not reported earlier. We observed that the typical sulfur deficiency marker genes were more highly expressed in the mutant background including, *BGLU28* (*At2g44460*, coding for a putative β- glucosidase) which implies that sulfur uptake is impeded in *gcc8* plants. Our experiments with the *gcc8 bglu28-KO* double mutant and enzymatic degradation of GS strongly reject the idea that BGLU28 could degrade the GS under sulfur starvation

conditions; rather, the high expression of BGLU28 might be a secondary effect of sulfur deficiency. Finally, we conclude that reduced sulfur uptake causes down regulation of key enzymes of GS biosynthesis.

LIST OF FIGURES

Introduction

Results

List of figures

List of figures

LIST OF ABBREVIATIONS

GS - glucosinolates

dsGS - desulfo-GS

JA - jasmonate

SA - salicylic acid

ET - ethylene

SULTR - sulfate transporter

SAT - serine acetyl transferase

APS - adenosine 5'-phosphosulfate

OPDA – Oxo-phyto dienoic acid

SAR- systemic acquired resistance

1. INTRODUCTION

a. Secondary Metabolites in Plant Defense Metabolism

There are numerous secondary metabolites present in plants, which have roles in defense, growth and development. In this way, plant secondary metabolism plays a pinnacle role in keeping all the plants' systems working properly. The common role of plant secondary metabolites is to fight against various herbivores, pests and pathogens (Hartmann, 2007). Several classes of secondary metabolites are induced in plant defense including phytoalexins produced upon induction of pathogen invasion, while others called phytoanticipins are constantly present in plant tissues. Major secondary metabolites include cyanogenic glucosides, glucosinolates (GS), non-protein amino acids, alkaloids, phenolics, terpenes, and sterols. Of the many different plant secondary metabolites (Bennett & Wallsgrove, 1994), we are focusing on the GS compounds.

In addition to their roles in plant defense, GS are cancer-preventing agents, biopesticides and flavor compounds. In recent years, certain GS have been identified as potential cancer-prevention agents in a wide range of animal models due to the ability of certain hydrolysis products to induce phase II detoxification enzymes, such as quinone reductase (QR), glutathione-S-transferase, and glucuronosyl transferases (Wittstock & Halkier, 2002; Halkier & Gershenzon, 2006).

b. Glucosinolates

Glucosinolates are S- and N-containing secondary metabolites, which are mainly involved in plant defense. When plant tissue gets damaged, GS are released from the vacuole and come into contact with myrosinase, a β-thioglucosidase. Hydrolysis by myrosinase yields the unstable intermediate, *O*-sulfonyl-thiohydroxamic acid, which can rearrange into various bioactive compounds such as isothiocyanates, thiocyanates, epithiocyanates and nitriles. Based on the precursor amino acid, GS are divided into aliphatic GS, derived from alanine,

leucine, isoleucine, valine, and methionine; aromatic GS, derived from phenylalanine and tyrosine; and indolic GS, derived from tryptophan. Indolic glucosinolates are mainly involved in plant defense against generalist herbivores (Muller, et al., 2010).

c. The Glucosinolate Biosynthetic Pathway

i. Side chain elongation

Biosynthesis of GS is divided into three major steps (Figure 1.1). First, chain elongation of precursor amino acids, in which methionine and other aliphatic amino acids can undergo a process similar to the conversion of the branched-chain amino acid valine to its chain-elongated homolog leucine. This process starts with a deamination by a branched-chain amino acid aminotransferase (BCAT), which gives rise to a 2-oxo acid. The 2-oxo acid enters a cycle of three successive transformations; condensation with acetyl CoA by a methyl thioalkyl malate synthase (MAM), isomerization by an isopropyl malate isomerase (IPMI) and oxidative decarboxylation by an isopropyl malate dehydrogenase (IPM-DH). The result of this series of reactions is addition of a methylene group to the 2-oxo acid (Sonderby, et al., 2010).

ii. Core biosynthetic pathway

The second step in GS biosynthesis is constructing the GS core structure. The precursor amino acids are converted into aldoximes by cytochromes P450 of the CYP79 family. CYP79B2 and CYP79B3 both metabolize tryptophan, CYP79A2 uses phenylalanine as a substrate, CYP79F1 converts all chain-elongated methionine derivatives and CYP79F2 only converts the long chain methionine derivatives (Hansen, et al., 2001; Chen, et al., 2003). Next, aldoximes are oxidized to activated compounds by cytochromes P450 of the CYP83 family. In Arabidopsis, the two members of this family display some promiscuity with regard to side-chain structure, but it is likely that *in vivo* CYP83B1 metabolizes both the tryptophan-

derived and phenylalanine derived acetaldoximes, and CYP83A1 converts aliphatic aldoximes. The substrate specificity of the CYP83 proteins is not absolute (Naur, et al., 2003). The sulfur donor that is conjugated to the activated aldoxime was long thought to be cysteine (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). However, recent investigations indicate glutathione (GSH) a more likely sulfur donor (Bednarek, et al., 2009). The initial conjugation product is processed by γ-glutamyl peptidase (*GGP1*) to produce cysteine-glycine conjugates (Geu-Flores, et al., 2011). The resulting *S*-alkyl-thiohydroximates are converted to thiohydroximates by the *C-S* lyase SUR1 (Mikkelsen, et al., 2004). Thiohydroximates are in turn *S*-glycosylated by glucosyltransferase UGT74B1 to form desulfo-GS (dsGS). The glycosylation gives rise to dsGS, which are finally sulfated by the sulfotransferases SOT16, 17 or 18 to form complete glucosinolate (Piotrowski, et al., 2004).

iii. Side chain modifications

The biological activity of GS is influenced by the structure of the side chains (Stotz, et al., 2011). Aliphatic GS can undergo oxygenation, hydroxylation, dehydrogenation, and benzoylation. Indolic GS can undergo hydroxylation and methoxylation. Arabidopsis typically contains four different indole GS: the unmodified indolyl-3-methyl GS (I3M) and its downstream relatives 4-hydroxy I3M (4OH-I3M), 4-methoxy I3M (4M-I3M), and 1-methoxy I3M (1M-I3M) (Kliebenstein, et al., 2001; Brown, et al., 2003). *CYP81F2* is responsible for the 4-hydroxlylation of I3M. Knockouts in *CYP81F2* contained less 4OH-I3M and 4M-I3M and also accumulate high levels of I3M (Sonderby, et al., 2010).

So far, over 200 different GS have been identified in *Arabidopsis thaliana* (Halkier & Gershenzon, 2006). There are still several unknown side chain modification genes. Side chain structure modulates the various biological activities and specificity in defense against plant pests and pathogens. Side chain modifications are co-evolved with pathogen action. When the

pathogen changes its mode of action against plant, then the plant must also change their mode of defense.

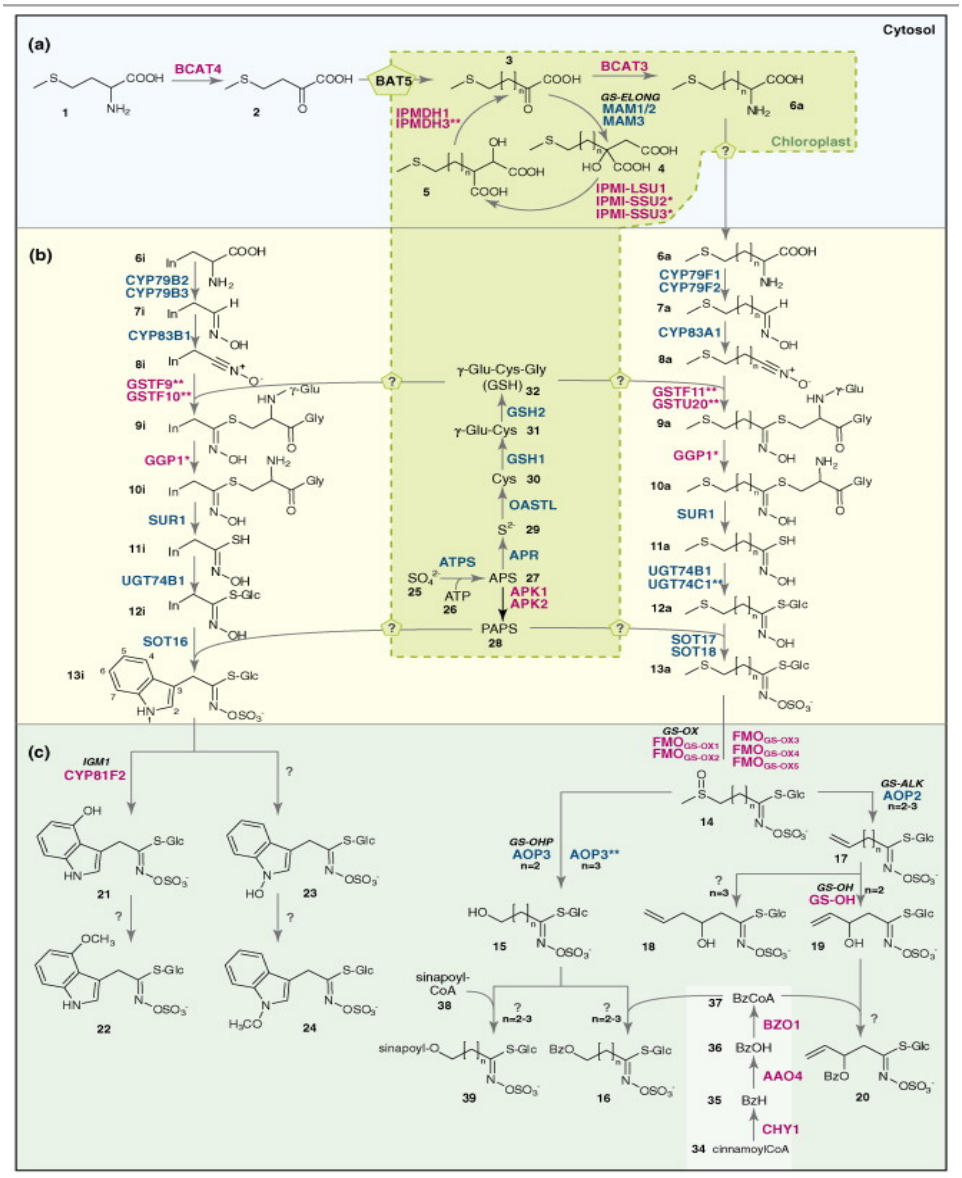

Figure 1. 1: The GS biosynthetic pathway in *Arabidopsis* sp. a) Chain elongation machinery, b) core biosynthetic pathway, c) secondary modifications. Figure reproduced from (Sonderby, et al., 2010).

d. MYB Transcription Factors

The MYB family of proteins is large, functionally diverse and present in eukaryotes. MYB proteins are key factors in development, metabolism and response to biotic and abiotic stresses (Oh & Reddy, 1999). Many MYB proteins function as transcription factors with varying numbers of DNA-binding MYB domains. R2R3-MYB comprises a large subfamily within the plant MYB family which is involved in plant specific processes (Stracke, et al., 2001). The R2R3-MYB proteins of subgroup 12 regulate GS biosynthesis and *AtMYB28*, *AtMYB29* and *AtMYB76* are regulators of aliphatic GS biosynthesis (Hirai, et al., 2007). However, *AtMYB34*, *AtMYB51* and *AtMYB122* regulate the production of indolic GS in roots and late stage rosette leaves (Dubos, et al., 2010).

The characterized MYB factors are obviously key players in the signal transduction chain, leading to biotic stress perception and increased GS biosynthesis. *MYB28, MYB29* and *MYB76* show positive reciprocal activation of aliphatic GS biosynthesis (Li, et al., 2013). *MYB28* overexpression caused an increase in *MYB29* and *MYB76* transcripts, whereas an increase in *MYB29* transcripts resulted in accumulation of *MYB76* transcripts (Sonderby, et al., 2010). *MYB76* is the weakest regulator of aliphatic GS biosynthesis (Sonderby, et al., 2010). Expression of *MYB28* cannot be induced by up regulation of *MYB28* and *MYB76* (Sonderby, et al., 2010). Regulation of indolic GS are induced by *MYB51*, *MYB122* and *MYB34*, a family of R2R3 MYB transcription factors (Celenza, et al., 2005; Gigolashvili, et al., 2007a; Malitsky, et al., 2008). On the other hand the positive regulators of aliphatic GS accumulation have been shown to down-regulate the expression of regulators of indolic GS accumulation i.e., *MYB34*, *MYB122* (Gigolashvili, et al., 2008b).

e. Jasmonic Acid and Plant Defense

Jasmonic acid (JA) is one of the three major plant defense hormones; the other two are salicylic acid (SA) and ethylene (ET). Apart from plant defense, JA has a role in fruit ripening, pollen development, root growth and tendril coiling. Early studies showed that exogenous JA or MeJA can promote senescence and act as a growth regulator. Subsequent research revealed that JA specifically alters gene expression and that wounding and elicitors could cause JA/MeJA accumulation in plants (Creelman & Mullet, 1997). The level of JA in plants varies as a function of tissue and cell type, developmental stage, and in response to several different environmental stimuli. JA modulates the expression of numerous genes and influences specific aspects of plant growth, development, and response to abiotic and biotic stress. JA induces tryptophan-derived indolic GS but not aromatic or aliphatic GS in plants (Brader, et al., 2001; Mikkelsen, et al., 2003; Sasaki-Sekimoto, et al., 2005). Some results are contradictory, for example sometimes aliphatic GS are affected and sometimes not. So our understanding of this relationship is clearly incomplete and even more interesting.

f. Salicylic Acid and Plant Defense

Salicylic acid (SA) has been shown to play a role in plant disease resistance, by triggering Systemic Acquired Resistance (SAR) and also local defense responses (Vlot, et al., 2009). This SAR is effective against necrotrophic as well as biotrophic pathogens (Glazebrook, 2005). SA induces the accumulation of GS in oil seed rape (Kiddle, et al., 1994); but results reported in Arabidopsis have been quite variable, with some groups seeing effects and others not (Halkier & Gershenzon, 2006). However, there is very good reason to believe that aliphatic GS may be involved in SA-mediated resistance against biotrophs: a recent paper demonstrated that the ability of *Pseudomonas* to infect Arabidopsis depends on its ability to detoxify isothiocyanates (Fan, et al., 2011).

In addition, the complex crosstalk between the JA and SA pathways, and the importance of JA for GS regulation discussed above, implicates SA in indirect regulation of GS accumulation at the very least.

g. Plant Sulfur Assimilation

In nature, sulfur is available in both organic and inorganic forms. It is essential for many living organisms being found in reduced form in amino acids, peptides and proteins, in iron-sulfur clusters and many other cofactors. Oxidized sulfur compounds act as terminal electron acceptors in respiration for sulfur-reducing bacteria (Truper & Fischer, 1982). Prokaryotes, yeasts and plants take up their sulfur in inorganic form. Then later by reduction to sulfide, which is then assimilated into organic compounds. Sulfur is one of the essential macro nutrients most commonly limiting plant growth (Leustek & Saito, 1999).

Plants take up inorganic sulfate through high affinity sulfate transporters (SULTR) and reduced form of sulfur such as sulfur dioxide and hydrogen sulfide from the atmosphere (Davidian & Kopriva, 2010). Plants have multiple SULTR with different functions and properties. The first SULTR were cloned from a tropical legume, *Stylosanthes hamate* by Smith et al. (1995), who performed functional complementation of a yeast SULTR mutant. Arabidopsis has 14 *SULTR* genes, divided into five subgroups based on their sequence similarity and function. All the subgroups except group 5 shows high sequence similarity with the originally identified *SULTR* cDNA clone *SHST1*, where the subgroup 5 shows much less sequence and structural similarity and the function is yet to be verified. High affinity SULTR, group 1, are responsible for uptake of sulfate from the soil into the root cells (Shibagaki, et al., 2002; Yoshimoto, et al., 2002). Low affinity SULTR, group 2, are required for translocation of sulfate within the plant; some are localized in the tonoplast and are responsible for sulfate efflux from the vacuole (Kataoka, et al., 2004b). Very little is known about the function of

SULTR of groups 3 and 5, except SULTR3;5 increases the rate of root-to-shoot sulfate translocation in Arabidopsis (Kataoka, et al., 2004a).

In past decade the pathway of sulfate assimilation in plants been studied intensively. Biochemical and molecular methods reveal that adenosine 5'-phosphosulfate reductase, which is the key enzyme of the pathway, reduces adenosine 5'-sulfate into sulfite (Koprivova, et al., 2000; Kocsy, et al., 2000; Bick, et al., 2001; Tsakralides, et al., 2002; Vauclare, et al., 2002). Intracellular sulfate is further metabolized into a wide variety of primary and secondary metabolites. For assimilation into amino acids such as cysteine and methionine, sulfate has to be transported into plastids and activated by adenylation to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (ATPS). APS is reduced to sulfite by APS reductase (APR); the electrons are derived from glutathione. Sulfite is further reduced by a ferredoxin-dependent sulfite reductase (SiR) to sulfide, which is incorporated by O-acetyl serine (thiol) lyase OASTL into the amino acid skeleton of *O*-aceyl serine (OAS) to form cysteine. OAS is synthesized by acetylation of serine with acetyl-Coenzyme A, catalyzed by serine acetyl transferase (SAT) (Leustek, et al., 2000; Suter, et al., 2000). SAT and OASTL from a multi-enzyme complex called cysteine synthase (Hell, et al., 2002). Cysteine can be directly incorporated into proteins or peptides, such as glutathione (GSH), the most abundant low-molecular weight thiol, with a plethora of functions in plant stress defense, redox regulation, and sulfur storage and transport. Alternatively, cysteine is further metabolized and serves as a donor of reduced sulfur for synthesis of methionine, iron-sulfur centers, and various coenzymes and secondary metabolites. An intermediate of the sulfate assimilation pathway, sulfite, is metabolized into sulfo-lipids, essential components of chloroplast membranes (Sanda, et al., 2001). Sulfur reduction is localized exclusively in plastids, whereas cysteine is

synthesized in all three compartments which can do protein synthesis: plastids, mitochondria and the cytosol (Leustek, et al., 2000).

Apart from its role in protein structure and function as S-containing amino acids and a structural component, sulfur is also present in plant metabolites in the oxidized state, as sulfated metabolites that play important roles in plant defense with respect to various biotic and abiotic stresses. Among various plant secondary metabolites, GS are well studied, as actors against herbivores and pathogens of various Brassicales (Mikkelsen, et al., 2002). Glucosinolates are also responsible for the flavor and odor of cruciferous vegetables, and their degradation products include isothiocyanates, some of which have anti-cancer activity (Mithen, 2001; Fahey, et al., 2001). Flavonoids are also another group of sulfated secondary metabolites present in more than 250 species of 32 families (Barron, et al., 1988), which are involved in regulation of plant growth by detoxifying reactive oxygen species (Varin, et al., 1997). Apart from those metabolites, jasmonate derived sulfated metabolites are also involved in plant defense in Arabidopsis (Gidda, et al., 2003), sulfated β 1, 3- glucan (Menard, et al., 2004), several other secondary metabolites undergo sulfation reaction, which is the transfer of a sulfo-group, by sulfotransferases (SOT) (Klein, 2004). 3'-phosphoadenosyl 5'-phosphosulfate (PAPS) acts as a sulfate donor in SOT reactions with compounds containing a free hydroxyl group as an acceptor. Eukaryotic organisms have multiple SOT isoforms in order to provide activity against a diversity of biological acceptors (Klein, 2004). APS kinase phosphorylates APS to form PAPS. This reaction diverts APS from the primary sulfate assimilation pathway in plastids (Leustek, et al., 2000).

h. Sulfur Assimilation in *Arabidopsis thaliana*

Sulfur assimilation studies in *Arabidopsis thaliana* were greatly facilitated a decade ago after the sequencing the whole genome project. Early molecular cloning experiments and

characterization of the encoded proteins revealed the complexity of the sulfur assimilation pathway (Hell, et al., 2002; Leustek, et al., 1994; Bruhl, et al., 1996; Gutierrez-Marcos, et al., 1996; Setya, et al., 1996; Noji, et al., 1998). Most of the sulfate assimilation pathway enzymes are encoded by small gene families (Takahashi, et al., 1997); an exception is SiR. Among these, the SULTR family is well understood (Takahashi, et al., 2000; Shibagaki, et al., 2002; Yoshimoto, et al., 2002; Kataoka, et al., 2004a; Kataoka, et al., 2004b). Every isoform of SULTR has a specific localization, affinity towards sulfate, and shows unique functions. The same is true of another family of sulfate assimilation genes which is seine acetyltransferase (SAT) gene family which is known to be involved in regulating cysteine biosynthesis (Kawashima, et al., 2005). For other gene families such as *ATPS*, *APR*, and *APS kinase*, are very little is known about specific functions of individual members. GSH biosynthesis in Arabidopsis is organized differently from other plant species. For example, the first enzyme of GSH synthesis, γ-glutamyl cysteine synthetase (γ-ECS), seems to be exclusively localized in plastids and the second one, GSH synthetase (GSHS), is dually targeted to plastids and the cytosol from a single gene (Wachter, et al., 2005). Other plant species have multiple copies of both genes and γ-ECS activity occurs also in the cytosol (Hell & Bergmann, 1990).

The presence of cDNA sequences helped greatly with the molecular level elucidation of sulfate assimilation regulation. Early reports showed induction of mRNA levels of sulfate assimilation. For example, low affinity *SULTR2;1* and an isoform of *APR* and *SAT* were strongly up-regulated by 2 days of sulfate starvation (Takahashi, et al., 1997) and others showed sulfate assimilation up-regulated by JA (Harada, et al., 2000). The first detailed study including enzyme activity, mRNA and protein accumulation, metabolites (Cys and GSH) and fluxes through the pathway was done on APR activity in a diurnal rhythm (Kopriva, et al., 1999). Sulfur assimilation is strongly interconnected with the nitrogen assimilation pathway

(Reuveny, et al., 1980; Brunold, et al., 1987). Three days of nitrogen starvation led to a specific decrease of APR2 activity in *Arabidopsis thaliana*, whereas OASTL and thiol contents were not affected (Koprivova, et al., 2000). Still other work focused on APR induction and protein activity in sulfur assimilation (Bick, et al., 2001).

There were several potential signals identified as regulators of sulfur assimilation, the most prominent among them is the cysteine precursor OAS. By inducing mRNA accumulation of all sulfur assimilation genes, OAS drastically increases flux through the sulfur assimilation pathway (Koprivova, et al., 2000). OAS acts as mediator of sulfur assimilation regulation by accumulating during sulfur deficiency, which affects cysteine synthase and sulfur assimilation genes (Hell, et al., 2002). OAS also up-regulates the activity of the β_{SR} sulfur-responsive region of the β subunit of soybean conglycinin (Awazuhara, et al., 2002; Ohkama-Ohtsu, et al., 2004). Another publication strengthened that idea when a transcriptome analysis suggested a role of OAS as a general regulator of gene expression (Hirai, et al., 2003). There were 850 genes whose mRNA level responded to 1 mM OAS treatment. Many other publications support the idea that OAS is the main regulator of sulfur assimilation, although many details of the mechanisms of regulation are yet to be elucidated (Kopriva, 2006; Davidian & Kopriva, 2010).

Another important compound that regulates sulfur assimilation is glutathione (GSH). Sulfate uptake and assimilation is strongly regulated by reduced forms of sulfur such as H_2S, cysteine or GSH (Brunold, et al., 1987; Lappartient, et al., 1999; Westerman, et al., 2001). Either cysteine or GSH feeding leads to reduced APR activity and mRNA levels in Arabidopsis root cultures (Vauclare, et al., 2002). The activity and the mRNA levels of APR are induced by carbohydrates in dark (Kopriva, et al., 1999; Kopriva, et al., 2002; Hesse & Hoefgen, 2003).

Sulfur assimilation control by phytohormones is less well-known, but a few recent publications show that phytohormones play an important role in sulfur nutrition (Ohkama, et al., 2002; Maruyama-Nakashita, et al., 2005; Maruyama-Nakashita, et al., 2004). Plants expressing GFP controlled by the promoter of the sulfur responsive gene β-conglycinin were treated with various phytohormones for a sulfur starvation response shows increased expression of sulfur response genes such as *APR1* and *SULTR2;2* in the presence and absence of sulfur (Ohkama, et al., 2002). The expression of the high affinity SULTR and sulfur uptake were both repressed by cytokinins in plants which expressed GFP fused to the promoter of *SULTR1;2* (Maruyama-Nakashita, et al., 2004). Cytokinins were previously known to regulate nitrogen assimilation (Samuelson, et al., 1995; Takai, et al., 2001; Collier, et al., 2003). More recent publications show cytokinins are involved in phosphate uptake (Martin, et al., 2000), which clearly shows cytokinins play a general role in uptake of nutrients. Auxin is not known to be directly involved in sulfur assimilation, but one of the genes, *NIT3* (nitrilase3) involved in biosynthesis of IAA is strongly induced by sulfur deficiency (Kutz, et al., 2002). The promoter sequence of *SULTR1;2* has a cis-acting element conferring sulfur starvation responsiveness (Maruyama-Nakashita, et al., 2005). This element resembles an Auxin Response Factor binding site.

Jasmonates also involved in sulfur assimilation but did not affect the expression via the sulfur responsive element (Ohkama, et al., 2002). Arabidopsis plants treated with methyljasmonate (MeJA) show a fast and transient increase in expression of mRNA levels of many sulfur assimilation genes and those involved in GSH biosynthesis (Xiang, et al., 2001; Harada, et al., 2000; Jost, et al., 2005). Jasmonate treatment does not induce the mRNA levels of high affinity SULTR, indicating that sulfate uptake may be independent of JA treatment. Sulfur starvation conditions induce many JA biosynthetic genes in Arabidopsis (Hirai, et al., 2003;

Maruyama-Nakashita, et al., 2003; Nikiforova, et al., 2003). Since JA is involved in signal transduction in stress responses, it is not surprising that it induces the expression of sulfur assimilation genes (Reymond & Farmer, 1998). The fact that Arabidopsis plants treated with either abscisic acid (ABA) or SA, show induction of GSH biosynthesis illustrates the relationship between sulfate assimilation and GSH biosynthesis in stress defense responses (Jiang & Zhang, 2001; Fodor, et al., 1997). Cytosolic OASTL mRNA is induced upon ABA treatment, indicating the hormone might control sulfur metabolism rather than having a direct effect on sulfur assimilation (Barroso, et al., 1999).

Global transcriptome analysis for the sulfur starvation response shows 2700 genes were differentially regulated. Among many other induced genes, *SULTR* and *APR* genes are the most prominent sulfur assimilation genes. JA and auxin biosynthetic genes are also up-regulated upon sulfur starvation (Hirai, et al., 2003; Maruyama-Nakashita, et al., 2003; Nikiforova, et al., 2003). In sulfur starvation conditions, Arabidopsis plants induce thioglucosidase genes (Maruyama-Nakashita, et al., 2003) and likely degrade GS and utilize the sulfur for primary metabolism (Wittstock & Halkier, 2002). Metabolome analysis of 13 day old sulfur starved plants showed 11.5% of the 6000 metabolites were significantly affected (Nikiforova, et al., 2003). Among those metabolites, the tryptophan concentration is 28 fold higher and while other compounds like thiols, lipids, and chlorophylls are decreased (Nikiforova, et al., 2005). Few recent reports have employed genetic approaches to study sulfur assimilation (Shibagaki, et al., 2002; Ohkama-Ohtsu, et al., 2004). Identification and functional characterization of *SULTR1;2* resulted from a screen for selenate resistance, revealing its major role in sulfate uptake (Shibagaki, et al., 2002).

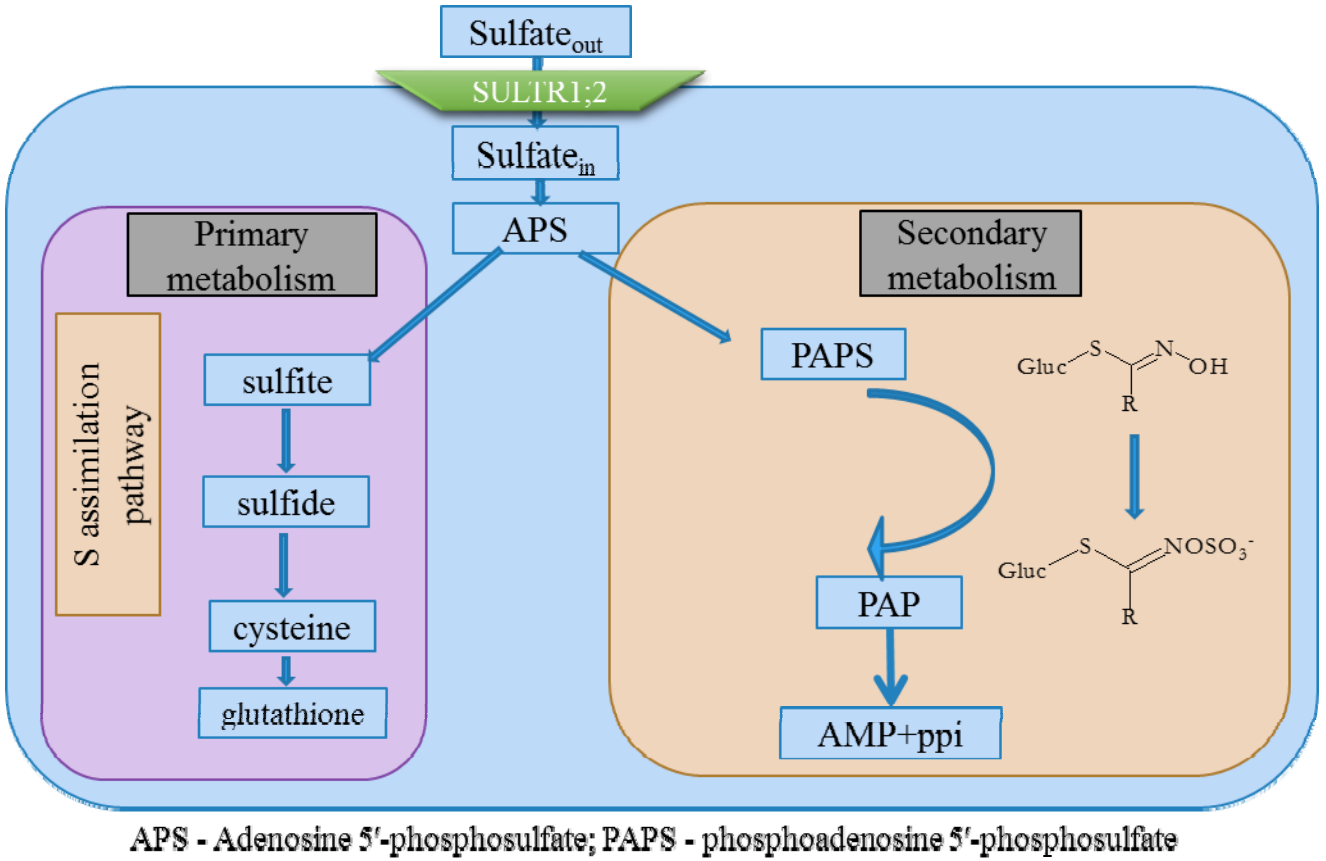

Figure 1. 2: Schematic representation of the sulfur assimilation pathway in *Arabidopsis thaliana*.

i. Sulfate Transporter Proteins

Based on similarity of the protein sequence, kinetic properties and tissue-specific localization, SULTR were classified into four groups (Grossman & Takahashi, 2001). Each group member has specialized functions in uptake and transport of sulfate from root to final destination. Group 1 SULTR encode high affinity SULTR and are mainly localized in roots. Their primary role is sulfur uptake from outside environment; they are the best studied SULTR (Smith, et al., 1995; Shibagaki, et al., 2002; Yoshimoto, et al., 2002). Group 1 SULTR genes differ in their responsiveness to changes in the nutritional status of the plant. *SULTR1;2* is always expressed (constitutive expression) irrespective of either sulfur availability or sulfur depletion conditions. In contrast, *SULTR1;1* is highly induced in sulfur depletion conditions. (Yoshimoto, et al., 2002). Identification of selenate resistance of *SULTR1;2* mutants (*sel1* alleles) shows its important role in sulfate uptake from environment to root cells (Shibagaki, et al., 2002). Group 2 transporters have low affinity towards sulfate and are expressed in vascular tissues, suggesting their role is internal translocation of sulfate (Smith, et al., 1995; Takahashi, et al., 1997; Takahashi, et al., 2000). Group 3 SULTR are expressed in leaf tissues and show less sequence similarity with other families. Their physiological function is not yet verified (Leustek & Saito, 1999). Group 4 transporters possess an N-terminal plastidial signal peptide sequence; they are localized in plastids and show high sequence similarity with Group 1 SULTR (Takahashi, et al., 2000).

j. Screening for GS-related Mutants in *Arabidopsis thaliana*

Natural isothiocyanates derived from aromatic and aliphatic GS are effective chemoprotective agents that block chemical carcinogens. Various studies have shown that isothiocyanates target mammalian drug-metabolizing enzymes and their coding genes, resulting in reduced carcinogen-DNA interactions and in increased carcinogen detoxification (Talalay & Zhang,

1996). For example, 4-methylsulfinylbutyl isothiocyanate inhibits Phase I enzyme-mediated activation of procarcinogens and induces Phase II detoxification enzymes such as quinone reductase (QR) and glutathione *S*-transferase (GST) in hepatoma cells (Barcelo, et al., 1998; Maheo, et al., 1997). It effectively blocks mammary tumor formation in rats and is among the most powerful natural inducers of chemo protective enzymes (Prestera, et al., 1993; Fahey, et al., 1997). The chemopreventive properties of natural isothiocyanates have renewed interest in GS biosynthesis.

In the GS biosynthetic pathway, discovery of the core synthesis genes is likely nearing completion. Some regulatory genes are known, but GS accumulation responds too many environmental and development cues: the complexity of this regulation indicates that (possibly many) other genes are likely involved in GS regulation. Other unknown genes include transporters and many side-chain modification enzymes. Another justification for a forward genetic screen is that identification of regulators can facilitate discovery of downstream genes.

To identify new regulatory genes of the GS pathway, the Abel group performed a forward genetic screen to identify *Arabidopsis* mutant plants (EMS-mutagenized plants and T-DNA activation tagged lines) with altered levels of GS (Grubb et al., 2002; Levy et al., 2005). The primary bioassay for leaf GS content was based on the ability of the derivative methylsulfinylalkyl isothiocyanates, generated upon leaf tissue wounding or destruction, to induce QR activity in murine hepatoma cells. Colorimetric detection of QR activity in hepatoma cells cultured in microtiter plates allowed for robust high-throughput analysis of leaf extracts of chemically mutagenized plants (see Figure 1.3).

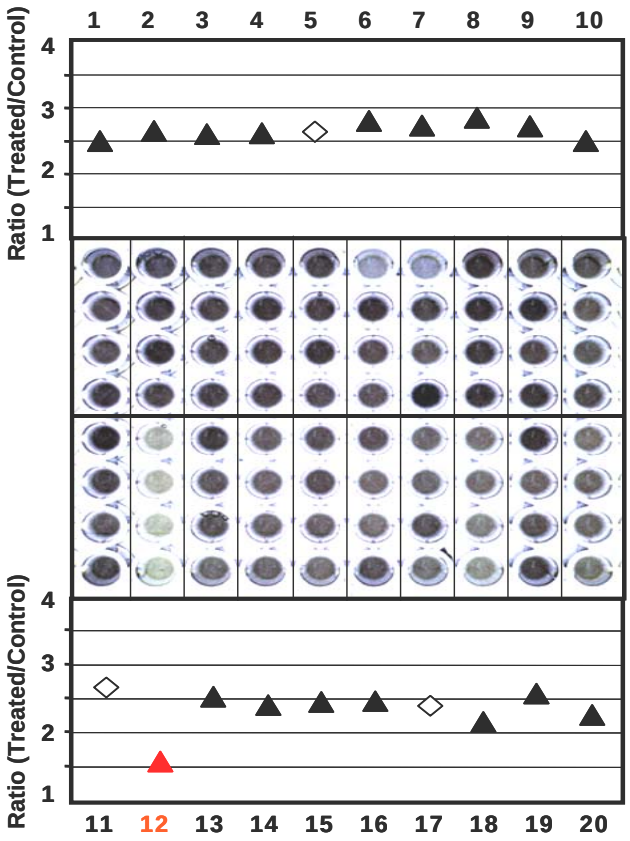

Figure 1. 3: Screening of EMS-mutagenized *Arabidopsis* plants for altered GS accumulation. Shown is an example of the primary colorimetric bioassay of QR activity in murine hepatoma cells induced by *Arabidopsis* leaf extracts. Open diamonds: wild-type (Col-0) plants. Black triangles: M2 progeny of EMS-mutagenized seeds. Red triangle: mutant with low QR inducer potency and low GS levels (Grubb, et al., 2002).

The screening of T-DNA activation-tagged lines (harboring a strong 35S CaMV promoter) resulted in the isolation of several insertion mutants with lower or higher GS accumulation due to insertional gene inactivation or *trans* gene activation by the 35S CaMV promoter, respectively. Among the high GS lines, overexpression of *IQD1* (At3g09710) was shown to be responsible for the GS chemotype (Levy et al., 2005). IQD1 is a calmodulin-binding protein that is localized to the cell nucleus and to the microtubules (Bürstenbinder et al., 2013). IQD1 overexpression upregulates genes coding for enzymes of the indolic GS

biosynthetic pathway and downregulates the aliphatic branch of the GS pathway (Levy et al., 2005; Abel et al., 2013).

The screening of EMS mutant lines resulted in the isolation of several lines with altered *glucosinolate content* and *composition*, which were named *gcc1* to *gcc9* (Grubb, et al., 2002). Of these lines, *gcc8* stands out because it accumulates very low levels of all GS. However, GS of all classes are detectable, and their relative proportions are similar to wild-type, indicating that *gcc8* may represent a new regulatory locus.

THESIS OBJECTIVES

Molecular identification of the *GCC8* locus was the primary objective of my PhD research, which I accomplished by a Next Generation Sequencing approach together with high-resolution mapping of the *gcc8* mutation. Because the *GCC8* gene encodes a sulfur transporter (*AtSULTR1;2*) the second objective was a more detailed characterization of the *gcc8* mutant in relation to sulfur metabolism in *Arabidopsis thaliana*.

2. RESULTS

a. The *gcc8* Mutant Phenotype

When we grow *gcc8* plants along with wild-type (Col-0) on ½ MS medium for 18 days we observed a very peculiar phenotype for the mutant line. The *gcc8* plants are small and appear to accumulate high levels of anthocyanins compared to wild-type plants (Figure 2.1).

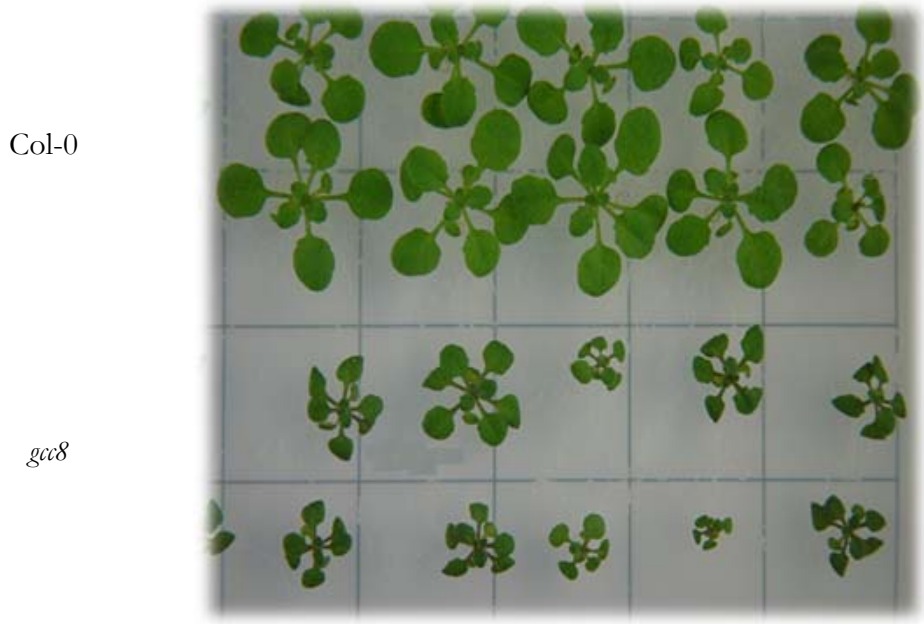

Figure 2. 1: Visual phenotype of wild-type (Col-0) and *gcc8* plants grown on ½ MS medium for 18 days.

b. Glucosinolate Profiling of *gcc8*

Wild-type (Col-0) and *gcc8* mutant plants were grown on horizontal round plates with ½ MS medium for 18 days and harvested for measurement of individual GS. HPLC chromatograms show the GS profiles of wild-type and *gcc8* plant in which the mutant has all the individual GS but their content is very low (about 10 % of Col-0; see Figure 2.2). The main objective of the project is to find out the causative mutation for this dramatic GS variation. To identify the mutation we followed fine mapping and whole-genome sequencing approach.

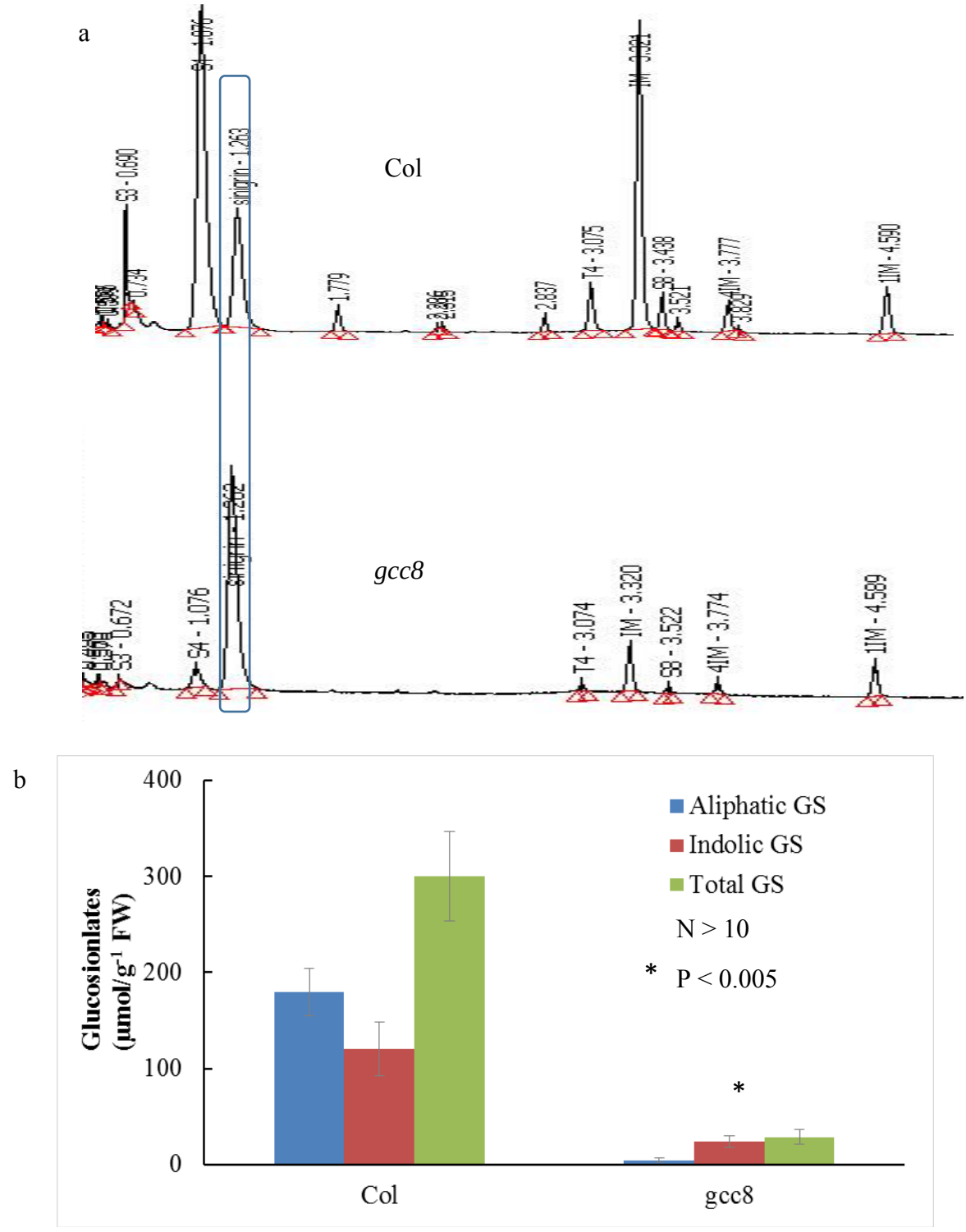

Figure 2. 2: (a) HPLC chromatogram show GS profiling of wild-type (Col-0) and *gcc8* plants. Individual peaks are various GS. The common peak highlighted in both is an internal standard added during processing of samples. In *gcc8*, all the individual GS are present but in far lower amounts. (b) The bar diagram shows quantification of wild-type and *gcc8* GS levels. S3-glucoiberin; S4-glucoraphanin; T4-glucoerucin; IM-glucobrassicin; S8-glucohirsutin; 4IM-methoxy glucobrassicin; 1IM-neoglucobrassicin.

c. Anthocyanin Measurement

The *gcc8* mutant plants accumulate four-fold higher anthocyanin levels than the Columbia wild-type (Figure 2.3).

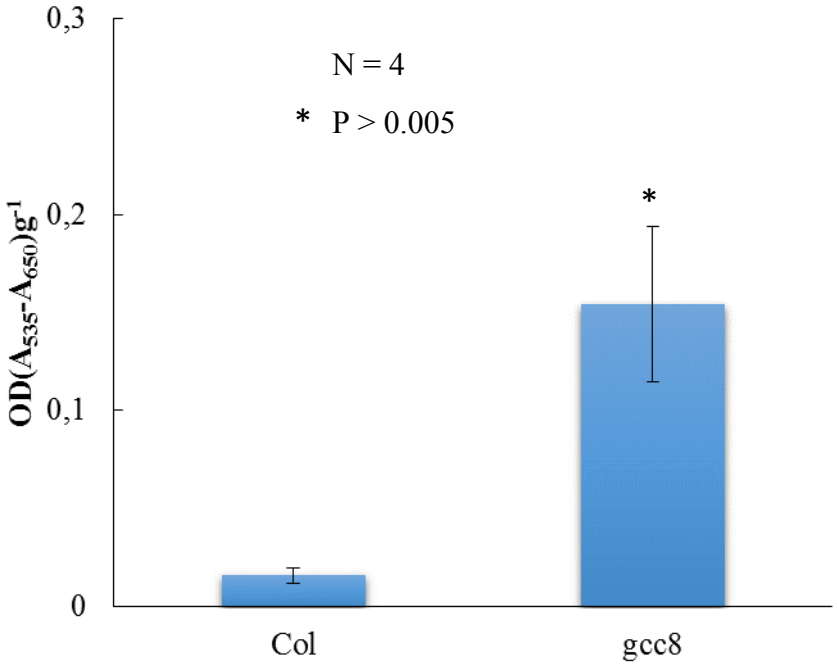

Figure 2. 3: Anthocyanin measurement of wild-type (Col-0) and *gcc8* plants. *gcc8* plants accumulate more anthocyanins (4-fold higher) than Col-0 plants, (*statistically significant).

d. Identification of the *gcc8* Locus by Map-based Cloning

A *gcc8* mutant plant was crossed with Landesberg *erecta* (Ler-0) for generating an outcross population and also to Columbia (Col-0) 3 times for creation of a back-crossed line 3 (BC3). When we measured the GS chemotype in the F_1 plants (of cross between *gcc8* X Ler_F_1 and *gcc8* X Col_F_1), we concluded that the GS chemotype is caused by a recessive *gcc8* mutation (Figure 2.4).

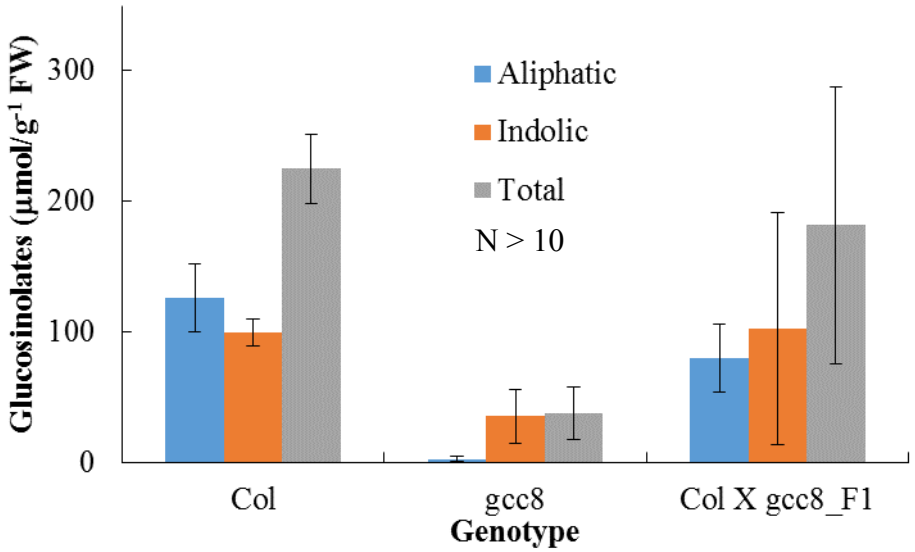

Figure 2. 4: GS content of Col-0 × *gcc8*_F_1 progeny (N>10). GS levels of F_1 plants are significantly higher than those of the *gcc8* mutant line. This experiment shows the *gcc8* phenotype is caused by recessive mutations in one or more genes

i. Reconfirmation of the mapping population

Plants of the earlier generated mapping population were reconfirmed for their low GS chemotype before pooling individuals for bulk DNA isolation for next generation sequencing. From each line of the mapping population, plants were grown in ½ MS media for 18 days, harvested and the GS extracted. Figure 2.5 shows the GS profiles of all plants of the mapping population which were included for bulk DNA preparation. Similar to the *gcc8* mutant, they are all low in GS.

ii. Next generation sequencing of the mapping population

The confirmed plants of the mapping population were pooled to prepare bulk DNA for next generation sequencing. Some important raw data statistics are given in sequence summary (Table 2.1), which shows that the raw data are quite good.

GC rate %	37.01
Q20 rate %	95.66
Reads (M)	83.84
Bases (G)	7.55

Table 2. 1: Summary of results from next generation sequencing

The raw data were mapped against the Col-0 reference genome (TAIR 10 release) to map the SNPs against chromosome positions. Recovered SNPs may arise from polymorphism between Col-0 and Landsberg accessions as well as from EMS mutagenesis. The alignment statistics are given in the mapping summary (Table 2.2).

Coverage rate %	99.82
Map reads rate %	93.17
Unique hits rate %	78.56
Effective depth (x)	56.88

Table 2. 2: Next generation sequencing results (mapping summary)

The SNPs were mapped against the Col-0 reference genome throughout the five chromosomes and the results are plotted against chromosome position (Figure 2.6). The horizontal axis represents chromosome position and the vertical axis represents the number of SNPs at a particular position (summed in a 50 Mbp window). The number of SNPs varies from one chromosome to another. We see a strikingly different pattern at the end of first chromosome, where the maximum number of SNP is almost zero for about 4 Mbp, which indicates that this region is entirely derived from the Col-0 genome in plants of the mapping population. Since we performed EMS mutagenesis in the Col-0 background, one can deduce the causative mutation is at end of chromosome 1. Therefore, we took a closer look at the chromosome 1 SNPs by calculating the frequency of the non-reference alleles. Figure 2.7

represents the proportion of best hit reads which represent the non-Columbia allele at a given position. At the end of the chromosome 1, the number of SNPs are reduced drastically, especially those with a frequency between 50 – 80% which are most likely Columbia – Landsberg polymorphisms. The rectangular box in this regions highlights ~20 apparent SNPs for which all SNPs (or nearly all) were the non-Columbia alleles. These were considered candidates for the mutation causing the *gcc8* phenotype.

To identify the causative mutation, we filtered the putative candidates according to gene annotations (whether the SNPs were located in genes or inter genic regions) and the nature of the mutations (whether or not they were synonymous). Eleven SNPs in intergenic regions of the genome were eliminated based on the idea that they were less likely to be responsible for the phenotype. The nine remaining SNPs fall in the genes listed in Figure 2.8.

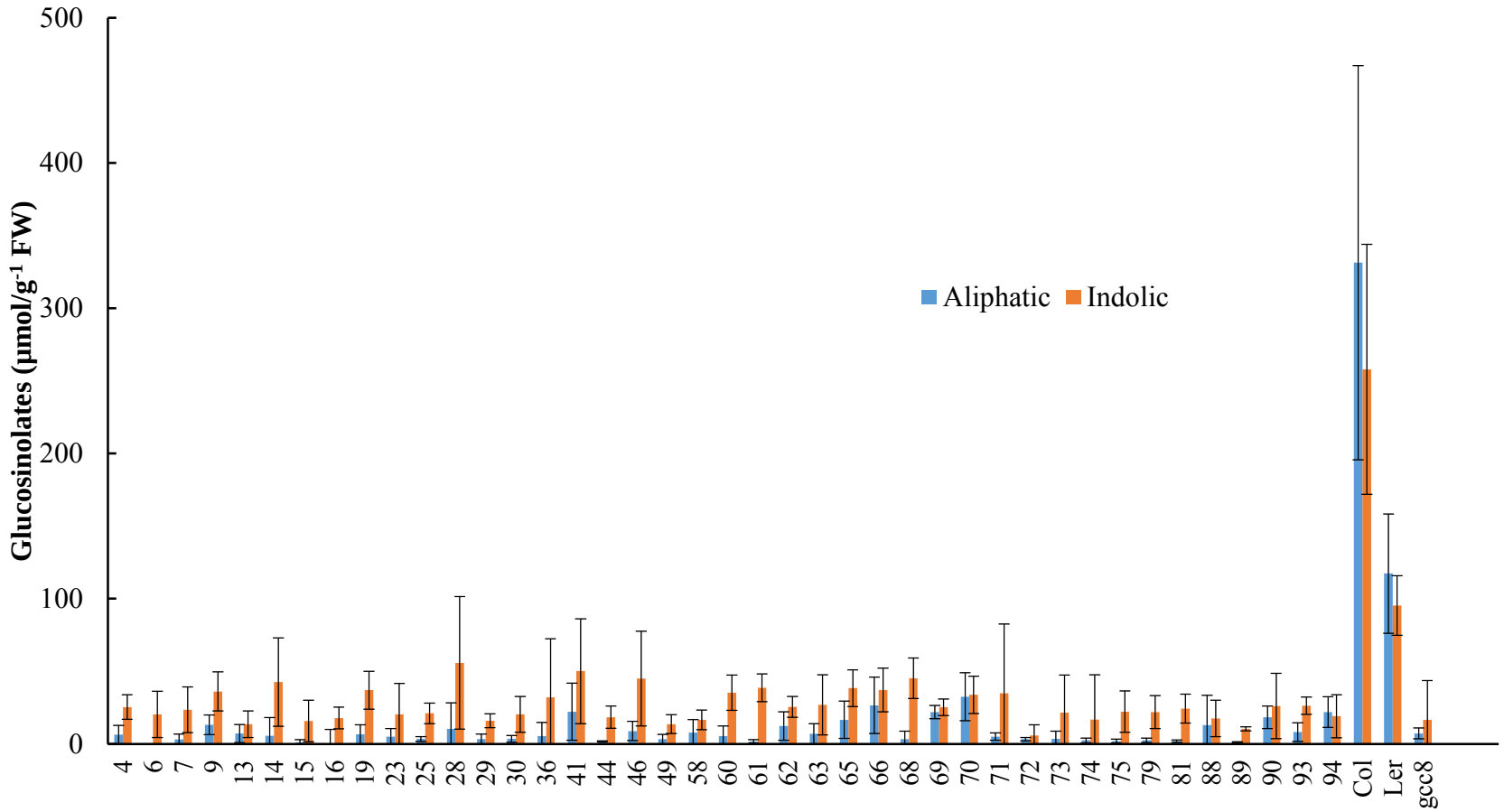

Figure 2. 5: GS analyses of individual lines from the mapping population along with Col-0 and Landsberg wild-type GS profiles. All mapping lines show the *gcc8* phenotype (n < 15).

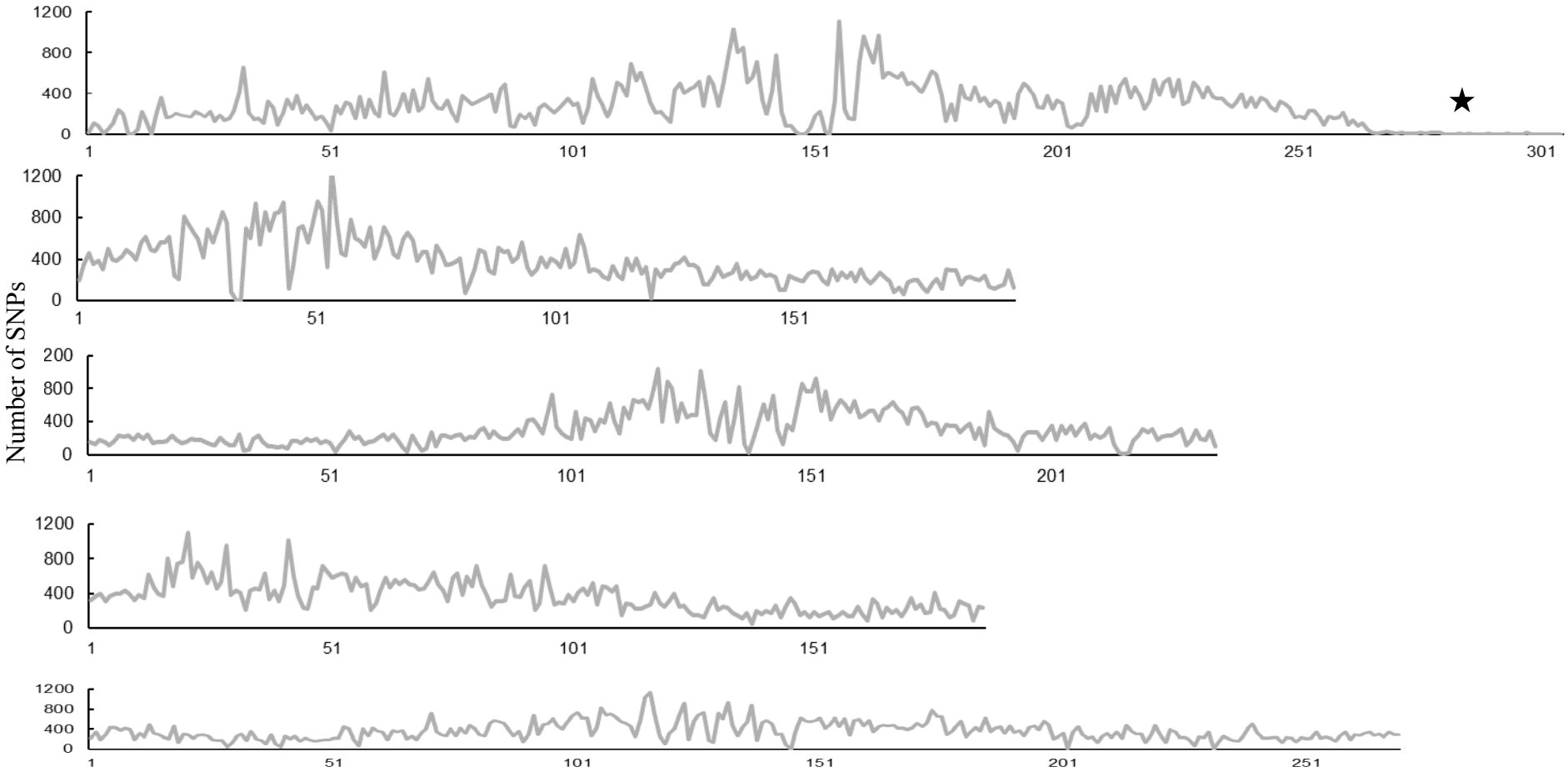

Figure 2. 6: All Single Nucleotide Polymorphisms (SNPs) identified by Next Generation Sequencing (NGS) are mapped against Col-0 (TAIR-10) reference genome throughout all 5 chromosomes. The vertical axis represents number of SNPs in sliding window of 50 Mbp and the horizontal axis represents base-pair positions in each chromosomes. At the end of the chromosome 1, we observed far fewer SNPs, which indicates the causative mutation is at this position.

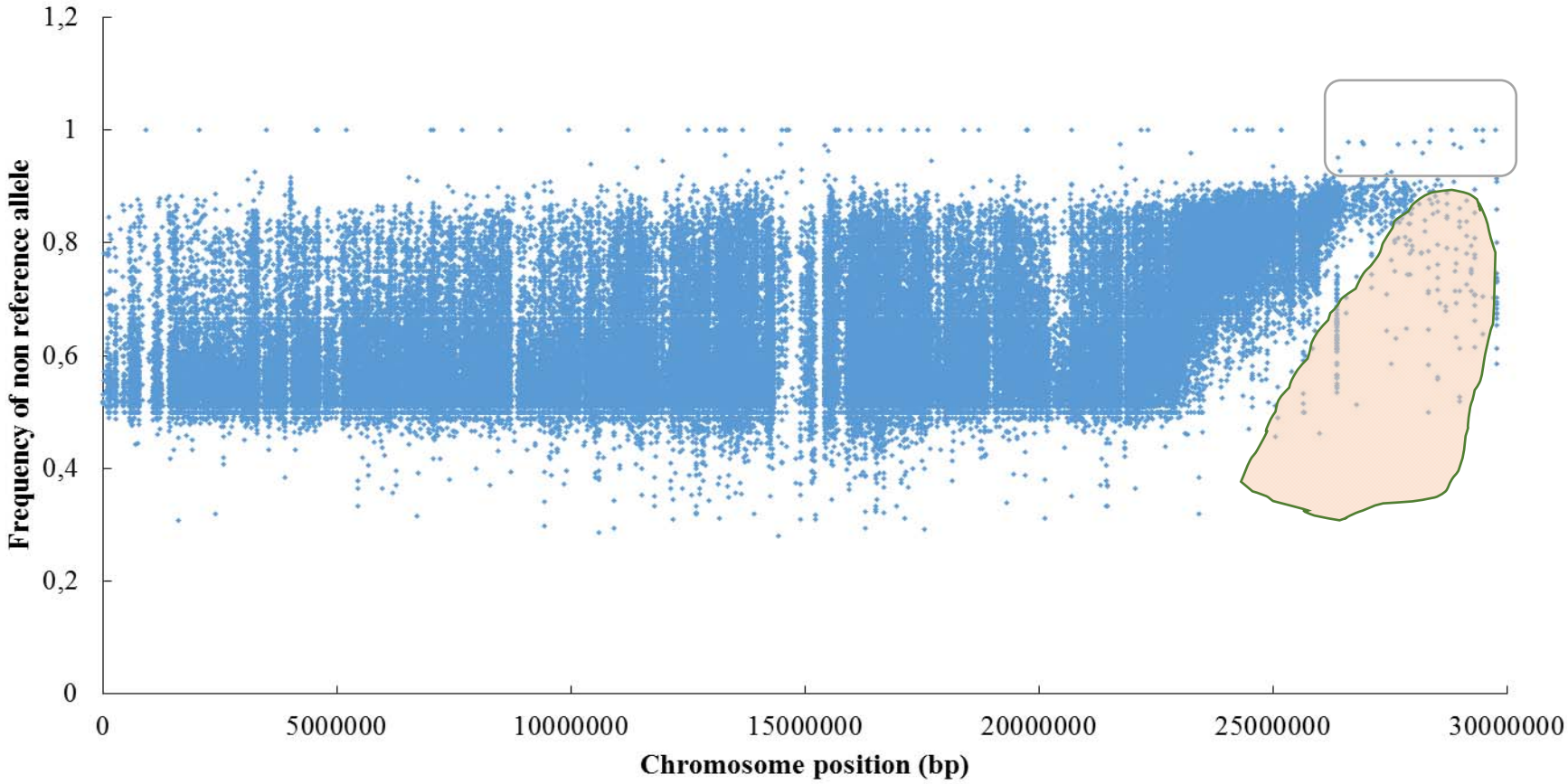

Figure 2. 7: Single Nucleotide Polymorphism (SNP) distribution of chromosome 1, shown as the frequency of the non-reference (Col-0) allele against plotted against the position along chromosome 1. The high frequency SNPs at the right are most likely to represent mutations caused EMS mutagenesis, as they are homozygous (or nearly so) in all mapping population members. SNPs between 50 - 80% are most likely to be natural polymorphisms between Columbia and Landsberg accessions. At the end of the chromosome 1 we observed drastic reduction of SNPs and above it a group of SNPs at very high frequency (grey rectangle) which we considered as putative candidates.

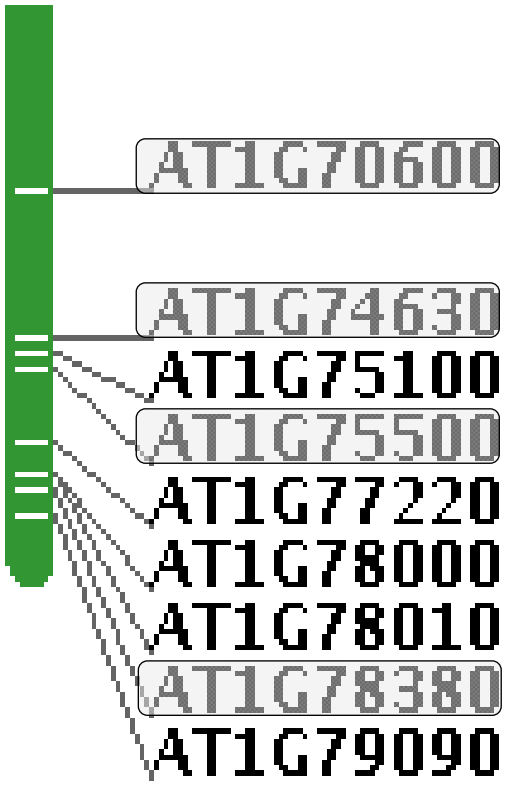

Figure 2. 8: Among the 20 candidate SNPs, nine of them are present in genes of *Arabidopsis thaliana* at the end of chromosome 1. Of these nine genes, only five carry non-synonymous mutations in coding regions; these are shown in black type.

Of those 9 genes, we examined the nature of the mutations such as whether they were non-synonymous or synonymous, and whether they affect the coding sequence. Two of the nine genes (*At1g75500* and *At1g78380)* carry mutations in introns; in such cases the mutation could be spliced out during transcription. Candidates *At1g70600* and *At1g74630* bore synonymous mutations. These are less likely to cause the phenotype and were eliminated from the candidate list. The other five genes carry non-synonymous mutations in the coding region and include *At1g75100*, *At1g77220*, *At1g78000*, *At1g78010* and *At1g79090*.

Genetic analysis (fine mapping) reduced the list further. The three times back crossed mutant line (*gcc8* (BC3)) has low GS content but it did not have the candidate mutation in *At1g75100* and *At1g77220* gene, eliminates the possibility of these two genes being causative for the

phenotype. The candidate SNP in *At1g78010* was eliminated after examination of a T-DNA knock-down line of the same gene which has GS content as high as the Col-0 wild-type (Figure 2.9).

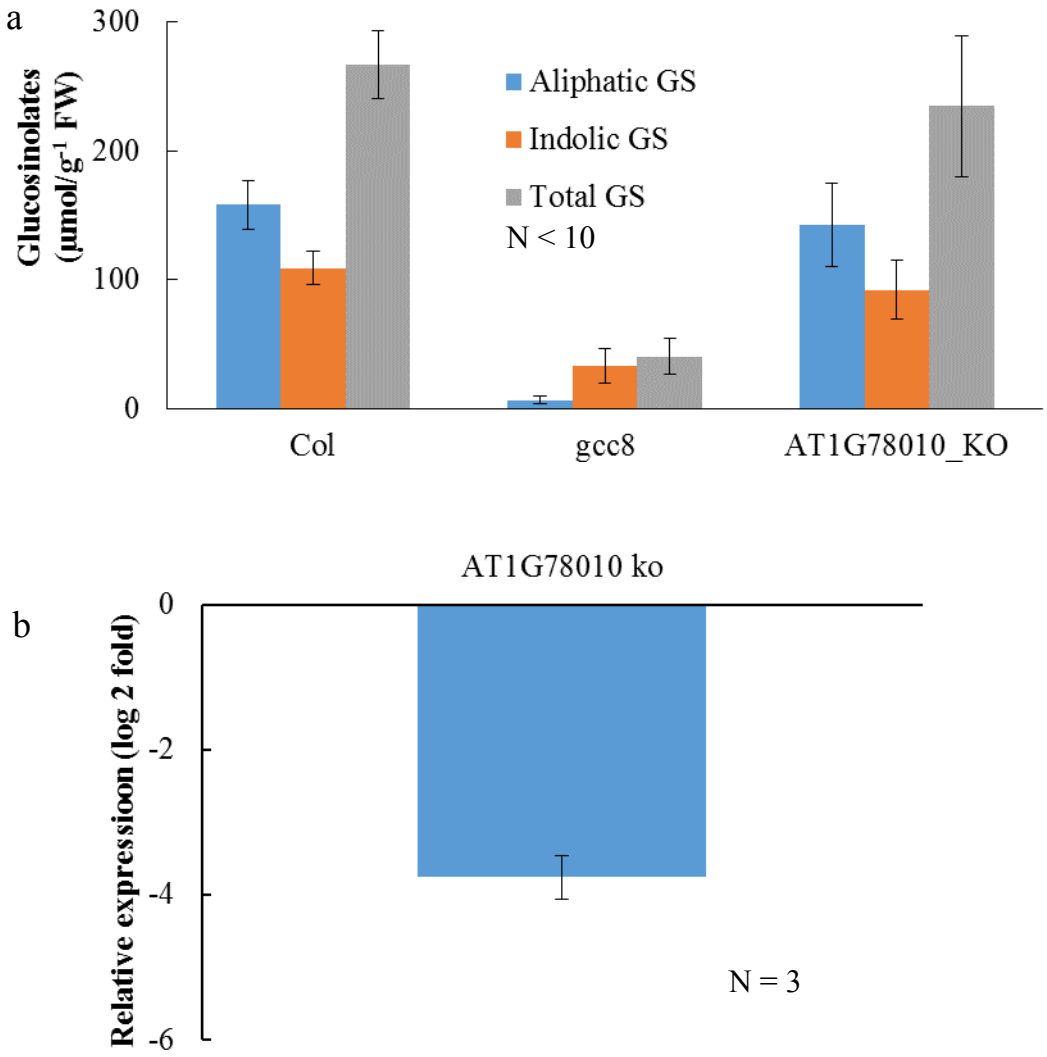

Figure 2. 9: (a) Glucosinolate analysis of the *At1g78010* T-DNA knock down line, which has GS levels indistinguishable from those of Col-0 wild-type, demonstrating that the candidate mutation in this gene is very unlikely to be responsible for the *gcc8* GS chemotype. (b) Expression analysis of *At1g78010* T-DNA line shows that the gene is knock down line.

The candidate mutation in the gene *At1g79090,* has been eliminated by analysis of an out crossed population (*gcc8* X Ler_F_2_7_14) line which is homozygous for the mutation in this gene and homozygous wild-type for the other four genes. This line had high GS content indistiguishable from wild type (Figure 2.10).

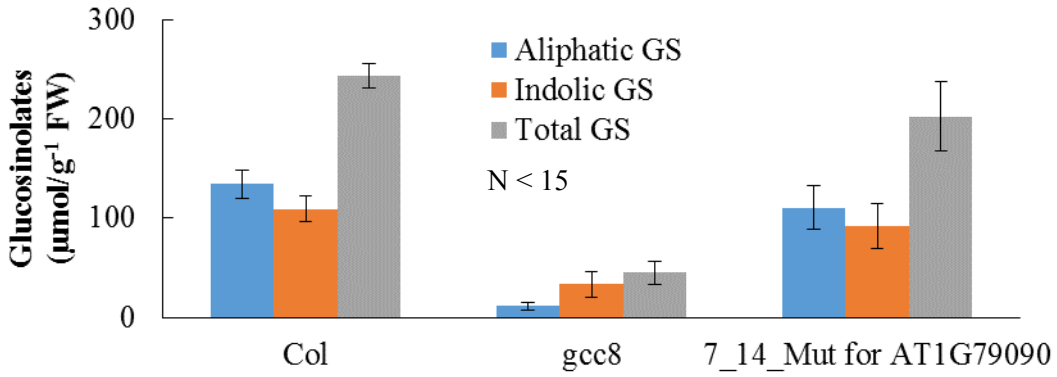

Figure 2. 10: GS profile of a member of the out crossed population which is homozygous for the mutation in *At1g79090* and wild-type for the other candidate genes. The recombinant line shows high GS similar to the wild-type (Col-0), demonstrating that this mutation by itself cannot cause the *gcc8* phenotype

iii. Fine mapping of the gcc8 locus

Hence, our genetic analysis produced evidence against four of our five candidates, with the exception of *At1g78000,* which encodes the known sulfate transporter *SULTR1;2*.

The *gcc8* line has a G→A mutation in this gene, leading to conversion of a tryptophan-encoding triplet a premature stop codon in the first exon of the gene (Figure 2.11). The resulting truncated protein is very likely nonfunctional. There are many isoforms of sulfate transporters which have been identified based on sequence similarity. *SULTR1;2* encodes one of the high-affinity SULTR of Arabidopsis, which is the major sulfate transporter with a function in sulfur uptake.

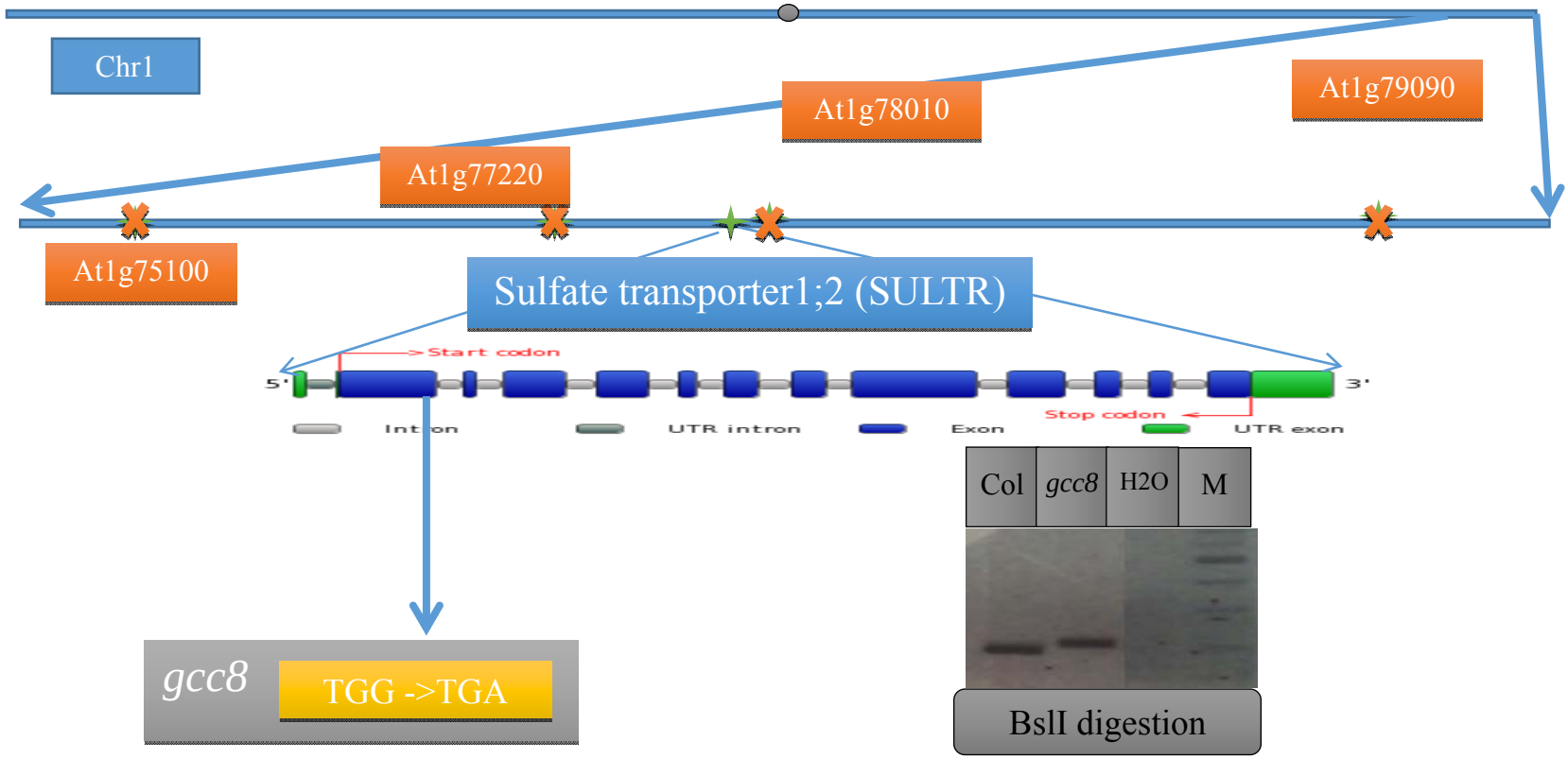

Figure 2. 11: Fine mapping of the *gcc8* mutation to distinguish the effects of five non-synonymous mutations in different genes. The two genes on the left were eliminated because the 3X backcrossed line of *gcc8* has low GS but is wild-type for these genes. The rightmost two candidates were eliminated because lines which are mutant only for these genes have high GS. Agarose gel image analyses of dCAPS marker which differentiates wild type and *gcc8* line by BslI digestion, where the enzyme cuts the wild type amplicon, but not that of the mutant line.

e. Various *sel1* Alleles Phenocopy the *gcc8* Mutant

AtSULTR1;2 (*At1g78000, sulfate transporter 1;2*) mutants were identified based on their resistance to selenate, a toxic structural analogue of sulfate. Sixteen so called *sel1* alleles were identified for this gene. We requested seeds of two *sel1* alleles which were published recently (*sel1-15* and *sel1-16*) and carry mutations in two different transmembrane helices. When we measured total GS of both *sel1* alleles along with *gcc8* and wild-type (Col-0) plants, we observed that the *sel1* alleles have GS profiles similar to *gcc8,* which further confirms that the mutation in *SULTR1;2* causes the low GS phenotype (Figure 2.12).

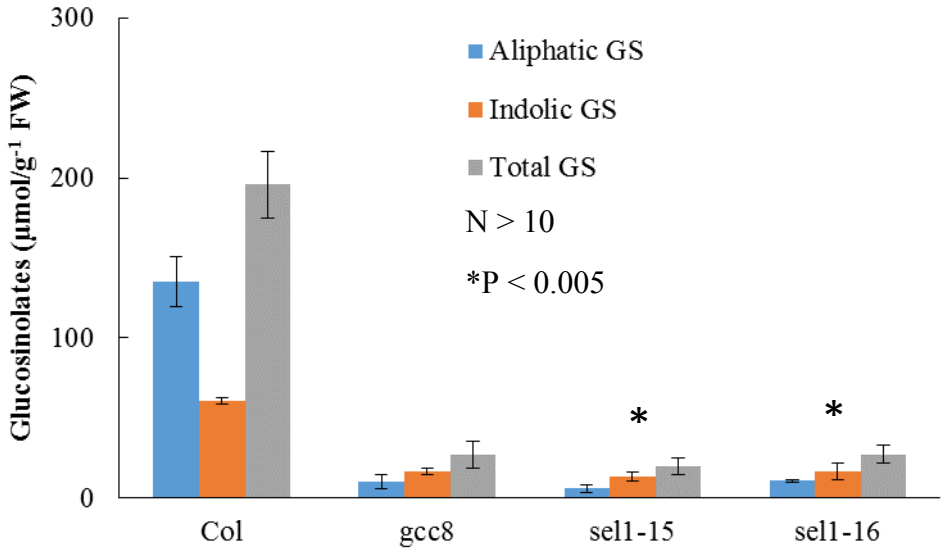

Figure 2. 12: GS analysis of *sel1* mutants along with the *gcc8* line and wild-type (Col-0) in 18 day old plants. The two *sel1* alleles have GS profiles similar to that of *gcc8*, providing strong additional support that mutations in *AtSULTR1;2* gene cause the low GS phenotype.

f. Non-complementation of *gcc8* with *sel1* Alleles

For genetic complementation tests, the *gcc8* line was crossed with both *sel1* lines to determine the GS profiles of the F_1 progeny. F_1 seeds were grown along with wild-type (Col-0), *gcc8* and *sel1* alleles for 18 days and the total GS content was measured (Figure 2.13). The total GS content of *gcc8*X*sel1-15*_F_1s and *gcc8*X*sel1-16*_F_1s was similar to those of *gcc8* and *sel1* alleles. Thus, the non-complementation provides further evidence that the observed mutations in *SULTR1;2* (*At1G78000*) cause the low GS phenotype of *gcc8*. One way ANNOVA was used to test for differences among all four genotypes with *gcc8* line (the two *sel1* alleles and F_1 progenies of their cross with *gcc8*) reveals that they are not different (P value is less than 0.005).

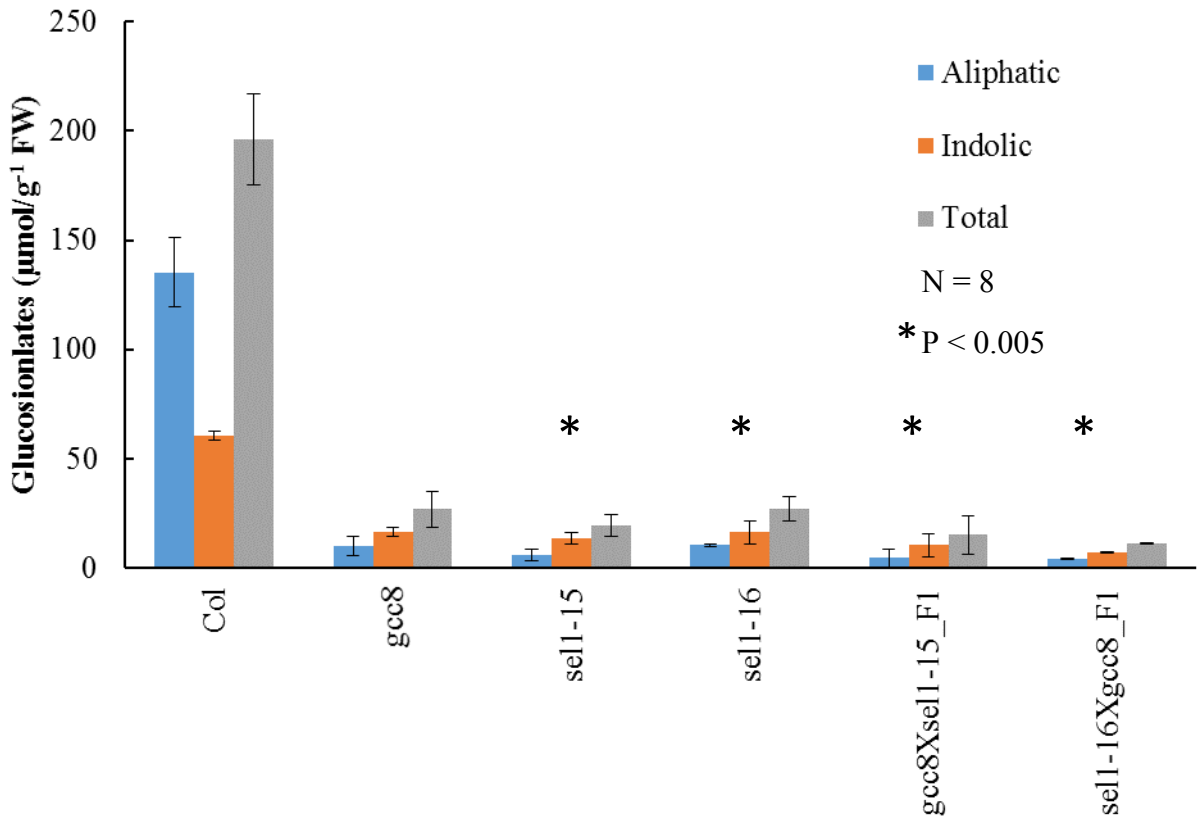

Figure 2. 13: GS analysis of F_1 progenies of *gcc8* and *sel1* mutants. Both the F_1 progenies (*gcc8* X *sel1-15*_F_1 and *sel1-16* X *gcc8*_F_1) have GS profiles similar to *gcc8* and *sel1* alleles, confirming that loss-of-function mutations in *SULTR1;2* cause the low-GS phenotype.

g. Selenate Resistance of *gcc8*

Null mutations in *SULTR1;2* are known to be selenite-resistant. Selenate is a toxic structural analogue of sulfate. Plants impaired in sulfate uptake are resistant, because they are also impaired in uptake of selenate. Hence, mutant plants that cannot take up sulfate because of a mutation in the major sulfate uptake protein can grow in selenate medium, but wild type plants cannot grow after certain period. Wild-type and *gcc8* plants were grown on ¼ MS medium with and without addition of 30 μM sodium selenate. After eight days of growth, plates were photographed and the root length was measured using ImageJ software. Wild-type seedlings had significantly shorter roots compared to *gcc8.* To eliminate the possibility that this effect was due to the additional sodium, the experiment also included control plates with 30 μM sodium chloride. The results show that wild type and *gcc8* plants had similar root length (Figure 2.14a). The results are shown quantitatively in Figure 2.14b. It could be formally argued that it is the 60 μM added sodium (which changes the total medium concentration of sodium from 100 μM to 160 μM) which causes the observed root phenotype. However, it is very well established in the salt resistance literature that at least 100 mM sodium is required to alter the development of wild-type Arabidopsis, so this idea can be safely rejected.

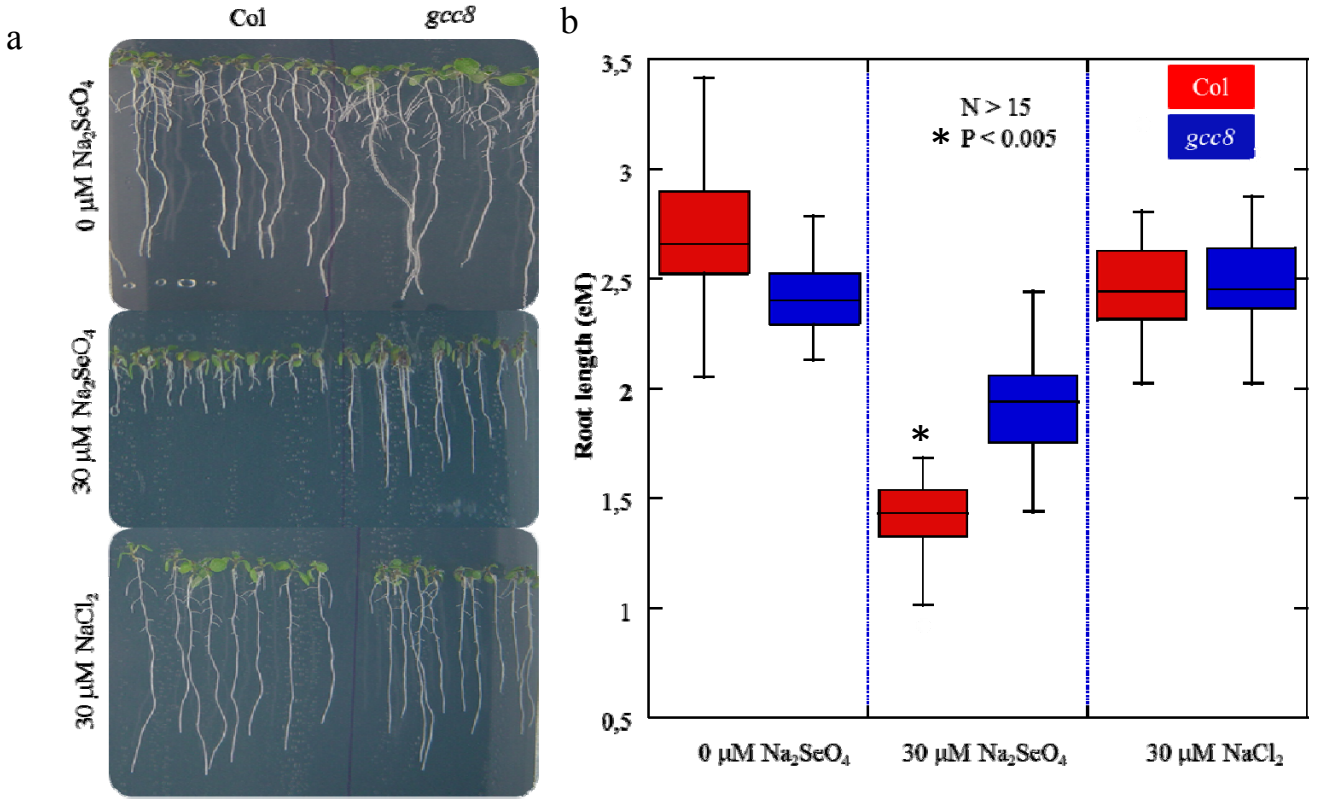

Figure 2. 14: (a) Selenate resistance of *gcc8* plants. In 30 µM sodium selenate *gcc8* seedlings are resistant compared to the wild-type. (b) Quantification of selenate resistance of mutant line in box plot.

h. Glucosinolate Analyses of *SULTR1;2* T-DNA Insertion Lines

We ordered T-DNA insertion lines of *AtSULTR1;2*, verified that the T-DNA insertions are homozygous, and measured total GS content. We ordered two T-DNA insertion lines, one in the promoter region and the other one at the end of the 3' UTR. Semi-quantitative reverse-transcription (RT) polymerase chain reaction (PCR) measurements confirmed that transcript levels were reduced compared to the wild-type, so they are knock-down lines. The total GS content of the T-DNA lines was intermediate between Columbia and *gcc8* (Figure 2.15), further supporting the idea that the T-DNA insertion lines are knock-down lines. Since these T-DNA insertion lines do not have a very strong phenotype like the EMS lines (*gcc8*, *sel1* alleles), they were not included in subsequent experiments.

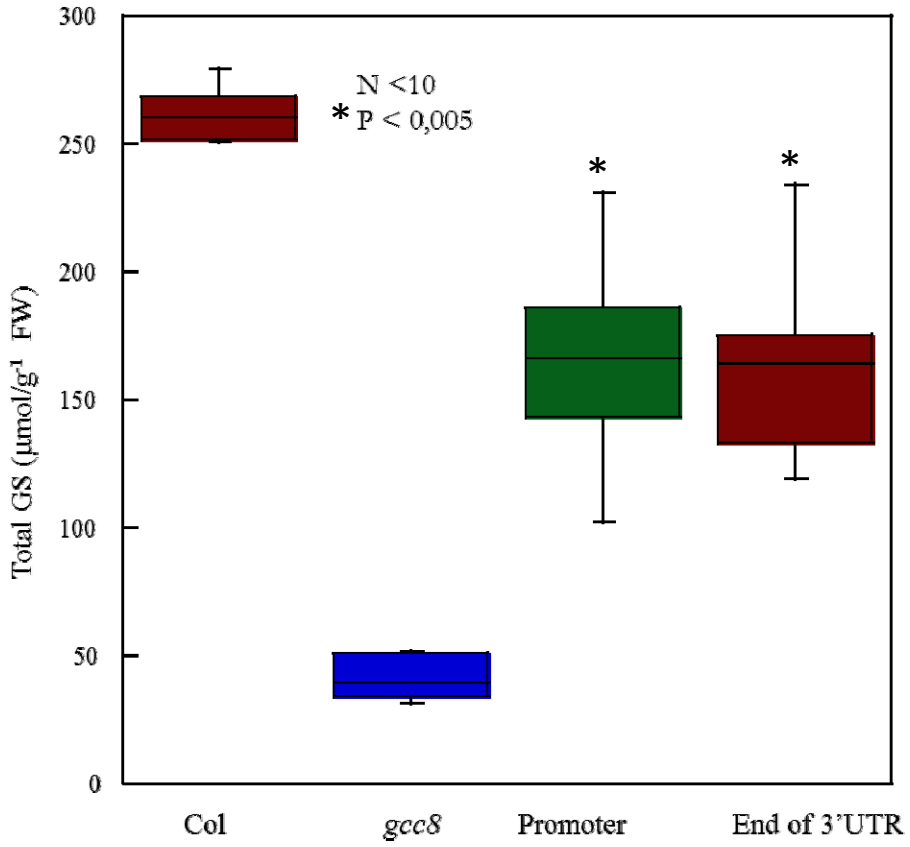

Figure 2. 15: GS analyses of *SULTR1;2* T-DNA insertion lines along with control plants (wild-type and *gcc8*). Both the T-DNA insertion lines have intermediate GS levels between those of Col-0 and *gcc8*.

i. Glucosinolate Analyses of Other High-affinity Sulfate Transporter Knockout Lines

In Arabidopsis, there are three high-affinity sulfur transporters (SULTR): *SULTR1;1* and *SULR1;2* are mainly localized in roots, and *SULTR1;3,* is present only in the tonoplast. To check the importance of these SULTR in GS metabolism, we obtained homozygous knockout T-DNA insertion lines for *SULTR1;1* and *SULTR1;3,* and we measured their total GS content. Neither of these transporters is required for the plant to produce wild-type GS levels at this growth stage (Figure 2.16).

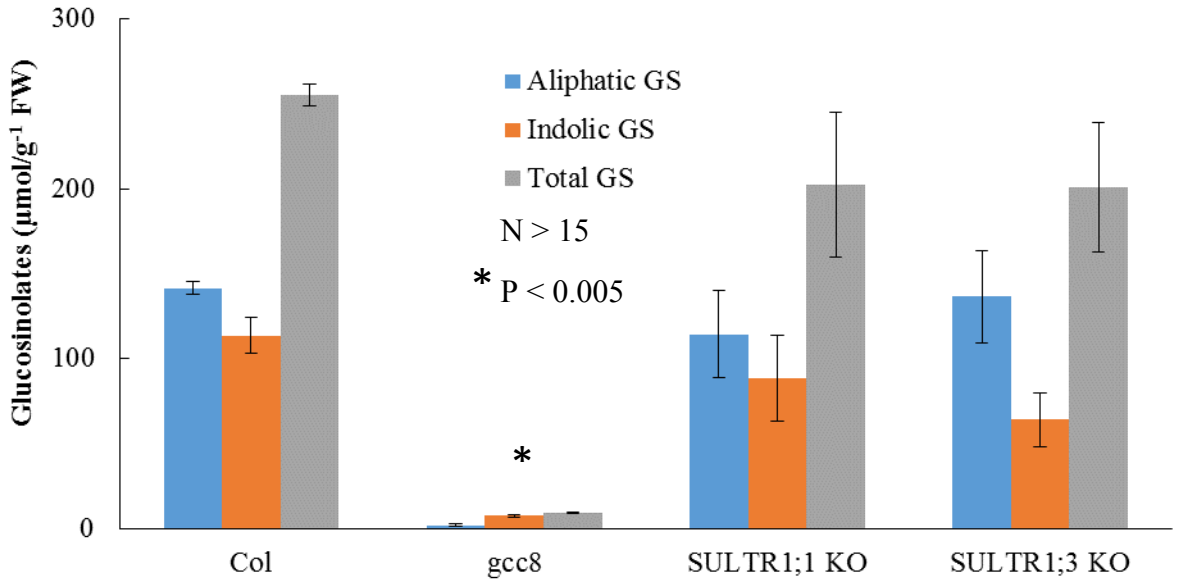

Figure 2. 16: GS analysis of T-DNA insertion lines of other high-affinity SULTRs. Total GS levels of *SULTR1;1* and *SULTR1;3* knockout lines show their absence does not have any effect in total GS content accumulation compared to *gcc8* line (*statistically significant).

j. Microarray Analysis of the *gcc8* Line

To obtain a better understanding of the effect of the *gcc8* mutation on global gene regulation, we performed microarray experiments to identify genes that are differentially regulated in the *gcc8* mutant background. There were 258 genes differentially expressed in *gcc8* compared to the wild-type (Col-0). Among those, 204 genes were up-regulated and 54 genes were down-

regulated. When we looked closer at the annotations of these differentially expressed genes, only four of them are GS-related.

i. Gene expression analysis of GS-related genes

Of four GS-related genes identified as differentially regulated on the microarray, two of them are down regulated: *MYB29*, a transcription factor that regulates aliphatic GS biosynthetic pathway (Gigolashvili, et al., 2008b), and the GS biosynthetic gene called *BCAT4*. The other two are up-regulated: *NSP5* and *NIT3* which are known to be involved in GS degradation (Kutz, et al., 2002). Apart from these four genes, no other GS-related genes are differentially expressed in the mutant background, as determined by the microarray experiment. Hence the microarray analysis provided no obvious explanation for our phenotype of interest. However, since the microarray experiment included only three replicates of each genotype, failure to detect changes in GS-related genes may just be a matter of low statistical power. We therefore performed qRT-PCR analysis for a large group of genes know to be involved in GS synthesis (Figure 2.17).

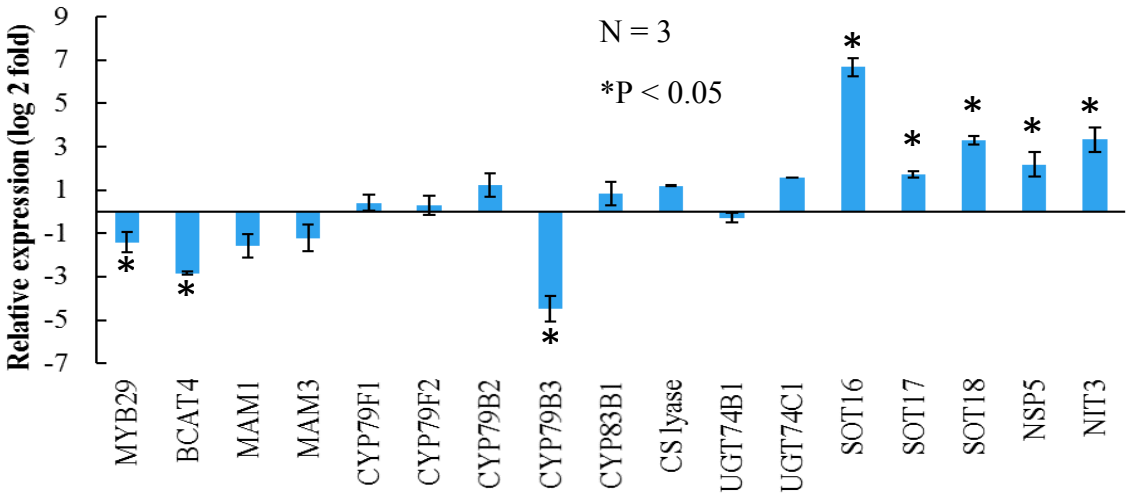

Figure 2. 17: qRT-PCR analyses of GS-related genes in the *gcc8* mutant compared to the wild-type (Col-0). Key biosynthetic genes are down regulated in the mutant background.

ii. Gene ontology analysis of micro array analysis

Gene ontology analyses were performed to identify the major functional categories which are significantly affected. The lists of differentially expressed genes were fed into the web based program AgriGo [http://bioinfo.cau.edu.cn/agriGO/] for gene ontology analysis. Figure 2.18 shows the functional categories of genes which are significantly overrepresented in our data set. GO terms toward the bottom of the figure represent more specific categories and those nearer the top show broader biological categories. The GO term "JA stimulus" stands out as the most specific term with extraordinarily strong overrepresentation. Of the 258 genes found to be significantly affected in our experiment, 206 are including in this GO term. This makes sense, given the role of JA in regulating sulfur assimilation and metabolism (Kopriva, 2006). Oddly, however, our analysis did not identify any of the numerous GO terms related to sulfur, sulfate, sulfur-containing amino acid metabolism, or sulfate transport.

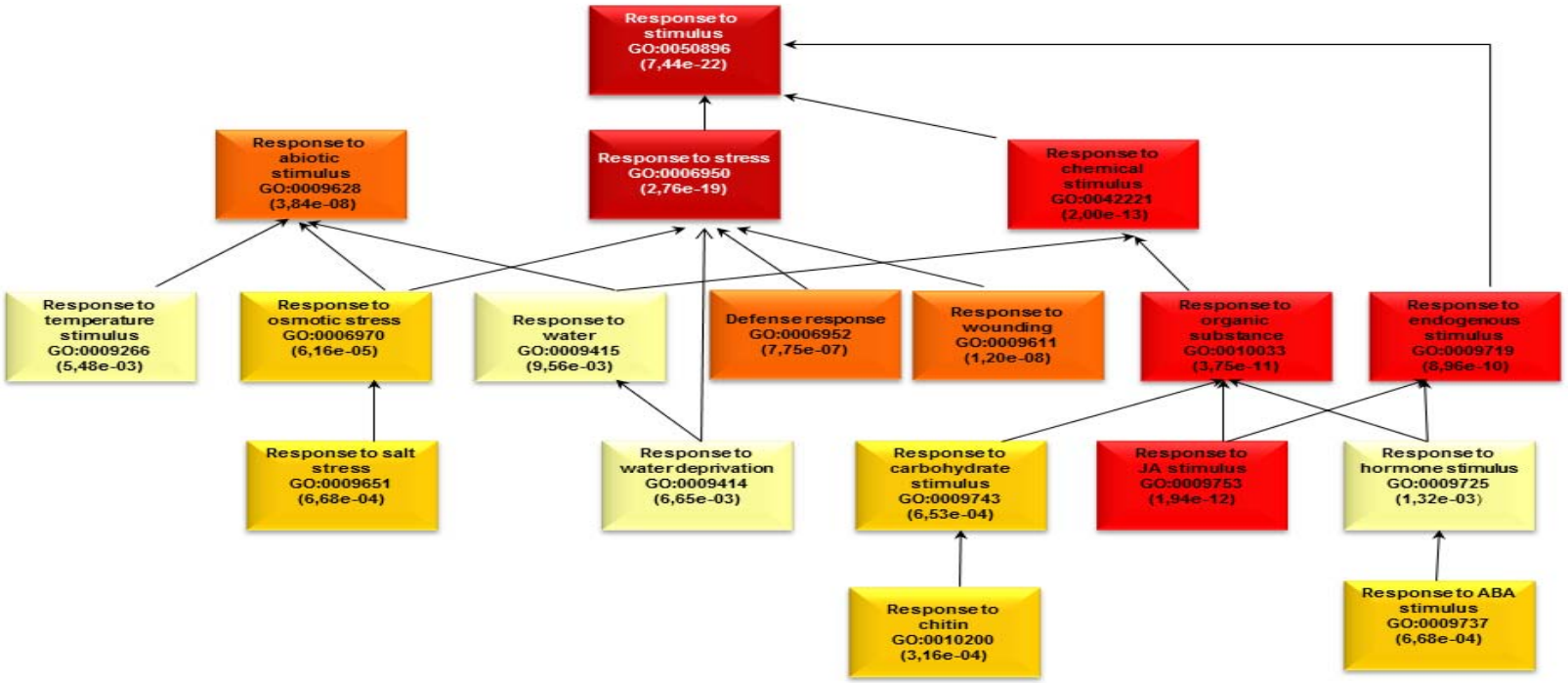

Figure 2. 18: Gene ontology analysis of differentially expressed genes in *gcc8* background. GO analysis reveals systematic perturbation of genes involved in response to JA stimulus are over represented with more number of genes compared to any other GO terms. Darker red corresponds to lower p-values, i.e more significant overrepresentation of the genes of a given category.

iii. Expression analysis of jasmonate-related genes

Gene ontology analysis shows that genes related to JA stimulus are strongly overrepresented in our microarray data set. Figure 2.19 shows expression of various JA-related genes which were induced both microarray and qRT-PCR analyses. The key biosynthetic genes of JA were significantly up-regulated in the *gcc8* mutant background. The remaining genes of JA biosynthetic pathway are normally constitutively expressed (Turner, et al., 2002).

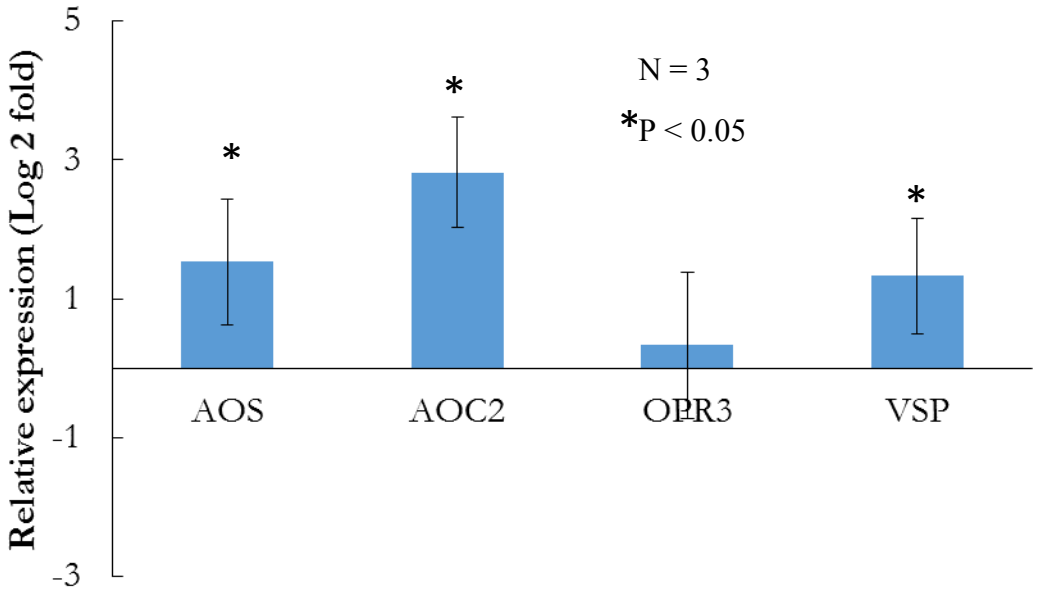

Figure 2. 19: qRT-PCR analyses of JA-related genes in the *gcc8* mutant compared to the wild-type (Col-0). All these genes are differentially expressed in micro array experiment; hence we validated with qRT-PCR except for *OPR3*.

iv. Expression analysis of sulfur starvation genes

When sulfur is limiting, the plants tend to induce sulfur starvation response by inducing JAs and reducing the levels of all sulfated secondary metabolites (Hirai, et al., 2003; Nikiforova, et al., 2005). *BGLU28 (At2g44460),* annotated as putative β-glycosidase 28, *LSU1* (*RESPONSE TO LOW SULFUR-1*, *At3g49580*) and *SDI1* (*SULFUR DEFICIENCY INDUCED-1*, *At5g48850*) are known to be induced under sulfur starvation conditions (Zhang,

et al., 2014). The functions of these genes are not currently known. In the *gcc8* background these genes are highly induced under normal growth conditions, as indicated by our microarray analysis. To confirm this result, we performed qRT-PCR analyses for these genes. Both microarray and qRT-PCR analyses confirm that these sulfur starvation genes are induced in the *gcc8* background (Figure 2.20).

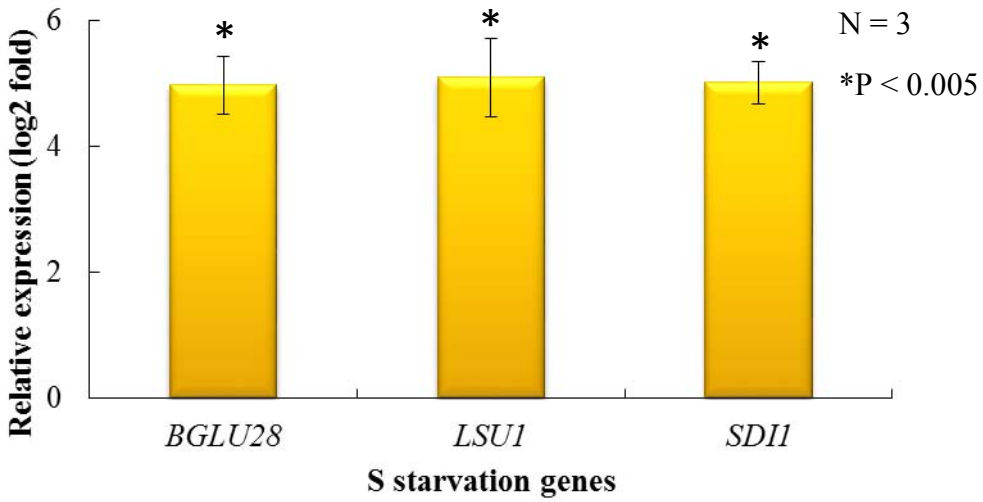

Figure 2. 20: qRT-PCR analyses of sulfur starvation genes in *gcc8*. Their microarray expression fold change values were as follows: *BGLU28*, 90.85; *LSU1*, 16.34; and *SDI*, 19.30.

k. Oxylipin Measurements

Hence we observed jasmonate biosynthetic genes are induced in the mutant we measured various oxylipins in wild-type and *gcc8* mutant plants (Figure 2.21). The results show only OPDA is significantly higher in the mutant line than the wild-type.

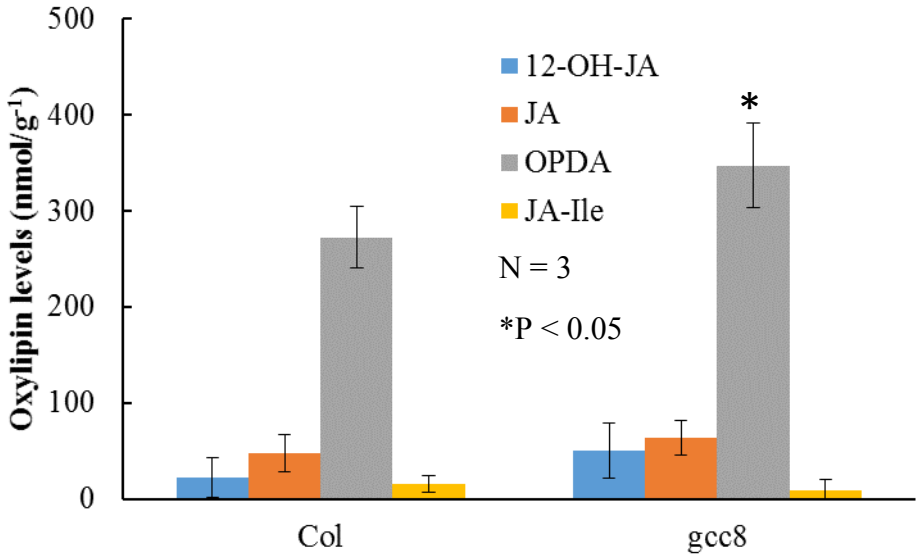

Figure 2. 21 Oxylipin measurements of wild-type (Col-0) and *gcc8* plants. OPDA is significantly different in mutant line than the wild-type.

l. Double Mutant Construction of *gcc8* and *bglu28-KO*

We observed induction of sulfur starvation genes in the *gcc8* background, likely because the lack of a functional *SULT1;2* limits sulfur availability. We hypothesized that *BGLU28*, annotated as putative β-glycosidase, could directly cause the low-GS chemotype by degrading GS in *gcc8* plants. To test this idea, we isolated a T-DNA insertion knock-down line for *BGLU28* and measured its GS profile, which was similar to the Col-0 wild-type. We further crossed the *BGLU28-KO* line with *gcc8* to obtain the double homozygous line. From the F_2 segregating population, we identified three F_2 plants homozygous for both mutations. Strikingly, their GS profiles are indistinguishable from that of the *gcc8* line (Figure 2.22). If *BGLU28* is the enzyme that degrades GS, we would expect higher GS in the double knockout plants, but we observed quite similar GS profiles to those seen in *gcc8* plants, providing clear genetic evidence that BGLU28 does not degrade GS in this mutant.

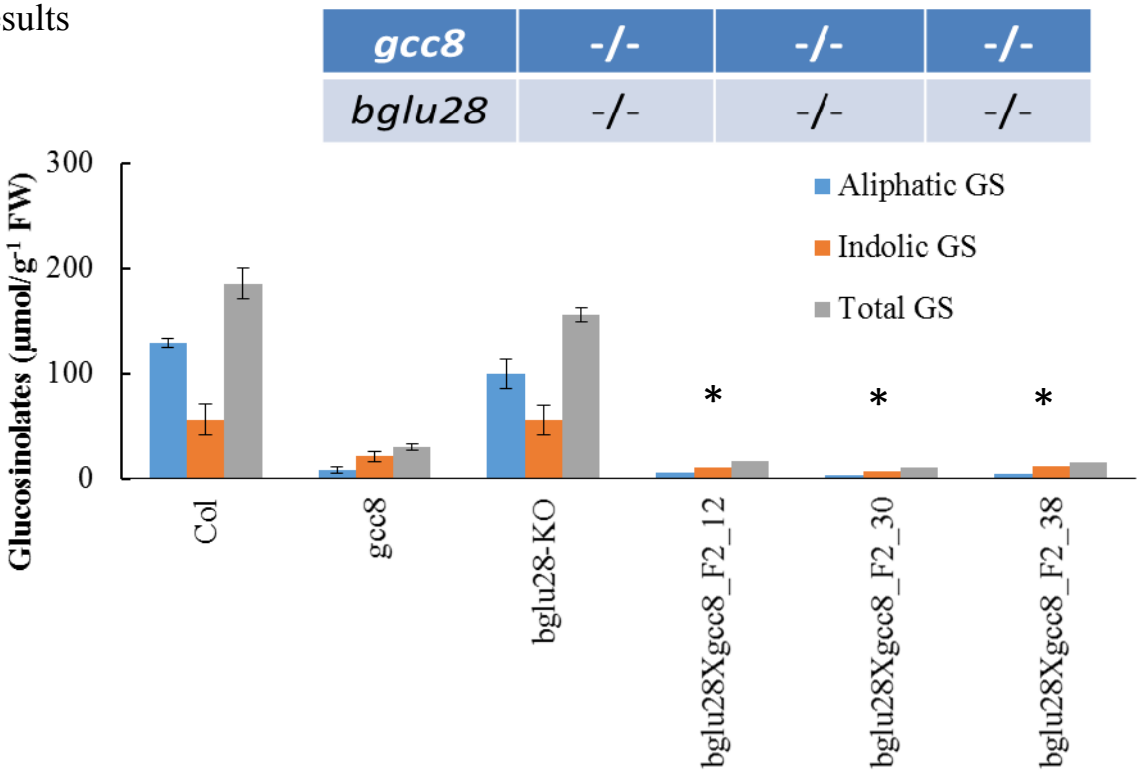

Figure 2. 22: GS analyses of three different double homozygous plants of *bglu28-KO* and *gcc8* along with wild-type (Col-0), *gcc8* and *bglu28-KO* GS GS profiles. The double mutant plants have GS profiles similar to *gcc8* plants. Genotyping results of the double mutant plants are indicated in the inset.

m. Benzyl Glucosinolate Degradation Assays with Plant Extracts

To further explore the relationship between myrosinase activity and the low-GS phenotype of *gcc8*, we performed an enzymological experiment in which soluble protein extracts of wild-type and mutant lines were used as an enzyme source, and benzyl-GS as a substrate, and we measured the GS degradation rate. We used protein extracts from wild-type, *gcc8* and *sel1* plants along with an extraction buffer (no protein) control (Figure 2.23). If BGLU28 degrades GS, we would expect the rate of degradation for the mutant plant extract would be higher than that from Columbia wild type, because the mutant line overexpresses BGLU28. Interestingly, we observed the plant extracts of the wild-type degrade benzyl GS faster than either *gcc8* or *sel1* plant extracts. This could indicate that BGLU28 is not a thioglucosidase, or that it is not a myrosinase, or that it is induced at the level of transcription, but not at the protein level, or that the activity of this enzyme is miniscule compared to the activities of other myrosinases present in the extract. It also indicates that, if increased GS degradation is responsible for the

low-GS phenotype of *gcc8*, it may proceed by a catabolic route which does not involve myrosinase. For example, degradation could begin with a sulfatase, with the glucose removed second by a thioglucosidase other than myrosinase.

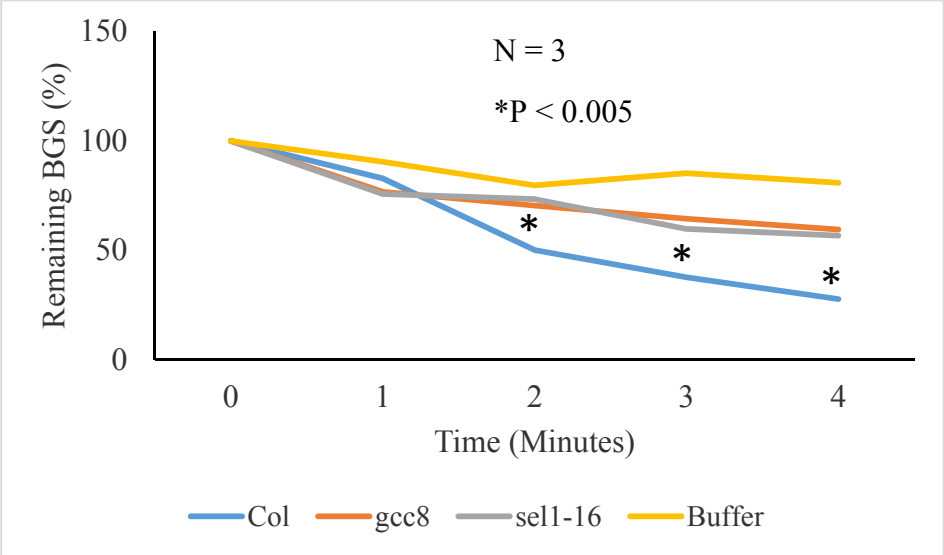

Figure 2. 23: Benzyl-GS degradation analysis of various plant extracts over time. Wild-type (Col-0) plant extracts degrade benzyl-GS faster than *gcc8* and other *sel1* alleles.

n. Metabolite Analysis

i. Total sulfate content

Hence, we know *gcc8* line has a mutation in one of the major SULTR gene (*AtSULTR1;2*) we prone to measure the total sulfate content of 18 day old plants of wild-type (Col-0) and *gcc8* line in both optimum and low sulfate concentration with ion-exchange chromatography (Figure 2.24). The results clearly show much less sulfate content in the mutant line compared to wild type.

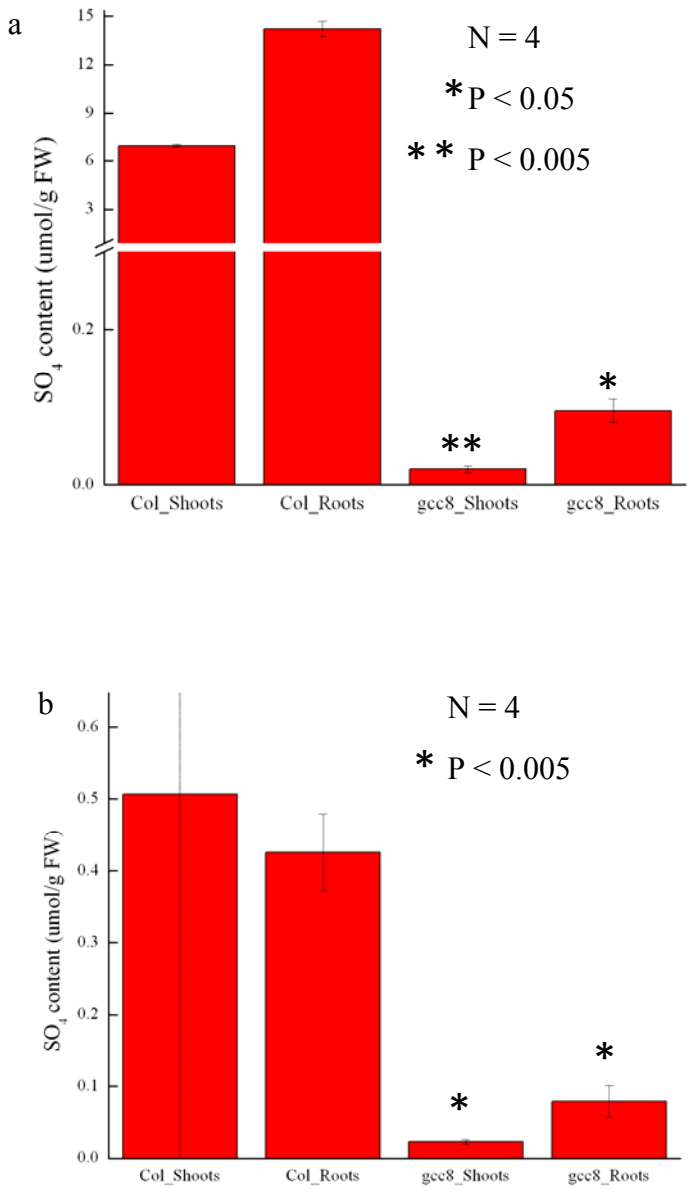

Figure 2. 24: Total sulfate measurements of *gcc8* and wild-type (Col-0) in both ½ MS (a) and 0.1 mM (b) sulfate in the medium.

ii. Glutathione measurement

When plants accumulate less sulfur, it could indirectly affect levels of sulfur-containing amino acids, as well as levels of other sulfur-containing metabolites. We measured glutathione content of 18 day old plants of wild type and mutant plants grown on ½ MS and

0.1 mM sulfate medium (Figure 2.25). We observed significantly less glutathione in shoots of mutant plants in both media.

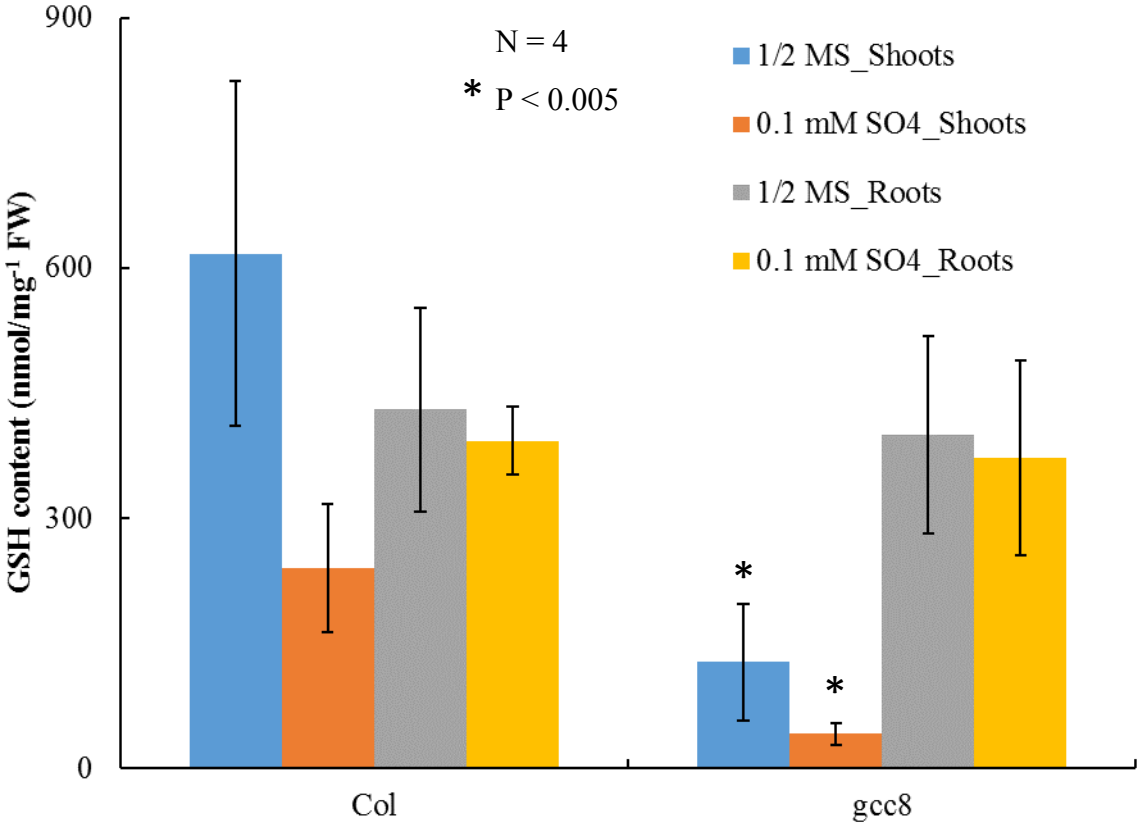

Figure 2. 25: Total glutathione measurements of wild-type (Col-0) and *gcc8* plants in both ½ MS and 0.1 mM sulfate.

iii. PAPS and PAP measurement

PAPS (3'-Phosopho Adenosine 5'-Phospho Sulfate) is the main sulfur donor for all sulfated secondary metabolites in Arabidopsis. Since we observed that the mutation in *AtSULTR1;2* affects the sulfate content of the plant, we measured the total PAPS and PAP levels in the wild type and mutant lines (Figure 2.26). The results revealed that *gcc8* has PAPS and PAP content similar to wild type in both media, which further suggests that the low GS content of *gcc8* is not due to a limitation in levels of the sulfate donor (PAPS).

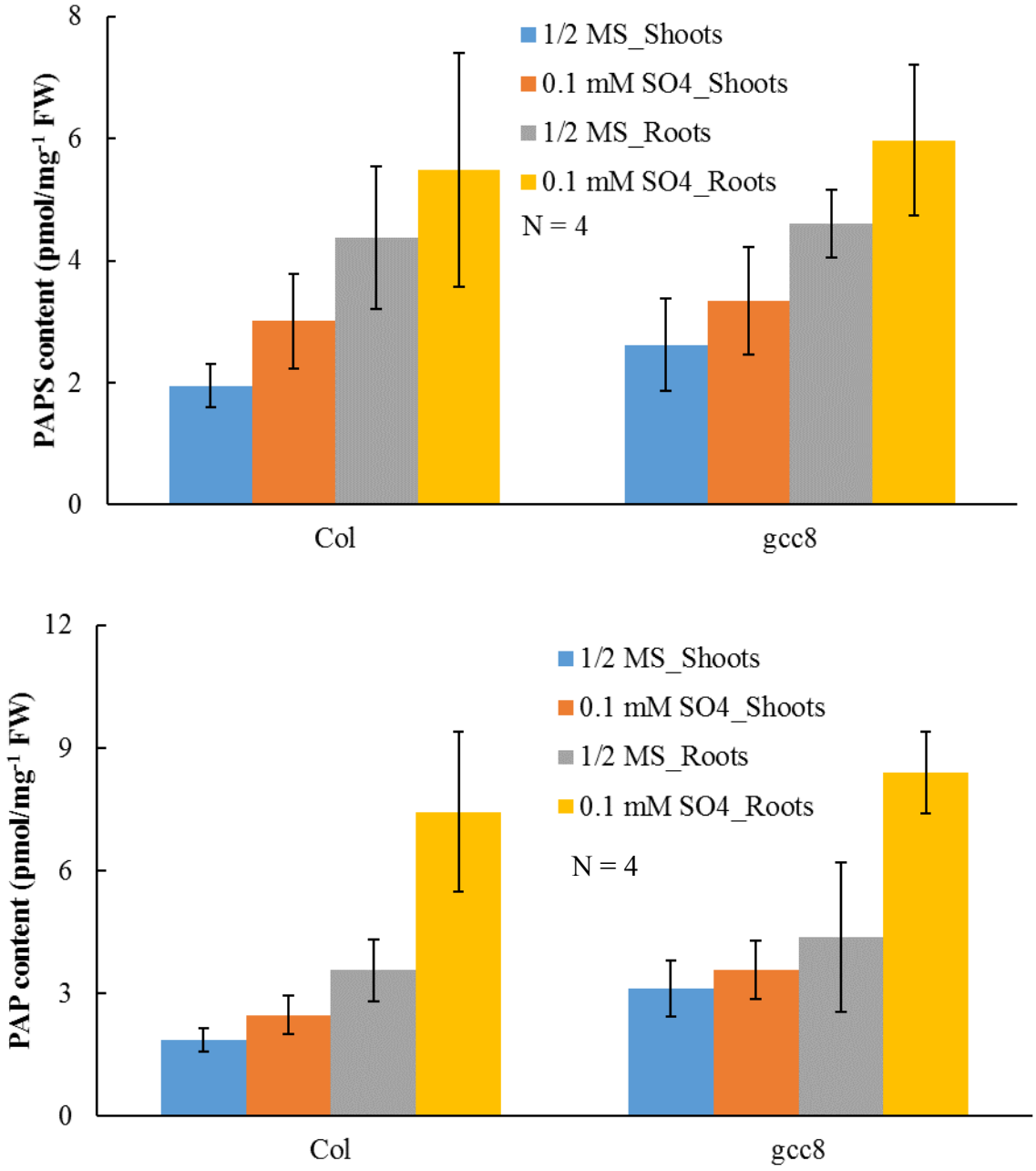

Figure 2. 26: Total PAPS and PAP content of wild-type (Col-0) and *gcc8* plants in both ½ MS and 0.1 mM sulfate.

o. Gene Cloning and Complementation

To verify that the EMS mutation in *AtSULTR1;2* is causative for the *gcc8* chemotype, we cloned the coding region of *SULTR1;2* under control of the 35S CaMV promoter and transformed this construct into *gcc8* plants to test whether it could complement the GS chemotype. Indeed, 18-day-old *gcc8* T_2 plants carrying transgene have wild-type levels of GS (Figure 2.27).

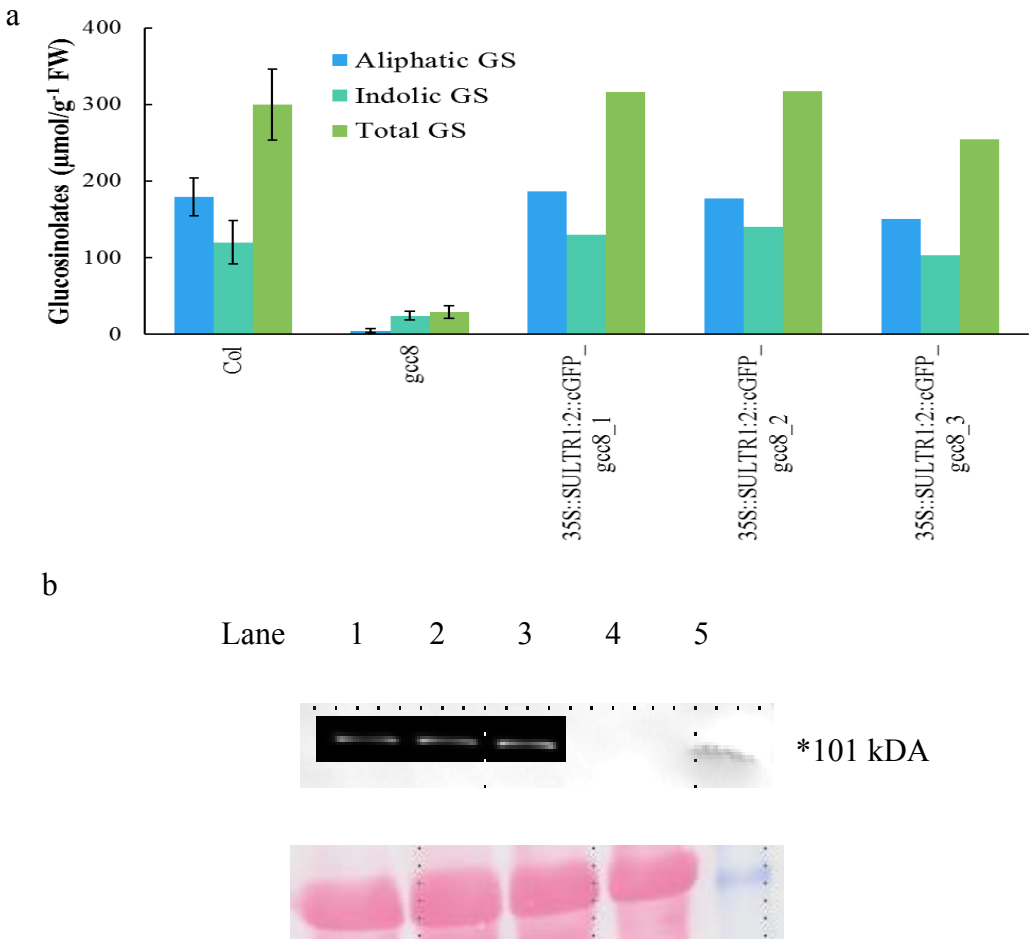

Figure 2. 27: a) GS analysis of *Arabidopsis* stable transgenic lines. *AtSULTR1;2* cDNA expressed under the control of the 35S CaMV promoter completely rescues the *gcc8* GS chemo type. b) Western blot analyses of the SULTR1;2 proteins loaded in the assay using anti-GFP antibody. Lane 1-3 shows the GFP tagged fusion protein (35S::SULTR1;2::GFP) of protein extracts of plants shown in GS analyses of stable transformed plants, lane 4-Col-0 and the last lane shows pre stained molecular weight marker. The bottom panel shows Ponceau S staining of the nitrocellulose membrane as the loading control.

3. DISCUSSION

Earlier establishment of a colorimetric bioassay for QR activity in murine hepatoma cells and subsequent HPLC analysis of desulfo-GS have established a strong positive correlation between leaf QR inducer potency and leaf content of methionine-derived GS. This assay allowed identification of several GS-related mutants, which were named *gcc* (*glucosinolate content* and *composition*) mutants (Grubb, et al., 2002). Among the various *gcc* mutants, the *gcc8* line was very interesting to us because it produces all the individual GS but in much lower quantity than the wild-type. The dramatically reduced GS content of the *gcc8* mutant led us to hypothesize that the line harbors a mutation affecting one of the major regulatory loci of GS biosynthesis in *Arabidopsis thaliana*.

a. Gene Identification

Using NGS and further fine mapping, we identified the causative mutation to affect *AtSULTR1;2*, a major sulfate uptake gene in Arabidopsis. This conclusion is strongly supported by the existence of other mutant alleles of this gene. Earlier, *AtSULTR1;2* mutants were identified by their resistance to selenate, a structural toxic analogue of sulfate (Barberon, et al., 2008; El Kassis, et al., 2007; Rouached, et al., 2008; Yoshimoto, et al., 2002). We found that two of these alleles, *sel1-15* and *sel1-16*, have GS content as low as that of the *gcc8* line, which indicates that all mutations are in the same gene.

In addition, the F_1 progenies of crosses between *gcc8* and *sel1-15* or *sel1-16* also have the low-GS phenotype which further confirms that *gcc8* and the other *sel1* alleles are allelic (Figure 2.13). The alternative explanation that both *sel1-15* and *sel1-16* harbor independent, dominant mutations in other genes that exactly mimic the *gcc8* phenotype, seems highly implausible.

Furthermore, we obtained two additional lines with T-DNA insertions, one in the promoter region of *AtSULTR1;2* and other in its 3'UTR. Both lines show GS levels intermediate to those of wild-type (Col-0) and the mutant *gcc8* line (Figure 2.15). Thus, these lines likely represent two additional independent and functionally distinct alleles of *AtSULTR1;2*. The selenate resistance of *gcc8* shows that the *gcc8* line is also a new member of the *sel1* mutant family—the fact that it also has a mutation in *sel1* can hardly be a coincidence. Finally, we performed the classic genetic complementation experiment, expressing wild-type *AtSULTR1;2* in the *gcc8* mutant background, and restoring wild-type phenotypes and the GS-chemotype (Figure 2.27). We are therefore convinced to have identified the correct *GCC8* locus.

b. Importance of *AtSULTR1;2* for GS Accumulation

Among the three high affinity sulfate transporters *AtSULTR1;1* and *AtSULTR1;2* expressed in root cortical cells (Smith, et al., 1995; Smith, et al., 1997; Takahashi, et al., 1997; Takahashi, et al., 2000; Hawkesford, 2003; Yoshimoto, et al., 2002) and *AtSULTR1;3* in the vacuoles (Yoshimoto, et al., 2003). To find the importance of these three different isoforms in providing sulfate to the GS pathway, we measured GS profiles of the T-DNA knockout lines of *AtSULTR1;1* and *AtSULTR1;3*. The results show, both *AtSULTR1;1* and *AtSULTR1;3* knockout lines have similar GS profiles as the Col-0 wild type. This demonstrates that of the three transporters only *AtSULTR1;2* is critical for both sulfate uptake and GS accumulation (Figure 2.16).

c. Gene Expression Analysis of GS-related Genes

Gene expression analysis of *gcc8* compared to the wild-type clearly revealed that few GS-related genes were differentially expressed in the mutant. These include the major aliphatic GS regulator *MYB29* and the some of the genes involved in methionine side chain elongation:

BCAT4, MAM1 and *MAM2.* All are down regulated, and this can explain why we have dramatic reduction of aliphatic GS (i.e., 4MSOB) in the *gcc8* mutant line. Down regulation of *CYP79B3*, the major indole GS biosynthetic gene (Zhao & Last, 1996; Zhao, et al., 1998) is an obvious factor that could be responsible for the strongly reduction in indolic GS (i.e., I3M, Figure 2.18). In case of *CYP79B2* is the second gene shown to be responsible for Trp oxidation (Hull, et al., 2000). However, much more work would be required to demonstrate that these expression changes are actually responsible for the metabolic difference between wild type and *gcc8.*

Alternatively, *gcc8* may have low levels of GS because GS degradation is increased. The known GS catabolic genes *NIT3* and *NSP5* are up-regulated in the mutant, suggesting that increased catabolism could be contributing to the observed phenotype. Previous work found that sulfur starvation induced both *NIT3* transcripts as well as active protein levels, which the authors suggested could be a response to the need to convert indole-3-acetonitrile (IAN) to indole acetic acid (IAA) in order to enhance the root growth of the sulfur-starved plants in order to take up more sulfate (Kutz, et al., 2002). In our mutants, the role of these enzymes may simply be detoxification of compounds produced when indolic GS are recycled to obtain sulfur.

d. Induction of Sulfur Starvation Genes

JA is a known inducer of GS biosynthesis (Brader, et al., 2001; Mikkelsen, et al., 2003) and also of the sulfur starvation response (Harada, et al., 2000; Jost, et al., 2005). Microarray analyses and qRT-PCR analyses of *gcc8* compared to Col-0 wild-type showed that some sulfur starvation genes are highly induced (Figure 2.20), and many JA-related genes are as well, and yet GS levels are low. None of the JA intermediates were induced in the mutant except OPDA (Figure 2.21), concludes induction of JA biosynthetic genes only at the

transcriptional level. We did not observe the induction of indolic GS because of the induction of the JA gene expression might be low sulfur in the mutant line in order to use the available sulfur into primary metabolism rather than secondary metabolites which used to play a role in plant defense (Mugford, et al., 2011).

e. The Function of BGLU28 in Sulfur Starvation Conditions

Earlier it was hypothesized that BGLU28, a putative β-thioglycosidase, degrades GS to release the sulfur moiety during sulfur starvation conditions (Maruyama-Nakashita, et al., 2003; Maruyama-Nakashita, et al., 2006; Hirai, et al., 2005; Dan, et al., 2007). *BGLU28* is one the marker genes whose expression is induced during sulfur starvation in many experiments (Maruyama-Nakashita, et al., 2006; Zhang, et al., 2014). In order to test this hypothesis, we performed two different experiments to examine this issue. Microarray analyses and qRT-PCR analyses confirm that in the *gcc8* background *BGLU28* expression is highly induced. If we assume these transcripts are translated into protein, then the abundance of BGLU28 protein would be more than the wild-type, and we might expect increased myrosinase activity in the *gcc8* line, yet we observe the opposite (Figure 2.23). In the benzyl-GS degradation assay, wild type extracts consistently had more activity. This experiment does not permit much interpretation, however, since there are many valid explanations: BGLU28 may have been denatured during extraction, or its activity may be unobservable because of a very high background activity from other myrosinase, or it may not be a thioglycosidase and so on.

Next, we performed double mutant analyses of *gcc8* and a *bglu28* T-DNA insertion knockout line. If BGLU28 participates in degradation of GS in *gcc8*, in the double knockout we might expect the GS content of wild-type, but all three independent double mutant plants had low

GS just like *gcc8*. This gives clear genetic evidence that BGLU28 is not critical for GS degradation in sulfur starvation conditions.

g. Why Does *gcc8* Have Low GS Content?

Halkier and coworkers have repeatedly shown that overexpression of CYP79s, which transform amino acids to aldoximes in the first committed step of GS synthesis, causes accumulation of the corresponding GS (Hansen, et al., 2001a; Hansen, et al., 2001b; Chen, et al., 2003; Mikkelsen, et al., 2000). This implies that these enzymes are rate-limiting for the GS pathway, but expression of these genes in *gcc8* is unaltered, except for *CYP79B3*. Reduction of *CYP79B3* levels may explain the low levels of Trp-derived GS in *gcc8*, but it is less obvious what is causing the reduction of aliphatic GS. While *MYB29* is the major regulator of aliphatic GS biosynthesis (Gigolashvili, et al., 2008b), and its expression is reduced, this does not affect *CYP79F1* or *CYP79F2*, whose products catalyze formation of Met-derived aldoximes, begging the question, what is limiting accumulation of aliphatic GS? One possibility suggested by our data is that flux through this branch of the GS pathway is limited by low expression of key enzymes of the chain-elongation pathway, and CYP79F1 and CYPF2 simply have no substrate.

It is also possible that GS synthesis is not changed much and the low GS levels might result from increased degradation. The *gcc8* mutant has low myrosinase activity, but this does not necessarily mean that degradation in intact plants is lower: degradation of GS in intact plants does not necessarily require myrosinase activity.

PAPS levels are unchanged, so the availability of PAPS for GS synthesis is unlikely to be limiting. It is still possible, though: the concentration of PAPS in the cells that are making GS could be limiting. Our measurements only reveal the concentration of PAPS in the whole leaf

tissue. The plastidic PAPS transporter plays an important role in synthesis of GS, as shown by the *PAPST1* mutants (Gigolashvili, et al., 2012). We conclude that *gcc8* has a block in sulfur uptake which causes reduced GS levels via the limited misregulation of GS-related genes.

4. CONCLUSION

We identified the causative mutation in the gene *AtSULTR1;2*, as a premature stop codon in the first exon of the gene, which likely leads to an absence of active protein in the mutant line, limiting import of sulfur from the environment. The block in sulfur uptake triggers a major downstream response called the sulfur starvation response, which includes induction and down regulation of several GS-related genes. The downregulation of *MYB29* (major aliphatic GS regulator) and *CYP79B3* (major indolic GS biosynthetic gene) could be enough to cause the low GS content of the *gcc8* line. Induction of transcript levels of *NSP5* and *NIT3*, whose products act in GS degradation, could indicate that GS catabolism also contributes to the phenotype.

5. MATERIALS AND METHODS

a. Plant Material

The *Arabidopsis thaliana* accessions used in this study were laboratory stocks: Columba-0 (Col-0), Landsberg *erecta* (Ler), and mutant line *gcc8* (Grubb et al., 2002). From the Nottingham Arabidopsis Stock Centre (NASC) we obtained *SULTR1;1* T-DNA insertion line (*SALK_093256*), *SULTR1;2* T-DNA insertion lines (*SALK_122974*, *SALK_133651*), *SULTR1;3* T-DNA insertion line (*SALK_018910*), *BGLU28* T-DNA insertion line (*SALK_002108*). The *sel1* alleles (*sel1-15* and *sel1-16*) along with their wild type GHF (Col-0 with *ProBGLU28*::*GUS*) were obtained from Dr Zheng ZL, Department of Biological Sciences, Lehman College, City University of New York, Bronx, USA.

b. Growth Conditions

Surface sterilized seeds were placed in a solid medium containing 8 g/L agar, 15 g/L sucrose and 2.16 g/L Murashige-Skoog medium including vitamins, pH 5.6 (Murashige & Skoog, 1962). Petri dishes (150 X 25 mm, gridded) containing 100 ml agar medium were used for plant growth. After 2-3 days of 4 ^{o}C treatment, plants were grown for 18 days at 22 – 24 ^{o}C under illumination with fluorescent and incandescent light (60 μM m^{-2} S^{-1}) with an 16/8 hour light/dark photoperiod.

c. Anthocyanin Quantification

Wild-type (Col-0) and *gcc8* seedlings were grown in vertical plates for five days and their weights were measured, followed by addition of 1 ml anthocyanin extraction buffer containing isopropanol: HCl: water (18:1:81). Eppendorf tubes were boiled for three minutes, covered with aluminum foil, and left overnight on a shaker at room temperature. The next day

we measured optical density at 535 and 650 nm. Total anthocyanins were determined by simple subtractive correction (A_{535}-A_{650}) (Schmidt & Mohr, 1981).

d. Glucosinolate Extraction

Plant materials were harvested and flash frozen in liquid nitrogen in 2 ml collection tubes along with 0.2 mm glass beads for tissue homogenization and stored at -80 ^{o}C. With the Mixer Mill MM 200 (Retsch GmbH, Germany) plant materials were homogenized and mixed with 450 µl of ice cold methanol-chloroform and sinigrin (20 mg/L) followed by addition of 150 µl of distilled water and placed on a rotor for one hour to mix the samples at room temperature. The samples were centrifuged at 4000 rpm for 15 minutes at 4 ^{o}C. Ion exchange columns were created by adding 200 µL of a 50% suspension of DEAE-sephadex 25 in 80% methanol to each well of a filtering 96-well plate. The plate was centrifuged briefly to remove excess methanol. Next, 200 µL of supernatant were added into the column and centrifuged for 1000 rpm for 4 minutes. Then the column was washed with 80% methanol 3 times and distilled water 3 times. Finally, sulfatase stock was diluted 1:4 with distilled water and added to each sample. Plates were kept in the dark for 16 hours for incubation. Desulfoglucosinolates were eluted and analyzed by HPLC.

e. HPLC Analysis of Glucosinolates

Glucosinolate samples were analyzed using Waters (USA) 600E HPLC with a Waters 486 absorbance detector (226 and 228 nm). Samples (10 µL) were separated using a C18 column with the particle size of 1.8 µm and operated at 400 µL/min at 40 ^{o}C. The HPLC peaks were identified using Empower software.

f. Genetic Mapping and Identification of the *GCC8* Locus

The *gcc8* line (Col-0) was crossed with the Landsberg wild-type accession to make use of genetic variation between the Columbia and Landsberg ecotypes. The resulting F_1 progeny of this cross was subsequently selfed to establish an F_2 generation and subsequently in the F_2, the low-GS phenotype of *gcc8* was used to select 41 individual plants. These were transferred to the soil to provide material for map-based cloning. F_3 populations from each of the parental groups of 41 individual F_2 plants were scored again for low GS content phenotype to confirm that the causal mutation was homozygous in all lines. All the 41 mapping population plant leaf tissues from all lines were pooled to isolate bulk DNA for next generation sequencing. DNA was sent to BGI, Hong Kong for the whole genome resequencing on an Illumina Hiseq2000 sequencing platform with 50X coverage. The sequencing reads were aligned onto the public data of the reference Col-0 genome (TAIR 10).

The Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/) application on the TAIR website (https://www.arabidopsis.org/) was used to map the insertion/deletion (INDEL) mutations between Col-0 and Ler at different chromosomal positions within the candidate region in order to obtain markers for genotyping lines of interest via markers derived from simple sequence length polymorphisms (SSLP). The presence of the SNP was confirmed by PCR amplified sequencing of the corresponding locus and by Cleaved-Amplified Polymorphic Sequences (CAPS) markers.

g. Root Growth Analysis

For root measurements, at least 20 sterilized seeds were placed in a single row on square plates containing solid media with or without 30 μM selenate. The plates were oriented vertically to allow for root growth along the agar surface. Root length was measured after

eight days of growth. Images of the roots were captured with a COHU high-performance CCD camera and quantitative analysis was done by Image J.

h. Generation of Transgenic Plants

The coding sequence of *SULTR1;2* without stop codon was amplified using primers (DM_935_OE_For & DM_936_NSTOP_Rev; Table S1) in combination with a high fidelity DNA polymerase and subsequently inserted into pENTR/D-TOPO plasmid via directional TOPO cloning (Invitrogen) to generate the *SULTR1;2*-pENTR/D-TOPO entry vector. The fidelity of the inserts was verified by DNA sequencing. To generate a complementation line with overexpressed GFP-tagged fusion protein, *SULTR1;2*-pENTR/D-TOPO entry plasmids were recombined in LR clonase reactions with the Gateway compatible binary destination vector pB7WG2 to generate the *CaMV* $35S_{pro}$*::SULTR1;2~GFP* plasmid construct. The resulting binary plasmid was transformed into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method, and *gcc8* plants were transformed via the floral-dip method (Clough & Bent, 1998). Transgenic plants (T_1) were selected by spraying BASTA (400 µL/L) on soil-grown seedlings. T_2 seedlings were grown on ½ MS plates along with Columbia and *gcc8* lines for 18 days and for GS measurement. Plants were genotyped for the WT copy of the *AtSULTR1;2* gene to detect insertion of the transgene in the *gcc8* background.

i. Microarray Analysis

Total RNA was extracted and purified using the Qiagen RNAEasy mini kit. The methods for cDNA synthesis, cDNA fragmentation, microarray hybridization, washing and detection are described in the GeneChip expression analysis technical manual.

j. Extraction, Purification and Estimation of Oxylipins

Fresh plant material (300 mg) was homogenized with 10 mL methanol and appropriate 100 ng of ($^2H_6$) JA, ($^2H_5$) OPDA, 11-($^2H_3$)OAc-JA and 12-($^2H_3$)OAc-JA as internal standards. The homogenate was filtered under vaccum on column with cellulose filter. Elute was evaporated and acetylated with 200 µl of pyridine and 100 µl of acetic acid anhydride at 20 ^oC overnight. The extract was evaporated and dissolved with 10 mL of methanol, and placed on a colum filled with 2 mL DEAE-Sephadex A25 (Amersham Pharmacia Biotech AB, Sweden) (Ac^- - form methanol); the column was washed with 3 mL methanol. After washing with 3 mL 0.1 M acetic acid in methanol, eluents with 3 mL of 1 M acetic acid in methanol and 3 mL of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC derivatized and analysed by GC MS. Derivatization: Evaporated samples were dissolved in 200 µl $CHCl_3$ / N, N diisopropylethylamine (1:1) and derivatized with 10 µl of pentaflurobenylbromide at 20 ^oC overnight. The evaporated samples 1 and 2 from HPLC were dissolved in 5 mL n-hexane and passed through a Chrombaond-SiOH-Column, 500 mg; (Machery Nagel, Germany). The pentafluorobenzyl esters were eluted with 7 mL m-hexane / diethyl ether (1:1). Elutes were evaporated, dissolved in 100 µl MeCN and analyzed by GC-MS.

k. Sulfate Content

Twenty mg of homogenized plant materials was added to 200 µl of water containing PVPP (Poly Vinyl Poly Pyrrolidone 25 mg^{-1} ml) and incubated on a shaker for 1 hour at 4 ^oC. Then the samples were heated to 95 ^oC for 15 minutes and centrifuged at 14000 rpm for 15 minutes at 4 ^oC. 100 µl of supernatant and 900 µl of distilled water were added to ion-chromatography vials and then subjected to ion-chromatography. We followed the program 1.2 mL/min of

buffer flow for a 13 minute isocratic run. Analysis of anions by ion-chromatography was performed as previously (Wirtz & Hell, 2007).

l. Glutathione Content

Plant materials were homogenized in 10 fold excess volume of 0.1 M hydrochloric acid and centrifuged for 10 minutes at 4 ^{o}C. In a new Eppendorf tube, 25 µL of supernatant was placed, along with 25 µL of 0.1 M NaOH and 1 µL of 100 mM freshly prepared dithiothreitol (DTT) for reducing disulfides. The tubes were mixed well by vortex, then spun down and incubated in 37 ^{o}C for 15 minutes in darkness. After incubation, 10 µL of 1 M Tris-HCl (pH 8.0), 35 µL of water, and 5 µL of 100 mM monobromobimane (Calbiochem, EMD Chemicals) in acetonitrile were added and samples were incubated for another 15 minutes in darkness. Finally, we added 100 µL of 9% acetic acid solution and then centrifuged for 15 minutes at 4 ^{o}C. The supernatant was placed in HPLC vials to for chromatographic separation. Analysis of thiols was performed by HPLC as previously described (Wirtz & Hell, 2003).

m. PAPS and PAP Content

Frozen plant materials were homogenized using 0.2 mm glass beads using Mixer Mill MM 200 (Retsch GmbH, Germany) and 500 µL of hot (80 ^{o}C) extraction buffer (62mM citric acid, 76 mM K_2HPO_4, pH 4) were added. Samples were incubated for 5 minutes at 80 ^{o}C. Samples were cooled on ice for 15 minutes and centrifuged at 14000 rpm for 10 minutes at 4 ^{o}C. Supernatants were transferred into new Eppendorf tube and used for derivatisation. As standards, 20 µM, 10 µM, 5 µM of ATP, ADP, AMP and PAP were used. Plant extracts (100 µL) were mixed with 355 µL extraction buffer and 45 µL of chloracetaldehyde mixture and mixed by vortexing and followed by incubation for 40 minutes at 60 ^{o}C. The derivatisation was stopped by cooling samples on ice for 15 minutes. Subsequently the samples were

centrifuged at 14000 rpm for 10 minutes at 4 ^{o}C. The supernatant was diluted 1:1 with distilled water and transferred to an HPLC vial for chromatographic analysis.

n. Real-time RT-PCR Analysis

For expression analysis of wild type and *gcc8* lines, RNA was extracted using the RNeasey Plant Mini Kit, including Dnase treatment (Qiagen). The Super Script First Strand Synthesis kit (Thermo Scientific, Pittsburgh, PA, USA, www.thermoscientific.com) was applied with 3 µg of total RNA as template and an oligo (dT) primer. Quantification relied on the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA, www.appliedbiosystems.com) applied with Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA www.appliedbiosystems.com). *PP2A* was used as the control in quantitative RT-PCR.

For semi-quantitative RT-PCR, total RNA was extracted from indicated genotypes with the Qiagen RNeasey kit and converted to cDNA with the Super Script First Strand Synthesis system (Invitrogen, Carlsbad, CA, USA). Oligomers designed to *SULTR1;1* or *SULTR1;3* were used to measure the transcript levels of particular mutant lines.

o. Primers Used in the Current Study

Primer name	Target	Primer sequence
SULTR1: 2_M_Forward	*gcc8* mutation	CGTCTTCCCGGCCTTCGATTG
SULTR1: 2_M_Reverse		TTCGCCAACTTAGCGTATCC
DM_940_002108_LP	Genotyping *bglu28* TDNA insertion line	AGATGGAGATTTCCCACCTTC
DM_941_002108_RP		TACATGCAAAAAGGGTTTTCG
DM_968__LP	Genotyping *sultr1: 1* TDNA insertion line	GCCCTAGGGACTATGAACGTC
DM_969__RP		TTTTCTTCAAGCCACAATTGC
DM_960__LP	Genotyping *sultr1: 3* TDNA insertion line	TCGATCAAGAAACCCAATCTG
DM_961__RP		ATTCTTAGGGGTGCTAATGGC
DM34_LBb1.3	Insertion primer	ATTTTGCCGATTTCGGAAC

Primers for GFP constructs

Primers name	Target	Primer sequence
DM_935_OE_For	*SULTR1: 2 ORF*	cacctTAGCTATGTCGTCAAGAGCTCA
DM_936_NSTOP_Rev		GACCTCGTTGGAGAGTTTTGG

Primers for semi-quantitative PCR

Primer name	Target	Primer sequence
BGLU28_For	*BGLU28*	TCACCTTCTTCTCGCTCATGCC
BGLU28_Rev		CCAATCTGACCGTCTTGAGTCTTG
SULTR1: 1_For	*SULTR1: 1*	AGATCGGTCTCTTGATCGCTGTG
SULTR1: 1_Rev		AACCGTGGTTCTTGGTCTCGTC

SULTR1: 3_For	*SULTR1: 3*	TTCTCAAGGGCTTCCGCATAGG
SULTR1: 3_Rev		TTCTTCCTATCGCTACAGCTTCCG

Primers for qRT-PCR genes

Primer name	Target	Primer sequence
DM_950_qRT_78000_F	*SULTR1: 2*	GAGCATTCTTTGGCGTCATCTTTG
DM_951_qRT_78000_R		ATCTTAGCAAACGAGATCGAGACG
Sulfur starvation genes		
BGLU28_F	*BGLU28*	CGCGTTACGTTGCTCATATT
BGLU28_R		GAGCTGATGATCGGTTACGA
LSU1_F	*LSU1*	GGATGAGCTAAGGAGGAGGA
LSU1_R		TTCGCTGCCACAACTTAATC
SDI1_F	*SDI1*	CAAATCTTTCCGTCCTCGTT
SDI1_R		CAACTCAACTTGCTCCTCCA
Jasmonate related genes		
AOC2_F	*AOC1*	CTCTCAGAACTTGGGAAATAC
AOC2_R		GATCTCCGAGACCAAACCTA
AOS_F	*AOS*	GAGGCATGTGTTGTGGTCGAA
AOS_R		CCGGCGCATTGTTTATTCC
OPR3_F	*OPR*	TCTCCGGCTATAGATCACTTGGA
OPR3_R		TGTAACGTGAAGGTAAGCGAGCT
VSP1_F	*VSP1*	CACTGTCGAGAATCTCAAGGCTG
VSP1_R		CGTTTGGCTTGAGTATGAGATGC
Glucosinolate related genes		
MYB29_For	*MYB29*	AGTTGTAGATTGCGATGGGCTAAC
MYB29_Rev		TGTCTCGCTATGACTGACCACTTG
BCAT4_For	*BCAT4*	GGATTCTGCTATTCCGACCA
BCAT4_Rev		TGATCGACCGAAGGATAAGG

MAM1_For	*MAM1*	CGGCTGAAAGAGTGGGGATATGA
MAM1_Rev		CGTTAGCGCCGTTTAATTTCTC
MAM3_For	*MAM3*	GAGAAATTGAACGCTGTCTTCTCAC
MAM3_Rev		AGCCGTTAGACTTTAAACCGTTAGC
CYB79F1_For	*CYP79F1*	CCATACCCTTTTCACATCCTACTAGTCT
CYB79F1_Rev		GTAGATTGCCGAGGATGGGC
CYP79F2_For	*CYP79F2*	ACTAGGATTTATCGTCTTCATCGCA
CYP79F2_Rev		CTAGGACGAGTCATGATTAGTTCGG
CYP79B2_For	*CYP79B2*	AACAAAAAGAAACCGTATCTGCCAC
CYP79B2_Rev		TCCTAACTTCACGCATGCTATCTC
CYP79B3_For	*CYP79B3*	CTCCTTCTTCCTTGCAAATGGA
CYP79B3_Rev		GAGAATCATCAAGAAGCAAAGGG
CYP83B1_For	*CYP83B1*	GGCAACAAACCATGTCGTATCAAG
CYP83B1_Rev		CGTTGACACTCTTCTTCTCTAACCG
CS lyase_For	*CS lyase*	CAAGACGAGCCGTTGCTGAT
CS lyase_Rev		GGACGTGGGAGCAAGATGTTT
UGT74B1_For	*UGT74B1*	TCCTAATCGAGAAATTCAAATCCAC
UGT74B1_Rev		CGCAACACAGAACAAACAGTGAGAT
UGT74C1_For	*UGT74C1*	CCTGACCGATTTCATCTCTAGTGC
UGT74C1_Rev		TGGCTATGTCCAATGCAAAGGG
SOT16_For	*SOT16*	CGAAGTCGTCGAACTCACAGAGTT
SOT16_Rev		AAAGACCTTCGAGGAGACATTCTTG
SOT17_For	*SOT17*	CCCTACCGAGTCACGACGAGA
SOT17_Rev		GGTAGCCACCAGTAACCACCATACT
SOT18_For	*SOT18*	GGAATCCAAAACCATAAACGACG
SOT18_Rev		CGGATCTTTTGGTCTCCAGCC
PP2A_For	*PP2A*	AGCCAACTAGGACGGATCTGGT
PP2A_Rev		GCTATCCGAACTTCTGCCTCATTA

6. REFERENCES

Abel, S., Bürstenbinder, K. & Müller, J., 2013. The emerging function of IQD proteins as scaffolds in cellular signaling and trafficking. *Plant signaling and behavior,* 8(6), pp. 243691-243696.

Abel, S., Savchenko, T. & Levy, M., 2005. Genome-wide comparative analysis of the IQD gene families in Arabidopsis thaliana and Oryza sativa. *BMC Evolutionary bioligy,* 72(5), pp. 1-25.

Awazuhara, M. et al., 2002. A 235-bp region from a nutritionally regulated soybean seed-specific gene promoter can confer its sulfur and nitrogen response to a constitutive promoter in aerial tissues of Arabidopsis thaliana. *Plant science,* 163, pp. 75-82.

Barcelo, S., Mace, K., Pfeifer, A. & Chipman, J., 1998. Production of DNA strand breaks by N-nitrosodimethylamine and 2-amino-3-methylimidazo[4,5-f]quinoline in THLE cells expressing human CYP isoenzymes and inhibition by sulforaphane. *Mutation research,* 402, pp. 111-120.

Barron, D. et al., 1988. Sulphated flavonoids—an update. *Phytochemistry,* 27, pp. 2375-2395.

Barroso, C. et al., 1999. Salt-specific regulation of the cytosolic O-acetylserine(thiol)lyase gene from Arabidopsis thaliana is dependent on abscisic acid. *Plant molecular biology,* 40, pp. 729-736.

Bednarek, P. et al., 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science,* pp. 101-106.

Bennett, R. & Wallsgrove, R., 1994. Secondary metabolites in plant defence mechanisms. *New phytologist,* 127, pp. 617-633.

Bick, J. et al., 2001. Regulation of the Plant-type 5‘-Adenylyl Sulfate Reductase by Oxidative Stress. *Biochemistry,* 40, pp. 9040-9048.

Brader, G., Tas, E. & Palva, E., 2001. Jasmonate-Dependent Induction of indole glucosionlates in Arabidopsis by culture filtrates of the nonspecific pathogen Erwiniacarotovora. *Plant physiology,* 126(2), pp. 849-860.

Brown, P., Tokuhisa, J., Reichelt, M. & Gershenzon, J., 2003. Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. *Phytochemistry,* 62, pp. 471-481.

Bruhl, A., Haverkamp, T., Gisselmann, G. & Schwenn, J., 1996. A cDNA clone from Arabidopsis thaliana encoding plastidic ferredoxin:sulfite reductase. *Biochemica biophysica acta,* 1295, pp. 119-124.

Brunold, C., Suter, M. & Lavanchy, P., 1987. Effect of high and low sulfate concentrations on adenosine 5′-phosphosulfate sulfotransferase activity from Lemna minor. *Physiologica plantarum,* 70, pp. 168-174.

References

Buchner, P., Takahashi, H. & Hawkesford, M., 2004. Plant sulfate transporters: co-ordination of uptake intracellular and long-distance transport. *Journal of experimental botany,* pp. 1765-1773.

Bürstenbinder, K. et al., 2013. Arabidopsis calmodulin binding protein IQ67-domain 1 localizes to microtubules and interacts with kinesin light chain related protein 1. *The journal of biological chemistry,* 288(3), pp. 1871-1882.

Celenza, J. et al., 2005. The Arabidopsis ATR1 MYB transcrition factor controls indolic glucosinolate homeostasis. *Plant physiology,* 13(1), pp. 253-262.

Chen, S. et al., 2003. CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in Arabidopsis. *The plant journal for cell and molecular biology,* 33, pp. 923-937.

Collier, M. et al., 2003. Regulation of nitrogen uptake by Fagus sylvatica on a whole plant level – interactions between cytokinins and soluble N compounds. *Plant cell and environment,* 26, pp. 1549-1560.

Creelman, R. & Mullet, J., 1997. Biosynthesis and action of jasmonates in plants. *Annual review of plant physiology and plant molecular biology,* 48, pp. 355-381.

Dan, H., Yang, G. & Zheng, Z., 2007. A negative regulatory role for auxin in sulphate deficiency resonse in Arabidopsis thaliana. *Plant molecular biology,* 63(2), pp. 221-235.

Davidian, J. & Kopriva, S., 2010. Regulation of Sulfate Uptake and Assimilation—the Same or Not the Same?. *Molecular plant,* 3(2), pp. 314-325.

Dubos, C. et al., 2010. MYB transcription factors in Arabidopsis. *Trends in plant science,* 15, pp. 573-581.

Fahey, J. W., Zalcmann, A. T. & Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry,* pp. 5-51.

Fahey, J., Zhang, Y. & Talalay, P., 1997. Brocoli sproutes: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *PNAS,* 94, pp. 10367-10372.

Fan, L. et al., 2011. Pseudomonas sax Genes Overcome Aliphatic Isothiocyanate–Mediated Non-Host Resistance in Arabidopsis. *Science,* 331, pp. 1185-1188.

Fodor, J. et al., 1997. Local and Systemic Responses of Antioxidants to Tobacco Mosaic Virus Infection and to Salicylic Acid in Tobacco (Role in Systemic Acquired Resistance). *Plant physiology,* 114, pp. 1443-1451.

Geu-Flores, F. et al., 2011. Cytosolic gamma-glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in Arabidopsis. *The plant cell,* 23, pp. 2456-2469.

Gidda, S. et al., 2003. Biochemical and molecular characterization of a hydroxyjasmonate sulfotransferase from Arabidopsis thaliana. *The journal of biological chemistry,* 278, pp. 17895-17900.

Gigolashvili, T. et al., 2007a. The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in Arabidopsis thaliana. *The plant journal,* 50(5), pp. 886-901.

Gigolashvili, T. et al., 2008b. HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosionlate biosynthesis in Arabidopsis thaliana. *New phytologist,* 177(3), pp. 627-642.

Gigolashvili, T. et al., 2012. The Arabidopsis Thylakoid ADP/ATP Carrier TAAC Has an Additional Role in Supplying Plastidic Phosphoadenosine 5′-Phosphosulfate to the Cytosol. *The plant cell,* 24, pp. 4187-4204.

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual review of phytopathology,* 43, pp. 205-210.

Grossman, A. & Takahashi, H., 2001. Macronutrient utilization by photosynthetic eucaryotes and the fabric of interactions. *Annual review of plant physiology,* 52, pp. 163-210.

Grubb, C. & Abel, S., 2006. Glucosinolate metabolism and its control. *Trends in plant science,* 11(2), pp. 89-100.

Grubb, C., Gross, H., Chen, D. & Abel, S., 2002. Identification of Arabidopsis mutants with altered glucosinolate profiles on isothiocyanate bioactivity. *Plant science,* 162, pp. 143-152.

Gutierrez-Marcos, J., Roberts, M., Campbell, E. & Wray, J., 1996. Three members of a novel small gene-family from Arabidopsis thaliana able to complement functionally an Escherichia coli mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and "APS reductase" activity. *PNAS,* 93, pp. 13377-13382.

Halkier, B. & Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. *Annual reviews of plant biology,* 57, pp. 303-333.

Hansen, C. et al., 2001b. CYP83B1 Is the Oxime-metabolizing Enzyme in the Glucosinolate Pathway in Arabidopsis. *The journal of Biological chemistry,* 276, pp. 24790-24796.

Hansen, C. et al., 2001a. Cytochrome p450 CYP79F1 from arabidopsis catalyzes the conversion of dihomomethionine and trihomomethionine to the corresponding aldoximes in the biosynthesis of aliphatic glucosinolates. *The journal of biological chemistry,* 276, pp. 11078-11085.

Harada, E., Kusano, T. & Sano, H., 2000. Differential expression of genes encoding enzymes involved in sulfur assimilation pathways in response to wounding and jasmonate in Arabidopsis thaliana. *Journal of plant physiology,* 156(2), pp. 272-276.

Hartmann, T., 2007. From waste products to ecochemicals. *Phytochemistry,* 68, pp. 2831-2846.

Hawkesford, M., 2003. Transporter gene families in plants: the sulphate transporter gene family - redundancy or specialization?. *Physiologia plantarum,* 117(2), pp. 155-163.

Hell, R. & Bergmann, L., 1990. λ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. *Planta,* 180, pp. 603-612.

Hell, R., Jost, R., Berkowitz, O. & Wirtz, M., 2002. Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant Arabidopsis thaliana. *Aminoacids,* 22, pp. 245-257.

Hesse, H. & Hoefgen, R., 2003. Molecular aspects of methionine biosynthesis. *Trends in plant science,* 8, pp. 259-262.

Hirai, M. et al., 2005. Elucidation of gene-to-gene and metabolite-to-gene networks in arabidopsis by integration of metabolomics and transcriptomics. *The journal of Biological Chemistry,* 280(27), pp. 25590-25595.

Hirai, M. et al., 2003. Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of O-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. *The plant journal,* 33, pp. 651-663.

Hirai, M. et al., 2007. Omics-based identification ofArabidopsis Myb transcription factors regulating aliphatic glucosinolatebiosynthesis. *Proceedings of the National Acadamy of Sciences of the United States of America,* 104(15), pp. 6478-6483.

Hull, A., Vij, R. & Celenza, J., 2000. Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *PNAS,* 97(5), pp. 2379-2384.

Jiang, M. & Zhang, J., 2001. Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant and cell physiology,* 42, pp. 1265-1273.

Jost, R. et al., 2005. Expression profiling of metabolic genes in response to methyl jasmonate reveals regulation of genes of primary and secondary sulfur-related pathways in Arabidopsis thaliana. *Photosynthesis reseach,* 86(3), pp. 491-508.

Kassis, E. et al., 2007. Characeterization of a selenate-resistant Arabidopsis mutant. Root growth as a potential target for selenate toxicity. *Plant physiology,* 143(3), pp. 1231-1241.

Kataoka, T., Hayashi, N., Yamaya, T. & Takahashi, H., 2004a. Root-to-shoot transport of sulfate in Arabidopsis. Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. *Plant physiology,* 136, pp. 4198-4204.

Kataoka, T. et al., 2004b. Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. *The plant cell,* 16, pp. 2693-2704.

Kawashima, C. et al., 2005. Characterization and expression analysis of a serine acetyltransferase gene family involved in a key step of the sulfur assimilation pathway in Arabidopsis. *Plant physiology,* 137, pp. 220-230.

Kiddle, G., Doughty, K. & Wallsgrove, R., 1994. Salicylic acid-induced accumulation of glucosinolates in oilseed rape (Brassica napus L.) leaves. *Journal of experimental botany,* 45, pp. 1343-1346.

Klein, M. a. P. J., 2004. The multi-protein family of Arabidopsis sulphotransferases and their relatives in other plant species. *Journal of experimental botany,* 55, pp. 1809-1820.

Kliebenstein, D., Gershenzon, J. & Mitchell-Olds, T., 2001. Comparative quantitative trait loci mapping of aliphatic, indolic and benzylic glucosinolate production in Arabidopsis thaliana leaves and seeds. *Genetics,* 159, pp. 359-370.

Kocsy, G. et al., 2000. Inhibition of glutathione synthesis reduces chilling tolerance in maize. *Pkanta,* 211, pp. 528-536.

Kopriva, S., 2006. Regulation of sulfate assimilation in Arabidopsis and beyond. *Annals of Botany,* pp. 479-495.

Kopriva, S. et al., 2012. Control of sulfur partioning between primary and secondary metabolism in Arabidopsis. *Frontiers in plant science,* 3(163), pp. 1-9.

Kopriva, S. et al., 1999. Light regulation of assimilatory sulphate reduction in Arabidopsis thaliana. *The plant journal,* 20, pp. 37-44.

Kopriva, S. et al., 2002. Interaction of sulfate assimilation with carbon and nitrogen metabolism in Lemna minor. *Plant physiology,* 130, pp. 1406-1413.

Koprivova, A. et al., 2000. Regulation of Sulfate Assimilation by Nitrogen in Arabidopsis. *Plant physiology,* 122, pp. 737-746.

Kutz, A. et al., 2002. A role of nitrilase 3 in the regulation of root morphology in sulphur-starving Arabidopsis thaliana. *The plant journal,* 30(1), pp. 95-106.

Lappartient, A. et al., 1999. Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. *The plant journal,* 18, pp. 89-95.

Leustek, T., Martin, M., Bick, J. & Davies, J., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annual review of plant physiology and plant molecular biology,* 51, pp. 141-165.

Leustek, T., Murillo, M. & Cervantes, M., 1994. Cloning of a cDNA encoding ATP sulfurylase from Arabidopsis thaliana by functional expression in Saccharomyces cerevisiae. *Plant physiology,* 105, pp. 897-902.

Leustek, T. & Saito, K., 1999. Sulfate transport and assimilatin in plants. *Plant physiology,* 120, pp. 637-643.

Li, Y. et al., 2013. Novel insights into the function of Arabidopsis R2R3-MYB transcription factors regulating aliphatic glucosinolate biosynthesis. *Plant & cell physiology,* 54(8), pp. 1335-1344.

Maheo, K. et al., 1997. Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. *Cancer research,* 57, pp. 3649-3652.

Malitsky, S. et al., 2008. The transcript and metabolite networks affected by the two clades of Arabidopsis glucosinolate biosynthesis regulators. *Plant physiology,* 148, pp. 2021-2049.

Martin, A. et al., 2000. Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. *The plant journal,* 24, pp. 559-567.

Maruyama-Nakashita, A. et al., 2006. Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. *The plant cell,* 18, pp. 3235-3251.

Maruyama-Nakashita, A. et al., 2003. Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. *Plant physiology,* 132, pp. 597-605.

Maruyama-Nakashita, A. et al., 2005. Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. *The plant journal,* 42, pp. 305-314.

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, pp. 1843-1849.

Menard, R. et al., 2004. Beta-1,3 glucan sulfate, but not beta-1,3 glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis. *The plant cell,* 16, pp. 3020-3032.

Mikkelsen, M., Hansen, C., Wittstock, U. & Halkier, B., 2000. Cytochrome P450 CYP79B2 from Arabidopsis Catalyzes the Conversion of Tryptophan to Indole-3-acetaldoxime, a Precursor of Indole Glucosinolates and Indole-3-acetic Acid. *The journal of biological chemistry,* 275, pp. 33712-33717.

Mikkelsen, M., Naur, P. & Halkier, B., 2004. Arabidopsis mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. *The plant journal for cell and molecular biology,* 37, pp. 770-777.

Mikkelsen, M. et al., 2003. Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. *Plant physiology,* 131(1), pp. 298-308.

Mikkelsen, M., Petersen, B., Olsen, C. & Halkier, B., 2002. Biosynthesis and metabolic engineering of glucosinolates. *Aminoacids,* 22, pp. 279-295.

Mithen, R., 2001. Glucosinolates – biochemistry, genetics and biological activity. *Plant growth regulation,* 34, pp. 91-103.

Mugford, S. et al., 2011. Control of sulfur partitioning between primary and secondary metabolism. *The plant journal,* 65(1), pp. 96-105.

Mugford, S. et al., 2009. Disruption of adenosine-5'-phosphosulfate kinase in Arabidopsis reduces levels of sulfated secondary metabolites. *The plant cell,* 21(3), pp. 910-927.

Muller, R. et al., 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *Journal of chemical ecology,* 36, pp. 905-913.

Naur, P. et al., 2003. CYP83A1 and CYP83B1, two nonredundant cytochrome P450 enzymes metabolizing oximes in the biosynthesis of glucosinolates in Arabidopsis. *Plant physiology,* 133, pp. 63-72.

References

Nikiforova, V. et al., 2003. Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. *The plant journal,* 33, pp. 633-650.

Nikiforova, V. et al., 2005. Systems Rebalancing of Metabolism in Response to Sulfur Deprivation, as Revealed by Metabolome Analysis of Arabidopsis Plants. *Plant physiology,* 138, pp. 304-318.

Noji, M. et al., 1998. Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from Arabidopsis thaliana. *The journal of biological chemistry,* 273, pp. 32739-32745.

Oh, H. & Reddy, E., 1999. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene,* 18, pp. 3017-3033.

Ohkama, N. et al., 2002. Regulation of sulfur-responsive gene expression by exogenously applied cytokinins in Arabidopsis thaliana. *Plant cell and physiology,* 43, pp. 1493-1501.

Ohkama-Ohtsu, N., Kasajima, I., Fujiwara, T. & Naito, S., 2004. Isolation and characterization of an Arabidopsis mutant that overaccumulates O-Acetyl-L-Ser. *Plant physiology,* pp. 3209-3222.

Piotrowski, M. et al., 2004. Desulfoglucosinolate sulfotransferases from Arabidopsis thaliana catalyze the final step in the biosynthesis of the glucosinolate core structure. *The journal of Biological chemistry,* 279, pp. 50717-50725.

Prestera, T., Holtzclaw, W., Zhang, Y. & Talalay, P., 1993. Chemical and molecular regulation of enzymes that detoxify carcinogens. *PNAS,* 90, pp. 2965-2969.

Rausch, T. & Wachter, A., 2005. Sulfur metabolism: a versatile platform for launching defence operations. *Trends in plant science,* 10(10), pp. 503-509.

Reuveny, Z., Dougall, D. & Trinity, P., 1980. Regulatory coupling of nitrate and sulfate assimilation pathways in cultured tobacco cells. *PNAS,* 77, pp. 6670-6672.

Reymond, P. & Farmer, E., 1998. Jasmonate and salicylate as global signals for defense gene expression. *Current opinion in plant biology,* 1, pp. 404-411.

Samuelson, M., Campbell, W. & Larsson, C., 1995. The influence of cytokinins in nitrate regulation of nitrate reductase activity and expression in barley. *Physiologica plantarum,* 93, pp. 533-539.

Sanda, S. et al., 2001. Recombinant Arabidopsis SQD1 Converts UDP-glucose and Sulfite to the Sulfolipid Head Group Precursor UDP-sulfoquinovose in Vitro. *Journal of biological chemistry,* 276, pp. 3941-3946.

Sasaki-Sekimoto, Y. et al., 2005. Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. *The plant journal,* 44, pp. 653-668.

Schmidt, R. & Mohr, H., 1981. Time-dependent changes in the responsiveness to light of phytochrome mediated anthocyanin synthesis. *Plant, Cell and Environment,* 4(6), pp. 433-437.

Setya, A., Murillo, M. & Leustek, T., 1996. Sulfate reduction in higher plants: Molecular evidence for a novel 5′-adenylylsulfate reductase. *PNAS,* 93, pp. 13383-13388.

Shibagaki, N. et al., 2002. Selenate-resistant mutants of Arabidopsis thaliana identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. *The plant journal,* 29, pp. 475-486.

Smith, F. et al., 1997. Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. *The plant journal,* 12(4), pp. 875-884.

Smith, F., Hawkesford, M., Prosser, I. & Clarkson, D., 1995. Isolation of a cDNA from Saccharomyces cerevisiae that encodes a high affinity sulphate transporter at the plasma membrane. *Molecular and general genetics,* pp. 709-715.

Sonderby, I. et al., 2010. A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. *Plant physiology,* 153(1), pp. 348-363.

Stotz, H. et al., 2011. Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against Sclerotinia sclerotiorum. *The plant journal,* 67, pp. 81-93.

Stracke, R., Werber, M. & Weisshaar, B., 2001. The R2R3-MYB gene family in Arabidopsis thaliana. *Current opinion in plant biology,* 4, pp. 447-456.

Suter, M. et al., 2000. Adenosine 5'-phosphosulfate sulfotransferase and adenosine 5'-phosphosulfate reductase are identical enzymes. *The journal of biological chemistry,* 275, pp. 930-936.

Takahashi, H. et al., 2000. The roles of three functional sulfate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana. *The Plant Journal,* pp. 171-182.

Takahashi, H. et al., 1997. Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in Arabidopsis thaliana. *PNAS,* 94, pp. 11102-11107.

Takahashi, H. et al., 2000. The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana. *The plant journal,* 23, pp. 171-182.

Takahashi, H., Yoshimoto, N. & Saito, K., 2006. Anionic nutrient transport in plants: the molecular basis of the sulfate transporter gene family. *Genetic engineering,* 27, pp. 67-80.

Takai, K., Sakakibara, H., Taniguchi, M. & Sugiyama, T., 2001. Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. *Plant cell and physiology,* 42, pp. 85-93.

Truper, H. & Fischer, U., 1982. Anaerobic Oxidation of Sulphur Compounds as Electron Donors for Bacterial Photosynthesis. *Biological sciences,* 298(1093), pp. 529-542.

Tsakralides, G. et al., 2002. Sulfate reduction is increased in transgenic Arabidopsis thaliana expressing 5'-adenylylsulfate reductase from Pseudomonas aeruginosa. *The plant journal,* 32, pp. 879-889.

Turner, J., Ellis, C. & Devoto, A., 2002. The jasmonate signal pathway. *The plant cell,* 14, pp. S153-164.

Varin, L., Marsolais, F., Richard, M. & Rouleau, M., 1997. Sulfation and sulfotransferases 6: Biochemistry and molecular biology of plant sulfotransferases. *FASEB journal,* 11, pp. 517-525.

Vauclare, P. et al., 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, pp. 729-740.

Vlot, A., Dempsey, D. & Klessig, D., 2009. Salicylic Acid, a multifaceted hormone to combat disease. *Annual review of phytopathology,* 47, pp. 177-206.

Wachter, A. et al., 2005. Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae. *The plant journal,* 41, pp. 15-30.

Westerman, S. et al., 2001. Atmospheric H2S as sulphur source for Brassica oleracea: Consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. *Plant physiology and biochemistry,* 39, pp. 425-432.

Wirtz, M. & Hell, R., 2003. Production of cysteine for bacterial and plant biotechnology: application of cysteine feedback-insensitive isoforms of serine acetyltransferase. *Amino acids,* 24(1-2), pp. 195-203.

Wirtz, M. & Hell, R., 2007. Dominant-negative modification reveals the regulatory function of the multimertic cysteine synthase protein complex in transgenic tobacco. *The Plant cell,* 19(2), pp. 625-639.

Wittstock, U. & Halkier, B., 2002. Glucosinolate research in the Arabidopsis era. *Trends in plant science,* 7, pp. 263-270.

Xiang, C., Werner, B., Christensen, E. & Oliver, D., 2001. The biological functions of glutathione revisited in arabidopsis transgenic plants with altered glutathione levels. *Plant physiology,* 123, pp. 564-574.

Yoshimoto, N. et al., 2003. Phloem-localizing sulfate transporter, sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis. *Plant physiology,* 131(4), pp. 1511-1517.

Yoshimoto, N. et al., 2002. Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. *The plant journal,* pp. 465-473.

Zhang, B. et al., 2014. Aberrant gene expression in the Arabidopsis SULTR1;2 mutants suggests a possible regulatory role for this sulfate transporterin response to sulfur nutrient status. *The plant journal,* 77(2), pp. 185-197.

Zhao, J. & Last, R., 1996. Coordinate regulation of the tryp-tophan biosynthetic pathway and indolic phytoalexin accumulation in Arabidopsis. *The plant cell,* 12, pp. 2235-2244.

Zhao, J., Williams, C. & Last, R., 1998. Induction of Arabidopsis tryptophan pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. *The plant cell,* 13(3), pp. 359-370.

CURRICULUM VITAE

Kalidoss Ramamoorthy

Weinberg 3
06120 Halle (Saale)
ramamoorthy.kalidoss@ipb-halle.de
Tel: +49 (0) 176 41 66 02 25

……..

DATE OF BIRTH	15TH FEBRUARY 1984
BIRTH PLACE	Sivakasi, India
EDUCATION	
2001 – 2004	Ayya Nadar Janaki Ammal College (Autonomas), Sivakasi Completion: Bachelor of Science (BSc) in Microbiology
2004 – 2006	Bharathiyar University, Coimbatore Completion: Master of Science (MSc) in Bioinformatics
PRACTICAL EXPERIENCE	
2006 – 2008	Research assistant In “Stem Cell and Molecular Biology Lab” at Indian Institute of Technology Madras (IIT-M), Chennai Reference: Prof. Rama Shanker Verma
2008 – 2011	Research assistant In “Proteomics division” at Center for Cellular and Molecular Biology (CCMB), Hyderabad Reference: Prof. R. Nagaraj
05/2011 – currently	PhD dissertation Works on the subject “Identification and Characterization of Glucosinolate Biosynthetic mutants in *Arabidopsis thaliana* “in the work group Regulation of Defense Metabolism at Leibniz-Institute of Plant Biochemistry, Halle
REFERENCE	Dr. Clayton Douglas Grubb Prof. Dr. Steffen Abel

PUBLICATIONS

.....

- Gautam P, Nair SC, **Ramamoorthy K**, Swamy CVB, Nagaraj R (2013) Analysis of Human Blood Plasma Proteome from Ten Healthy Volunteers from Indian Population. PLoS ONE 8(8)

- Gupta MK, Polisetty RV, **Ramamoorthy K**, Tiwary S, Kaur N, Shiras A, Sirdeshmukh R., Secretome analysis of Glioblastoma Cell-line HNGC-2., Molecular BioSystems (2013)

- Polisetty RV, et al, Glioblastoma Cell Secretome: Analysis of Three Glioblastoma Cell Lines Reveal 148 Non-Redundant Proteins, J.Prot(2011)

- Rakesh K.S, Dawood D, Nagaraja R, Minoru S.H.Ko, Yulan Piao, David Schlessinger, **Ramamoorthy K**, Sirdeshmukh R. Mouse Embryonic Stem Cell Proteome:High Confidence Identification Of 9370 Expressed proteins by Combining Mass Spectrometry and Transcript Profiling,….in communication Journal of Proteomic Research (JPR)

- Singh S K, Rakesh K S, **Ramamoorthy K**, Partha sarathi AV, Idris M M, Proteome Profile of Zebrafish Brain Based on Gel LC-ESI MS/MS Analysis. J Proteomics and Bioinformatics, 3: 135-142. doi:10.4172/jpb.1000132 (2010)

- **Ramamoorthy K**, Verma RS, Convergence pattern studies of folate receptor, Bioinformation., 3(4),168-172 (2008)

- Seenivasagan R, Kasimani R, Parthiban M, **Ramamoorthy R**, Shanmughavel P, Comparative Modeling of Viral Protein R from Human Immunodeficiency Virus 1 (HIV-1), Journal of Proteomics and Bioinformatics, 1:073-076. doi:10.4172/jpb.1000012

- **Ramamoorthy R**, Sirisha P, Verma RS, Insilco analysis of functionally important residues in folate receptors, Bioinformation 2(4), 157 (2007)

LANGUAGE SKILLS

……

English: fluent in speaking and writing

Halle, 6th. August 15

Ramamoorthy Kalidoss