Molecular genetic studies of sulfur nutrient response in

Arabidopsis thaliana

BY

HANBIN DAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2012

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

	Dr. Zhi-Liang Zheng
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Date	Chair of Examining Committee
	Dr. Laurel A. Eckhardt
---	---
Date	Executive Officer
	Dr. Hai-Ping Cheng

	Dr. Amy Litt

	Dr. Timothy W. Short

	Dr. Miguel Cervantes-Cervantes

	Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

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Adviser: Professor Zhi-Liang Zheng

Sulfur (S) is an important plant macronutrient for the biosynthesis of two S-containing amino acids, cysteine and methionine. In general, S is not limited in the soil, and therefore, sulfate is not frequently added to the fertilizer package that mainly includes nitrogen, phosphate and potassium. However, sulfate distribution is not even in the soil or very limited in non-polluted areas, and therefore the lack of sulfate in those areas will decrease crop yield and quality. Furthermore, how S nutrient status is perceived by plants remains unknown. To improve S nutrition, it is essential to understand the mechanism of plant nutritional responses, in particular the mechanisms by which plant sense the status of S and its balance with carbon (C) and nitrogen (N).

The aim of this study was to isolate the components that are critical for the S nutrient response in plants. We used *Arabidopsis thaliana* as a model system. We first developed a S deficiency-responsive promoter:GUS reporter system. We confirmed that At2g44460 encoding a putative thioglucosidase exhibits the strongest induction by S deficiency. Interestingly, At2g44460

induction by S deficiency was suppressed by the application of auxin, a plant hormone. Together with other physiological and genetic evidence, we showed that auxin plays a negative regulatory role in S deficiency response. Furthermore, we found that S deficiency-induced expression of At2g44460 and a sulfate transporter gene (*SULTR4;2*) is dependent on the availability of C and N, which exhibit a synergistic interaction. Therefore, we designed a genetic screen by using the At2g44460 promoter:GUS reporter line (designated GHF1) with an aim of isolating the mutants that alter the expression of At2g44460 in response to C,N and S status.

Screen of the mutants resulted in the isolation of two allelic mutations on the *SEL1* gene, which encodes a high-affinity transporter called SULTR1;2. SULTR1;2 is mainly responsible for transporting sulfate from the soil into the root. The two alleles, designated *sel1-15* and *sel1-16*, have distinct missense mutations on the putative transmembrane domains, but they did not seem to cause mislocalization of the protein. As expected, these two mutations, like a *SULTR1;2* null allele (*sel1-10*), abolish the sulfate uptake in both yeast and plant systems. They also reduced the accumulation of internal sulfate. However, a dose response study indicates that expression of the S deficiency-upregulated genes, At2g44460, *SULTR4;2, LSU1* and *SDI1*, is higher in the mutants than that in WT under either the high sulfate treatment or under different sulfate treatments that result in similar levels of internal sulfate. Furthermore, these mutants reduced the sensitivity to external application of the high concentration of sulfate metabolites, suflite, Cys and GSH. Taken together, these results indicate that besides the sulfate transport function, SULTR1;2 likely acts as a sensor for S nutrient, adding this transporter to the growing list of nutrient transceptors.

ACKNOWLEDGMENTS

First and foremost I would like to sincerely thank my advisor, Dr. Zhi-Liang Zheng, for his guidance, support and encouragement during the past seven years. I am grateful to his patience in guiding my project and correcting my dissertation. Without his generous aids, this thesis would not have been accomplished.

I also wish to express my deepest gratitude to my committee members, Dr. Hai-Ping Cheng, Dr. Amy Litt, Dr. Timothy W. Short, and Dr. Miguel Cervantes-Cervantes. Thank you for your time in reading my dissertation, and thanks for your insightful suggestions. Special thanks to Dr. Cheng and Dr. Litt, who have been my advisory committee members from the beginning of my project. I'm also grateful to Dr. Dominick Basile and Dr. Edward B. Tucker, who served as my second exam committee members at 2007.

I would like to thank the following experts for their critical help in this project: Haiyang Wang (Boyce Thompson Institute) for providing the *axr1-3* seeds, Erwin Grill (Tubingen University) for the P_{RD29B}*::GUS* line, Zhenbiao Yang (University of California at Riverside) for the *DR5::GUS* line with the permission of the use from Thomas Guilfoyle (University of Missouri-Columbia), Dominique Thomas for providing the yeast CP154-7A strain, and Françoise Gosti for *sel1-8* seeds. In particular, Dr. Thomas Leustek at Rutgers University, who has collaborated with our group in this project, has provided tremendous experimental help and insightful discussion.

Many thanks to the faculty both at the Graduate School and University Center and Lehman College: Richard Chappell, Joan Reid, Douglas Ewing, Edward Kennelly, Joseph Rachlin, Liesl Jones, Dwight Kincaid, Eleanore Wurtzel, Maryam Bamshad, Martin Muntzel, Stephen Redenti, Renuka Sankaran, as well as the staff at Lehman College: Dolores Vitanza, Christina Murillo, Patricia Carver, James Lee, Mike Baxter, Katherine Lieberman and David Cain (who has left us). Thanks for the countless helps, for the friendly atmosphere, and for the synergistic cooperation.

Accolades should also go to my CUNY fellows past and present: Qingping Wu, Hsinjung Juan, Yan Xue, Jiabu Ye, Guohua Yang, Peng Gao, Xuesong Zhang, Haiyang Lu, Damaris-Lois Lang, Shari Walcott, Yu Chen, Maria Shumskaya, Rena Quinlan, James Lyles, Amy Keller, Hui Yang, Kiyo Mondo, and Jing Zhou. I would like to extend my heartfelt thanks to all of you for your assistance and comfort during the past years.

Especially deep thanks to my friend, Dr. Changyong Zhou at the Southwest University of China, Dr. Huaiyou Gao at the Tianjin Bureau of Quality and Technical Supervision, and my former mentor, Professor Wenbo Yang at Nankai University of China. Your encouragement and understanding is far beyond a valuable gift to me.

This dissertation has been voluntarily revised by Dr. Zhi-Liang Zheng, Dr. Miguel Cervantes-Cervantes, Dr. Amy Litt, Miss Christina Murillo and Miss Shari Walcott. I am in honor to credit all the improvement in this paper to them, but thoroughly understand that I am the only one who's responsible for any possible mistake in this dissertation.

Finally, I want to acknowledge and thank my wife, Wenjiao Liu and my lovely daughter, Priscilla Dan. Thank you for your tremendous helps and your warm company during these years. This dissertation is dedicated to you and all our family members: our parents Zaiyuan Liu, Fangda Cao, Yunguang Dan, and Qianfen Tang, our sister Hanzhen Dan, and brother-in-law, Gangji Tang.

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ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
APK	APS kinase
APR	APS reductase
APS	Adenosine 5'-phosphosulfate
ARF	Auxin Responsive Factor
ATPS	ATP sulfurylase
Aux/IAA	*Auxin/Indole-3-Acetic Acid*
BA	benzyladenine
BR	brassinolide
C	carbon
-C	carbon-deficient
CDS	CoDing Sequence
CRC	Cysteine Regulatory Complex
Cys	Cysteine
EIL3	Ethylene-Insensitive 3-Like 3—a protein encoded by *SLIM1*
EMA	ethyl-methane sulfonate
GA	gibberellins
GHF1	Glucoside Hydrolase, Family 1
Glc	glucose
Gln	Glutamine
GLS	Glucosinolate
GSH	glutathione
GSSG	GSH dimmer, the oxidized form of GSH
GUS	β-glucuronidase
HAST	high-affinity sulfate transporter
IAA	indole-3-acetic acid
IAN	indole-3-acetonitrile
JA	jasmonic acid
LAST	low affinity sulfate transporter
LR	lateral root
LRP	lateral root primordia
Met	Methionine
MS	Murashige and Skoog
MSD	membrane-spanning domain
MYB	myeloblastosis
N	nitrogen
–N	N-deficient

NAA	naphthalene-1-acetic acid, another auxin
NiR	Nitrite reductase
NIT3	*Nitrilase 3*
NR	nitrate reductase
NTR	Nitrate transporter
OAS	O-acetylserine
OASTL	OAS thiol-lyase
OE	over-expression
PAPS	3'-phosphoadenosine 5'-phosphosulfate
Phe	phenylalanine
PM	plasma membrane
q RT-PCR	quantitative RT-PCR
ROS	reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S	Sulfur
-S	sulfur deficiency
SA	salicylic acid
SAT	Serine acetyltransferase
SDC	S-containing defense compound
Ser	Serine
SiR	sulfite reductase
slim1	*sulfur limitation1*--a mutant
SRP	S-rich protein
SSLP	Single Sequence Length Polymorphism
STAS	sulfate transporter and antiSigma antagonist
Suc	sucrose
SULTR	Sulfate transporter
SURE	SUlfur-Responsive Element
TM	transmembrane
Trp	tryptophan
Tyr	tyrosine
WT	wild type
Z	zeatin

Chapter 1

Literature review and introduction

1. 1 Importance of sulfur as a nutrient

Sulfur (S) is an essential plant macronutrient, and makes up 0.1% of the whole plant dry weight, which is about 15 and 450 times smaller than that of nitrogen and carbon respectively (Leustek et al., 2000). Despite its relatively small amount in plant, S plays an important role both inside and out of the cell.

The amino acid cysteine (Cys) is the first organic S-containing compound synthesized immediately after the assimilation of an inorganic S source (predominantly sulfate) from the environment. The disulfide bond (-S-S-) formed by oxidation of two thiol groups (-SH) between two Cys serves an important role in maintaining tertiary and quaternary protein structure and hence protein function. In an enzyme, the reduction of this disulfide bond will seriously impact the three-dimensional structure of a protein and its activity (Maathuis, 2009). The reversible redox of thiol group in Cys and another S-containing amino acid, methionine (Met), is thus an important mechanism in enzyme activity regulation (Leustek et al., 2000). Met is an essential amino acid for animals. Common seed crops are in shortage of Met content as related to nutrient value for animals. Genetically modified plants with a higher content of Met or other S-containing compounds have a high impact on animal breeding or even human health (Saito, 2004).

Other small S-containing molecules, such as vitamins and cofactors such as biotin, thiamine, coenzyme A, and S-adenosyl-Met play key roles in a variety of processes in cellular metabolisms and energy production (Saito, 2004).

The S-containing oligopeptides, glutathione (GSH), is a well-studied compound that functions in buffering the intracellular redox environment keeping it in a reduced state, which is essential for plants and other aerobic organisms to escape damage from those noxious chemicals such as reactive oxygen species (ROS) (Rausch and Wachter, 2005). The mechanism of this buffering is based on the reversible redox conversion between the reduced form of GSH and its oxidized dimer of GSSG. Cells maintain more than 90% of the reduced form of GSH in order to get a reduced cellular environment for normal enzyme activity. Beside this, GSH also serves as the major storage and transporting form of reduced S, to be involved in control of S assimilation, protein folding and even the cell cycle. GSH is also involved in detoxification of plants by direct binding to heavy metals (Leustek et al., 2000; Zhang et al., 2004).

The significance of S nutrient studies is partly based on the molecular mechanism of S metabolisms, but more so on the possible application of S-containing secondary defense compounds (SDCs), among which are glucosinolates (GLSs) of Brassicaceae. It is clear that glucosinolates can be decomposed to produce a variety of volatile hydrolysis products against plant pathogens. *In vitro* experiments have shown their toxic effects on plant pathogens (Rausch and Wachter, 2005).

Instead of the above compounds, many other secondary products such as the S-rich proteins (SRPs), phytoalexins, are all reported to posses the anti-microbial activity in plant immunity. SRP-transgenic plants are reported to have improved resistance to plant pathogens (Rausch and Wachter, 2005). Glutathione-based phytochelatins have high affinity to heavy metals and impact a phytoremediation potential in environmental protection (Maathuis, 2009).

1.2 Sulfur uptake and the four families of sulfate transporters in *Arabidopsis*

Although there are multiple varieties of S nutrient sources for plants, such as H_2S and FeS in the soil (especially under flooded conditions), and gaseous SO_2 in the atmosphere, the predominant S nutrient for plant growth is soil sulfate (Maathuis, 2009).

Plants assimilate sulfate from soil to the root system using two high-affinity sulfate transporters (HASTs), SULTR1;1 and SULTR1;2. The proposed mechanism for this process involves a proton-coupled co-transport possibly with a $3H^+$/sulfate stoichiometry (Buchner et al., 2006). Despite lack of direct evidence of the protein localization, these two HASTs are proposed to be localized on the plasma membrane of root epidermis and cortical cells based on data of spatial gene expression (Shibagaki et al., 2002; Rouached et al., 2009).

Although both SULTR1;1 and SULTR1;2 are HASTs and share more location and functional similarities, these two transporters are not wholly functionally redundant. SULTR1;2 is responsible for more than 80% of sulfate transport, and it works constitutively both under +S and

–S conditions. On the other hand, *SULTR1;1* is expressed less and the gene expression can not be detected under +S condition. Further, *SULTR1;1* failed in compensating the growth of plant missing *SULTR1;2* (Yoshimoto et al., 2002; Buchner et al., 2006; Rouached et al., 2009).

There is another HAST gene *SULTR1;3*, which is proposed to be expressed in the phloem and functions in source-to-sink S transport (Yoshimoto et al., 2003; Davidian and Kopriva, 2010).

In contrast to *SULTR1;1* and *SULTR1;2*, *SULTR2;1* and *SULTR2;2* are two low affinity sulfate transporter (LAST) genes that are expressed in the xylem parenchyma and pericycle cells of the root. By synergistically working with SULTR3;5, these two LASTs function in long distance (root-to-shoot) sulfate transportation (Rouached et al., 2009 ; Takahashi et al., 2000). It has been demonstrated that SULTR2;1 is necessary for sulfate redistribution from old to young leaves. This capability is impaired in the *sultr2;1* loss-of-function mutant (Liang et al., 2010).

Currently, there are 4 families (groups) with 12 members of sulfate transporters identified in plants. Besides the above three group I HASTs, two group II LASTs and SULTR3;5, the other six sulfate transporters are: another four in group III (SULTR3;1, 3;2, 3;3 and 3;4), and two in group IV (SULTR4;1 and SULTR4;2. Group IV sulfate transporters are located on the tonoplast membrane and are responsible for vacuolar sulfate remobilization to the cytosol (Rouached et al., 2009; Davidian and Kopriva, 2010).

All 12 members of group I-IV sulfate transporters are predicted to be a polypeptide with 12 membrane-spanning domains and a STAS (sulfate transporter and antiSigma antagonist) domain at their carboxyl-terminus, which extends into the cytosol (Buchner et al., 2006).

1.3 Sulfur metabolism

Plant S metabolism starts with the immediate transport (influx) of sulfate from the outside environment into a cell and follows the following basic steps to synthesize the first S-containing organic chemical, Cys (Leustek et al., 2000; Hawkesford and De Kok, 2006; Davidian and Kopriva, 2010).

Step 1: Activation of sulfate to produce adenosine 5'-phosphosulfate (APS) via the enzyme ATP sulfurylase (ATPS).

Step 2: APS reduction by APS reductase (APR) to produce sulfite. The electron donor is glutathione (GSH), which is oxidized into its dimer GSSG.

Step 3: Sulfite is further reduced by sulfite reductase (SiR) to produce sulfide. The electron donor is ferredoxin, which loses 6 electrons to form its oxidized form.

Step 4: Sulfide is incorporated into the amino acid skeleton of O-acetylserine (OAS), which then becomes the first S-containing amino acid, Cys. The enzyme involves is OAS thiol-lyase (OASTL).

Through the synthesis of Cys, S nutrient is incorporated into proteins thus impacting multiple subsequent enzymatic or regulatory functions. Additionally, Cys is also the precursor for the

synthesis of a large number of biologically important molecules, such as the amino acid Met, the multifunctional oligo-peptide GSH, and a variety of coenzymes.

Many secondary S-containing molecules such as glucosinolates, flavanol, gallic acid gluoside, are synthesized by the sulfation branch pathway, where APS is catalyzed by APS kinase (APK) to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The later serves as the activated sulfuryl donor for subsequent biosynthetic reactions (Leustek et al., 2000). Besides this, GSH is known to be the precursor of phytochelatin synthesis, and sulfite is the intermediate for synthesis of sulfolipids. Phytochelatins are important in detoxification of heavy metals, and sulfolipids are essential components of chloroplast membranes (Kopriva, 2006). Glucosinolates, an important class of defensive S-containing compounds, can be alternatively synthesized from other precursors such as Met, phenylalanine (Phe), tyrosine (Tyr), or tryptophan (Trp) (Wittstock and Halkier, 2002).

Although Cys can be synthesized in all the three sub-cellular compartments that are capable of protein synthesis (namely cytosol, plastids, and mitochondria), sulfate reduction steps are believed to be confined to the plastids (esp. chloroplasts). Evidence for this proposal is that the sulfite reductase is exclusively localized in chloroplast (Grossman and Takahashi, 2001; Hawkesford and De Kok, 2006). In another experiment, a cloned *APS* from Arabidopsis was also localized to chloroplast (Leustek et al., 1994).

Figure 1-1 is a summary for the present understanding of plant S metabolism, mainly focused on the steps of primary assimilations. To date, it is still difficult to draw the whole picture of sulfate

assimilation in plants. The basic understanding is that, sulfate is firstly absorbed by the two HASTs (SULTR1;2 and SULTR1;1) into root cells, and followed by storage into the vacuoles if temporarily in excess. There may be limited synthesis of Cys in the root, but the majority of sulfate is pumped up by the two LASTs and SULTR1;3, with possibly other synergistic transporters involved in long-distance transportation. Sulfate reduction and Cys synthesis is mainly carried out in shoot chloroplasts, then assimilated into other S- containing products, and finally re-distributed to other organs or to roots. The ratio of S-assimilation between shoot and root is reported to exceed a factor of 2-5 (Hawkesford and De Kok, 2006).

1.4 Sulfur nutrient sensing and signaling

The most easily observed phenotypes when plants encounter sulfur deficiency stress are stunted growth, chlorosis, and decrease in plant yield. At the sub-cellular level, the concentration of many of the important S-containing compounds such as Cys, Met, GSH, and glucosinolates (GLSs) also decrease drastically (Maruyama-Nakashita et al., 2003). Nevertheless, metabolomic experiments revealed that even long-term treated plants with under 2% of the normal sulfate content, decrease their intra-cellular sulfate concentration at most half. In comparison to the control, sulfur-stressed plants decrease their total amino acids very slightly (Hirai et al., 2004). This strongly suggests that plants have a well-managed sensing and regulatory machine, which may apply a variety of mechanisms to help them adapt to any environmental changes, including S stresses.

1.4.1 Regulation of sulfate transporters (SULTRs)

Although there is no current report on how plants sense the environmental S stress, sulfate transporters may possibly serve as sensors. Some of them (SULTR1;1, SULTR1;2) are the first group of proteins in the S uptake and assimilation pathway that directly contact the environmental sulfate thus sensing its change. Multiple experiments have reported that sulfur deficiency (-S) cause increased gene expression of sulfate transporters, SULTR1;1, SULTR1;2, SULTR2;1, SULTR3;4, SULTR4;1 and SULTR4;2 (Grossman and Takahashi, 2001; Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003; Hirai et al., 2004; Maruyama-Nakashita et al., 2006). Ion sulfate (SO_4^{2-}) is transported from the environment into a cell by the two HASTs (SULTR1;1, SULTR1;2) utilizing a symport mechanism with protons. The driving force for this process is a proton gradient across the membrane, and the transported molecules move against their concentration gradient (Yi et al., 2010). It is obvious that environmental sulfate is transported into a cell in an active manner, instead of a passive one by simple diffusion into a cell from higher to lower concentrations. Two important questions about this condition are: Firstly, how do plants perceive the S content so as to decide whether to activate the transporters or not? Secondly, how do plants up-regulate or down-regulate the expression of these transporter genes after they perceive the stress? These two questions may be the very hot frontier topics in the following years.

The above topics can be similarly addressed to other sulfate transporters instead of SULTR1;1 and SULTR1;2, but there is very little information about them. One difference between the two HASTs and other transporters is that HASTs are driven by a pH gradient across the plasma

membrane, but organelle-localized transporters may not have this driving force at their disposal, because generally there is no obvious pH difference inside-outside a plastid or a mitochondrion. At this point, ATP is needed and possibly transporters follow an alternative mechanism, e.g. membrane potential, for transportation (Yi et al., 2010). Another difference is that S transportation inside a cell is much more complicated than outside, because sulfate is the predominant (if not the 'only') form of molecule that is transported from outside of a plant root epidermis, but when inside a cell, not only sulfate, but several other biologically important S-containing compounds need to be correctly distributed. So what are these responsible transporters, and what are the driving mechanisms? How do plants manage to finely adjust each of these mechanisms so as to turn-on and turn-off the correct one in the right time?

The identification of a novel gene *SLIM1/EIL3* may provide a clue for understanding gene regulation that possibly connects with the transporters (Maruyama-Nakashita et al., 2006). The *slim1 (sulfur limitation1*) mutant was generated from a EMS-mutagenized reporter system, which contained a transformed construct of $P_{SULTR1;2}$-*GFP*. The *SLIM1* gene encodes the transcription factor EIL3 (Ethylene-Insensitive 3-Like 3), which is ubiquitously found in the plant kingdom and localized in the nuclei exclusively. GeneChip analysis revealed a list of –S-responsive genes are *SLIM1*-dependent, among which are several of the sulfate transporter genes *SULTR1;1, SULTR1;2, SULTR3;4*, and *SULTR4;2* that are up-regulated by –S stress. It is worth noticing that not all sulfate transporter genes are *SLIM1*-dependent. For example, *SULTR2;1* is reported to be strongly up-regulated in the absence of S in both roots and leaves of *slim1* mutant, suggesting that *SULTR2;1* induction is regulated by a *SLIM1*-independent mechanism. It is also

possible that more transcription factors are involved in regulation of sulfate transporters (Kawashima et al., 2008).

It is of great importance that all of the 12 group I to IV sulfate transporters, including the above *SLIM1*-dependent sulfate transporters, are reported to contain a STAS (sulfate transporter and anti-sigma antagonist) domain at their carboxyl-terminus, and proposed to be involved in protein-protein interaction and thus potentially able to regulate sulfate transporter activity (Shibagaki et al., 2006; Davidian and Kopriva, 2010). This provides the possibility that EIL3, or other transcription factors, may regulate or fine-tune sulfate transporters by direct or indirect binding with each other.

Another piece of evidence for the possibility of SULTR-related signal transduction pathways, is the identification of a *cis*-acting element, SURE (SUlfur-Responsive Element), on the promoter of *SULTR1;1* (Maruyama-Nakashita et al., 2005). This 16-bp sequence is necessary and sufficient for –S-response of *SULTR1;1*. Other *SUTR*s tested in this work are *SULTR2;1* and *SULTR4;2*, which were found to be both –S up-regulated and identified to contain the 5-bp core sequence (GAGAC) of SURE.

MicroRNA has been observed to be involved in the regulation of sulfur metabolism (Jones-Rhoades and Bartel, 2004). To date, the only identified involved microRNA species is miR395, which was identified to be specifically responsive to –S stress, but not to other nutrient stresses

tested, such as -Pi, -N, -K, -Cu, and –Fe (Hsieh et al., 2009). miR395 was observed to be induced under –S condition in a *SLIM1*-dependent manner, and found to be localized to the phloem companion cells (Kawashima et al., 2009). In this same reported work, miR395 was identified to be able to cleave 3 of the 4 *ATPS* (ATP sulfurylase) mRNAs, i.e., *ATPS1, ATPS3, ATPS4*, and the gene of SULTR2;1, which is localized in the xylem parenchyma. In a real-time quantitative RT-PCR test, over-expression of miR395 in plants inhibited the expression of the above three *ATPS* genes in both shoots and roots, and under both +S and –S conditions, but the expected inhibition of *SULTR2;1* was identified only in shoots (both +S and –S). In roots, the inhibition happened only under –S conditions, enhanced gene SULTR2;1 mRNA level was observed on +S conditions (Liang et al., 2010). Instead of the impact on *SULTR2;1*, miR395 over-expression plants also displayed the enhanced gene expression level of four other –S-responsive transporters, *SULTR1;1, SULTR1;2, SULTR4;1* and *SULTR4;2*. But the enhancement was identified only under +S condition, except for *SULTR1;1*, which was found to be induced under both +S and –S conditions (Liang et al., 2010). Although there are some proposed regulatory models that explain the role of miR393 in sulfur metabolism, especially in the regulation of sulfate transporters, the relationship between them is far from clear, an include "non-canonical" model as well (Liang et al., 2010; Kawashima et al., 2011). The most obvious "non-canonical" observation is that miR395 and *SULTR2;1* were both observed to be up-regulated by –S stress, but other experiments showed that the –S inducible miR395 can actually cleave *SULTR2;1* (Kawashima et al., 2009; 2011).

1.4.2 Regulatory role for S metabolic multi-enzyme complexes

The first characterized and most frequently cited multi-enzyme complex with regard to S metabolism is the CRC (cysteine regulatory complex) in plants. CRC is a bi-enzyme complex with a SAT (serine acetyltransferase) tetramer and two OASTL (O-acetylserine thiol-lyase) dimers, and is located in plastids, cytosol and mitochondria. It is interesting that CRC is not the direct enzyme responsible for the synthesis of the first organic S compound, Cys. Instead, it is an enzyme complex involved in the synthesis of a Cys precursor, OAS (O-acetylserine), which is both a 'signal' molecule and a rate-limiting compound in S-assimilation. Cys is synthesized from the two precursors, OAS and sulfide, by the catalysis of OASTL. The two substrates of OASTL, sulfide and OAS, antagonistically work to activate or decompose the CRC complex, respectively. Through this mechanism, a cell maintains an appropriate OAS concentration to unsure a balanced Cys synthesis under modified S status (Boddanova and Hell, 1997; Leusteck et al., 2000; Yi et al., 2010).

The above model appropriately explains the mechanism of how a plant cell regulates OAS production so as to acclimate to modified S availability. The model has also been verified by *in vitro* and *in vivo* experiments and well-explained thermodynamically (Boddanova and Hell, 1997; Droux et al., 1998). The molecular hypothesis underlying this model is that, when plants encounter –S, intracellular sulfide concentration will decrease and excessive OAS is made. OAS is supposed to serve as a 'signal' molecule to induce the activation of SULTRs and sulfate reductive enzymes. This hypothesis is supported by experiments feeding plants directly with OAS, and activation of SULTRs and sulfate assimilation enzymes, such as ATPS, APR, APK, has been observed (Hirai et al., 2003; Yi et al., 2010). However, a major argument about this

model is how does OAS serve as a 'signal' molecule, and what is the mechanism by which this molecule interacts with either or both of the regulated proteins?

A recent study on the interaction of OASTL with the high-affinity sulfate transporter SULTR1;2 may provide a support for the above model. Direct binding between these two proteins have been demonstrated and it is clear that OASTL negatively impacts on SULTR1;2 by binding to the STAS domain. This binding complex is promoted by OAS but decomposed by sulfide (Shibagaki and Grossman, 2010).

Other proposed enzyme complexes are the ATPS-APR (or ATPS-APK) complex. Both of these two enzyme complexes are theoretical proposals, rather than experimentally demonstrated arrays, but they address the thermodynamic problem of the first step of sulfate activation by ATP. As the enzyme ATPS which catalyzes this step, is extremely energetically unfavorable (K_{eq} is about 10^{-7} to 10^{-9}), it is necessary to keep a very high concentration of ATP and sulfate, as well as a very low concentration of product, APS, so as to draw the reaction forward. The possible co-ordination between ATPS and the next step enzymes, i.e. APR for further S reduction, and APK for further S activation, can easily remove the excessive APS so to draw the flux forward (Yi et al., 2010; Davidian and Kopriva, 2010).

1.4.3 Glucosinolate (GLS), a chemical, a reservoir, or simply a reservoir?

GLSs are a family of S-containing secondary compounds, which serve plants with anti-pathogenic immunity, and anti-herbivores' capacity (Wittstock and Halkier, 2002; Rausch and Wachter, 2005). In addition, GLSs molecules store 6% of total S inside their molecular structures. It is clear that GLSs will be decomposed under –S stress and release sulfate to compensate the –S stress from environment, or some other hormone precursors so as to possibly link –S to hormonal responses (Nikiforova et al., 2003; Artmann and Armengaud, 2009).

Multiple experiments have revealed that, the most responsive gene to –S stress is neither a *SULTR* gene, nor an S-assimilation gene, but a putative thioglucosidase gene encoded by At2g44460 (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Dan et al., 2007). Thioglucosidases decompose GLSs to release S-containing intermediates. By this mechanism, GLSs are incorporated into the net work of S-responses. Given the evidence that At2g44460 is strongly up-regulated in –S response, and more importantly, it is *SLIM1*-dependent (Maruyama-Nakashita et al., 2006), it is reasonable to propose that GLSs are molecules involved in the S-signaling inside a plant cell.

1.5 Involvement of hormones

Phytohormones have been increasingly shown to participate in specific metabolic pathways. Among the commonly recognized classes of phytohormones, cytokinin seems to be the most important hormone is involved in plant nutrient regulation.

1.5.1 Cytokinins

In an experiment reported by Ohkama et al. (2002), of all the 6 tested phytohormones, Zeatin (Z, a natural cytokinin), ABA, IAA, ACC, GA3, and JA, only Z enhanced the fluorescence signal of a –S responsive reporter line, which harbored a 35S::βSR-GFP (SR stands for Sulfur Responsive element). Cytokinin up-regulated βSR element not only under –S stress, but also under +S condition. Instead of that, cytokinin also increased the transcription of two endogenous –S-responsive genes, *APR1* and *SULTR2;2* under +S condition (Ohkama et al., 2002).

In another reported work, cytokinin played a negative role by inhibiting the two high-affinity sulfate transporters, SULTR1;1 and SULTR1;2. In a treatment of a transgenic reporter line, which contained the $P_{SULTR1;2}$-*GFP*, all other tested phytohormones include ABA, 2,4-D, NAA, GA, JA, ACC did not obviously change the GFP strength, but the two tested cytokinins, BA and Z both decreased the GFP strength and the mRNA level of *SULTR1;2*. Furthermore, application of cytokinin severely decreased sulfate uptake capability (Maruyama-Nakashita et al., 2004). This negative impact of cytokinin on the –S-responsive genes was further confirmed in our laboratory. In a test of transgenic plants that contained the $P_{AT2G44460}$::*GUS*, and P_{APR2}::*GUS*, application of 1μM BA strongly inhibited the GUS strength of both reporter lines (Dan et al., 2007).

Multiple reports have demonstrated the importance of cytokinins on plant growth and development. The regulatory role of cytokinins has been reported to be involved not only in S

metabolism, but also in that of other nutrients, such as carbon, nitrogen and phosphate (Takei et al., 2002; Franco-Zorrilla et al., 2005). The regulatory mechanism of cytokinin in reponse to different nutritional states in plants is not fully clear. The two-component signal system, which contains a sensor histidine kinase and a response regulator has been well studied in bacteria and provides clues for studying of cytokinin signal transduction in plants (Hutchison and Kieber, 2002). The identification of cytokinin response 1 (*CRE*) gene was later proved to be a cytokinin receptor that triggers phosphorelay upon perception of cytokine. It is biochemically a hybrid histidine protein kinase (Maruyama-Nakashita et al., 2004).

1.5.2 Auxin

Using the P_{NIT3}*::GUS* reporter system, Kutz et al., (2002) showed the possible linkage between sulfur starvation and indole acidic acid (IAA) biosynthesis. *NIT3* (*Nitrilase 3*) is one of the four genes in Arabidopsis that encode the enzyme nitrilase, which is able to convert indole-3-acetonitrile (IAN) to IAA. The largely existed S-storage compounds, GLSs, provide the substrate source for IAN synthesis. Under the –S treatment, *NIT3* promoter activity was induced 35 fold over that of the control with the constitutively active CaMV 35S promoter, and the IAN nitrilase activity was also increased. As predicted, contents of GLSs, esp. glucobrassicin, which is the substrate of IAN nitrilase for synthesizing IAN, also decreased rapidly. All above evidence seem to hypothesize that –S stress induces the biosynthesis of auxin, at least in Arabidopsis.

The linkage between –S stress and auxin synthesis was further proved by transcriptome and metabolome array analysis (Nikiforova et al., 2003; 2005b). In the reported experiments, not only *NIT3* was observed to be up-regulated by S starvation, but also the upstream gene (Myrosinase) and inducible compound (OAS) were observed increased. Further evidence for this linkage proposal is that -S stress also increased contents of Serine (Ser), Trp, and the Trp synthase β-subunit, and all are important elements for the Trp-dependent auxin synthesis pathway, which is an alternative pathway of the previous glucosinolates *NIT3* pathway.

A major question about the hypothesis described above is the lack of IAA increase under –S treatment. Some data even gave the opposite evidence (Kutz et al., 2002). Although the authors provided S-starving root growth evidence, such as longer primary root and more lateral root branching, which are considered to be an 'auxin phenotype', different results were obtained by other authors (Dan et al., 2007) to be discussed in detail in Chapter 2. So, as to the impact of –S on auxin synthesis, more evidence, especially direct IAA content measurement, is necessary.

An approach to understanding the impact of auxin to –S stress responses was carried out in our laboratory (Dan et al., 2007). By using an auxin reporter line *DR5::GUS*, both GUS strength and lateral root development (the 'auxin phenotype') were inhibited by –S treatment. Even stronger evidence is that, under –S treatment, 1μM auxin treatment almost totally diminished the GUS staining of a –S-responsive reporter line, $P_{At2g44460}$*::GUS*. In another experiment, the developmental response was abolished in the auxin signaling mutant *axr1*. These observations strongly suggest a negative role of auxin in the S starvation responses.

Regulatory mechanism and the possible signals connecting the S stress and auxin are far from clear at this time. However, we did identify some possible signaling molecules, or genes that are connected between S metabolism and auxin action. Among these are members of the Arabidopsis *Aux/IAA* (*Auxin/Indole-3-Acetic Acid*) gene family, which encode Aux/IAA proteins that transcriptionally regulate auxin-induced genes (Rogg et al., 2001). So far, a total of 29 Aux/IAA transcription factors been identified, among which, IAA9, IAA17, IAA18, and IAA 28 were activated by –S treatment (Falkenberg et al., 2009; Nikiforova et al., 2003). Transcriptome and metabolome analysis revealed that IAA28 may play a predominant role, because it has 28 connections with other genes or metabolites and can be considered as a regulatory 'hub' (Nikiforova et al., 2005a). Falkenberg et al., (2008) tested 21 -S-responsive genes and found all of them were also responsive to the IAA transcription factor expression. Among these, -S-induced expression of *SULTRs, ATPS, APK, OASTL* was down-regulated in *IAA28* and *IAA13* over-expression lines, but up-regulated in *IAA28* knock-down plants.

Other evidence for the role of auxin in –S response includes reports for the role of BIG transcription factor, and the auxin responsive factors (ARFs) (Kasajima et al., 2007; Falkenberg et al., 2008).

Based on the above discussion, a model regarding the role of auxin in –S stress response can be proposed: -S deficiency potentially activates auxin biosynthesis, which in turn activates

transcription factors such as IAA28, which in turn inhibits the –S-activated genes through a feedback loop. For example, IAA28 can form a dimer with ARF so as to form an inactivated body; once IAA28 undergoes degradation, ARF is released to activate expression of those auxin-responsive genes. (Rogg et al., 2001; Nikiforova et al., 2003; Weijers and Jurgens, 2004; Nikiforova et al., 2005; Falkenberg et al., 2008; Rubio et al., 2009).

1.5.3 Other hormones

Several phytohormones, namely ABA (abscisic acid), 2,4-D (a synthetic auxin), NAA (naphthalene-1-acetic acid, another auxin), GA (gibberellins), JA (jasmonic acid), ACC (1-aminocyclopropane-1-carboxylic acid), and the cytokinin BA (benzyladenine), were applied to treat transgenic plants containing $P_{SULTR1;2}$*::GUS* reporter construct. While all other hormones showed no obvious differences with the control, BA heavily decreased the *SULTR1;2* promoter activity (Maruyama-Nakashita et al., 2004). In our own experiments, not only IAA, but also BA and ABA, inhibited the promoter activity of another –S response gene almost completely, At2g44460 under S starvation (Dan et al., 2007). Present observation about the regulatory role between cytokinins and S responses may be defined as varied and complex, no clear patterns emerge.

There is much less information about other hormones involved in S metabolism. A gene of the homocysteine S-methyltransferase 3, an enzyme involved in the Met biosynthesis pathway, was reported to be up-regulated in a microarray experiment with 1μM ABA treated Arabidopsis

plants (Xin et al., 2005). In addition, ABA plays an important role in keeping plant sub-cellular sulfur homeostasis by increasing the level of GSH under –S stress. By a web-accessed GENEVESTIGATOR survey, GA is found to down-regulate about 90% of the –S-induced genes (Rubio et al., 2009).

1.6 The involvement of carbon (C) and nitrogen (N) in S response

Because they are photoautotrophic organisms, plants make their C source by photosynthesis. C sources, such as glucose (Glc) and sucrose (Suc), serve later on as substrates for energy production, but also provide a large supply of metabolic intermediates, which are used as backbones for the biosynthesis of cell structural or functional molecules or compounds, such as starches, cellulose, amino acids, proteins, lipids, and nucleic acids. In addition, many regulatory molecules such as hormones, vitamins, and transcription factors, are all synthesized from different carbon backbones. This may partly explain why C constitutes nearly half of the plant dry mass. And may also explain why –C stress impact not only C metabolism, but also N, S, and many metabolic pathways, while all other types of nutrient stress directly affect only one or a few pathways (Amtman and Platt, 2009).

N takes only 1.5% of the plant dry mass, but it is the most important fertilizer in agriculture. It is also a key limiting nutrient for plant growth. The primary N source from soil is nitrate, which is transported into the plant cell, reduced and incorporated into the first amino acid, glutamine (Gln). Following this, the plant can synthesize all other types of amino acids, different species of

proteins, nucleic acids, and other biological important compounds, such as hormones and vitamins (Coruzzi and Bush, 2001; Zheng, 2009).

1.6.1 The synergistic interaction of C and N in S deficiency response

The diurnal change of the two reductases for N and S assimilation, nitrate reductase (NR) and APR, were later proved to be C-stress-induced in the experiments of Lillo, 1994; Kopriva et al., 1999. These experiments mimicked the light-induced increase of the enzyme activity and mRNA level by adding sucrose in the dark treatment. So the addition of C source, not the diurnal rhythm, induced the gene expression and enzyme activity of the key enzyme in N and S reduction respectively. Further experiments in which glucose was applied, also lead to similar results: i.e. addition of sugar up-regulated the two reductases, NR and APR, on gene expression level, protein level, and enzyme activity level (Hesse et al., 2003). A novel observation in Hesse's experiment is that glucose increased NR only under N-sufficient, but not under N-deficient (–N) conditions. On the other hand, glucose increases APR under –N conditions, which suggests that glucose regulates APR expression independently from nitrate assimilation. Sugar addition was also reported to up-regulate two S transporters, SULTR1;1 and SULTR1;2 under –S stress. In another experiment, it up-regulated two N transporters, NRT1.1 and NRT2.1 (Amtman and Platt, 2009). In our experiment, we tested another S stransporter, SULTR4;2, which has been shown as vacuole-localized, and found that, sucrose increased endogenous gene expression of the transporter (Dan et al., 2007). An experiment on *Lemna minor* tested the effect of gaseous C nutrient (CO_2), on N and S metabolism (Kopriva et al., 2002), where plants incubated in a CO_2-free atmosphere and it was found that NR and APR activity and the Mrna

level were severely down-regulated. But addition of Suc and OAS compensated the effect to some extent.

In a DNA microarray analysis of a total of 1176 genes from shoot material, and 183 genes from root material, were found to be nitrate –responsive (Wang et al., 2003). Experiment applied a treatment of low nitrate (250μM) for 20 minutes before sampling for microarray. Among those N-responsive genes, those for nitrite reductase (NiR), NRT1.1, and NRT2.1 were up-regulated. Similarly, S-metabolic gene *APR, SULTR1;1 and SULTR1;2* were also up-regulated, just like –S stressed. Instead of this, low nitrate treatment also altered a few important genes involved in glycolysis process (Wang et al., 2003). In our experiments, addition of N increased the *promoter::GUS* activity of the putative thioglucosidase gene, At2g44460, under –S conditions. And more importantly, it did the same on *SULTR4;2* gene in a RT-PCR reaction (Dan et al., 2007).

-S stress affect not only on its own assimilation, such as through the increased activity of SULTRs, APR and accumulation of OAS, decrease of Cys and GSH, but also affects the whole plant generally by decreasing the total biomass, total chlorophyll, total RNA and total proteins (Nikiforova et al., 2005). Despite of the overall decrease under –S stress, some biologically important amino acids were identified to be up-regulated, such as Ser, Trp, which lead to auxin synthesis; Gly is connected to photorespiration. The decrease of photosynthesis and activation of photorespiration under –S is believed to contribute to the accumulation of excessive ureides and ammonia inside cell, which leads to a severe low S/ high N imbalance (Nikiforova et al., 2005).

1.6.2 C/N balance response and regulation of C-N-S interaction.

Plants encounter nutritional imbalance whenever they face a nutrient stress, such as –C, -N, or –S. Under this condition, a new balanced state should be reached so as to minimize the possible accumulation of toxic metabolites, and to achieve optimal plant growth. It has been reported that C and N are tightly coordinated and a few monitoring molecules have already been identified and characterized (Zheng, 2009). N is also tightly balanced with S metabolically, with a deprivation of one leading to a disruption of the other (Wang et al., 2003). One example of C-N-S interaction is the shifting of metabolic pathways when plant cells are under –S stress. It is clear that –S down-regulates the major energy-producing pathways of Glycolysis and TCA cycle, but it increases the synthesis of Ser and Trp, which are linked with C-metabolism through the precursor, 3-phosphoglycerate, and lead to enhanced auxin synthesis. Another alternative pathway is from enhanced Ser to Gly, which releases ammonia in the process of photorespiration by Gly decarboxylation. The released ammonia, combined with the ureides species accumulated under –S stress, can be assimilated into purine metabolism so as to avoid ammonia toxicity and achieve a new C-N-S balance (Nikiforova et al., 2005).

There are three genes proposed to be involved in the signaling of C/N balancing response: genes for the putative glutamate receptor GLR1.1, the putative nitrate transporter NTR2.1, and the putative methyltransferase OSU1 (Kang et al., 2003; Little et al., 2005; Gao et al., 2008; Zheng, 2009). Among these, GLR1.1 and NTR2.1 were both responsive to the high C/low N imbalanced nutritional state, while OSU1 wild type (WT) displayed no phenotypic difference in

the two imbalanced C/N states (high C/low N, and low C/high N). Instead, *osu1* mutants did show obviously short root length and accumulation of anthocyanin (Gao et al., 2008; Zheng, 2009). It is interesting to notice that all three present identified signaling molecules are not traditionally studied 'signal molecules', which are characterized not only by their smaller molecular size, but also by their functions—i.e. they don't involve metabolically or structurally inside a cell, instead, they function only regulatorily. The three signaling compounds cited here are both functional and regulatory molecules. These novel discoveries may provide new concepts and approach to understand and identify of more signal molecules in the future. In addition to the isolation of the signal molecule OSU1, Gao et al., (2008) further tested the involvement of another signal molecule, the transcription factor MYB75. By using an epistasis approach, experiments not only confirmed the participation of MYB75 in the OSU1 signaling cascade, but also proved that the *MYB75* gene works in the downstream of *OSU1*.

S metabolism is connected to N metabolism by the synthesis of Cys, the two substrates of which are sulfide (S source) and OAS (N source), a derivative of Ser. The substrate for Ser synthesis is 3-phosphoglycerate (C source), through which N metabolism is incorporated into C metabolism (Nikiforova et al., 2005). The key regulatory enzyme during this process is the CRC complex, which is up-regulated when sulfide is sufficient, but down-regulated when OAS or Cys (N source) are excessive. Therefore, CRC is proposed as a N/S balance sensor by some authors (Hawkesford et al., 2006; Yi et al., 2010), although there is still a long way before we accumulate adequate experimental evidence for this hypothesized mechanism.

Taken together, it is rational to propose and practical to observe the nutritional balance, especially the three major nutrients, C, N, S, inside a plant cell. And it is also understandable that a balanced nutrient state is essential for optimal plant productivity. As stated above, progresses have indeed been achieved during the past decades, in understanding the molecular mechanism of plant nutrient balancing, or acclimation when plant cells encounter a nutrient stress. **But we still have little knowledge regarding how plants perceive C-N-S status, and how the interaction is regulated so as to reach a new more balanced state. Published work focused mainly on the C/N interaction, it is important and worthwhile to start a new project on S signaling and integrating S signaling into the network of C-N-S balancing.**

1.7 Project and experimental approaches

Plant nutritional balance optimizes plant metabolism, growth and development, while imbalanced nutrient status induces drastic problems for agriculture, environment and human health. I proposed to study the C-N-S cross-talk and S sensing mechanisms through a forward genetic approach. Although the signaling mechanism governing this process remains poorly understood, several preliminary studies concerning the basic C, N and S stress responses had provided some candidate genes for me to choose as a reporter on the crosstalk of C, N and S nutrient responses. The reporter line was mutagenized and this work lead to the identification of some important signaling components that integrate the C, N and S responses, expecially in S sensing and signaling. This work may open a new window in understanding C, N and S nutritional signal interaction and ultimately contribute to the improvement of agricultural production

Specific aims:

1) Construction of a S deficiency-activated promoter:GUS reporter system (Chapter 2)

Candidate reporter genes for choices were either be transcription factors, such as *MYB41*, which was reported to be –S up-regulated (Maruyama-Nakashita et al., 2003); or S metabolic enzyme genes such as *APR2, APR3*, which were all identified to be –S-responsive (Leustek, 2002; Hirai et al., 2003; Maruyama-Nakashita et al., 2003). *SAT1* encodes an enzyme, Serine acetyltransferase, which plays a key role in the CRC (Cysteine Regulatory Complex) bi-enzyme system and regulates the C-S crosstalk (Hirai et al., 2004). The locus At2g44460 encodes a putative thioglucosidase / glycocyl hydrolase family 1, which is strongly –S-responsive and responsible of releasing storage sulfate into the cell when cell encountered –S stress (Maruyama-Nakashita et al., 2003; Kopriva and Rennenberg, 2004). Plasmid constructs with the fused promoter::GUS reporter system were introduced into *Agrobacterium tumefaciens* and transformed into Arabidopsis Col plants by the floral dip method (Clough & Bent, 1998). The transgenic reporter lines were identified and characterized before gene cloning.

2) Isolation of S response mutants and mutant gene cloning (Chapter 3)

One reporter line, GHF1, which harbors the $P_{At2g44460}$*::GUS*, was well characterized and chemically mutagenized by treatment with ethylmethane sulfonate (EMS). Following mutant screening and growing to the M3 homozygous generations, physiological and genetic

characterization was carried out. The mutants with altered C-N-S, especially S –responsive pattern was used for gene cloning by a map-based cloning approach.

3) Cloned gene characterization and functional analysis (Chapter 4)

Three mutants (11p34h, 11p101h, and 11p201h) were map-cloned but all corresponded to 2 alleles of a single gene, *SULTR1;2*, the high-affinity sulfate transporter. Physiological and genetic characterizations include the allelism tests, complementation tests, and the transporter functional confirmation. A set of *35S-SULTR1;2-GFP* constructs has been used to detect the protein localization.

As *SULTR1;2* is a previously reported gene, the protein of which was specially localized in the root epidermis and cortical layers for primary sulfate absorption from the soil. It was identified in a system that's specially designed for screening those signaling molecules involved in the C-N-S interaction or S signaling. So, instead of the transporting function, does it posses any signaling capability? *Promoter::GUS* tests revealed that *SULTR1;2* mutation decreased the sensitivity of –S response. RT-PCR and qRT-PCR tests further confirmed this observation not only on the endogenous gene At2g44460, but also on other –S –responsive genes, such as *SULTR4;2, LSU1 and SDI1*.The two mutants*, sel1-15, sel1-16* were severely defective in responding to two reduced S-compounds, Cys, and GSH, further proving that the mutation of SULTR1;2 not sole lead the loss of transport function, but also induce the loss of other functions such as the sensing of S –source presence.

4) Alteration of C-N-S crosstalk and hormone responses in *sel1* alleles (Chapter 5)

In order to test the possible alteration of C-N-S signaling, we performed 8 combinatory nutritional treatments with the WT, and the two mutant alleles (three mutants). In Chapter 2, we provided multiple evidences that support a negative regulatory role for auxin in –S responses. Our results also indicate the similar regulatory role for two other phytohormones, ABA and BA. Therefore, more experiments will be carried out in this part of work to investigate the hormonal response in the *sel1* mutants.

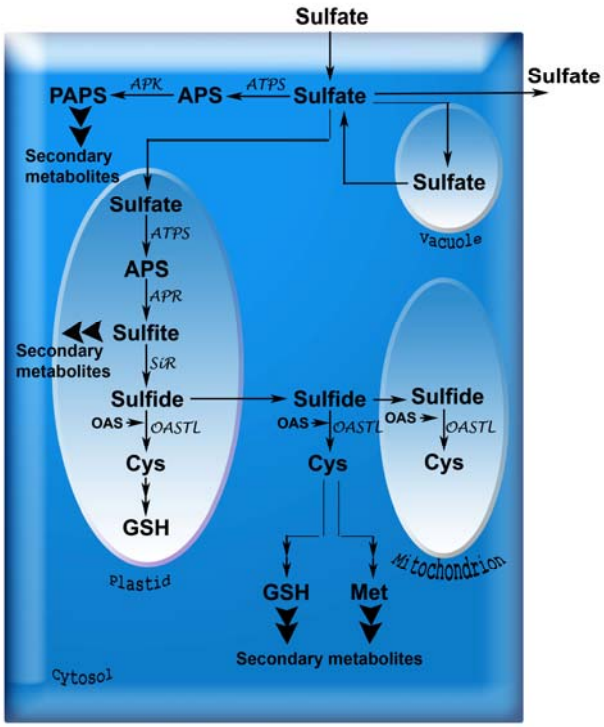

Figure 1.1 S metabolism and sub-cellular compartmentalization inside a plant cell*

Sulfate is absorbed from the environment and will be 1) pumped up to the shoot for the majority of metabolism and re-distribution; 2) transported into plastids for sulfate reduction and primary S-metabolism, i.e., synthesis of Cys and related S-containing chemicals. Cys is not only synthesized inside plastids, but also in cytosol and mitochondria; 3) transported into vacuoles for storage.

Enzymes for those major metabolic steps are italicized. For meaning of each abbreviation please see the corresponding text or the abbreviation table.

S-metabolism produces several of S-containing defensive secondary products, or chemicals with other important functions. This picture shows the three major pathways that lead to secondary metabolism: the first starts with the activation of sulfate and uses PAPS as a S-donor for sulfation of a large variety of intermediates to form S-containing secondary products, among which is glucosinalates; the second pathway is branched from sulfite, which can be incorporated into sulfolipids; the third pathway is branched from GSH and Met, which lead to synthesis of multiple secondary products such as phytochelatins and glucosinalates.

* Diagram developed on the basis of Kopriva, Annals of Botany 97: 479–495, 2006.

Chapter 2

A negative regulatory role for auxin in sulfate deficiency response in *Arabidopsis thaliana*[1]

Sulfate is a major macronutrient required for the synthesis of the sulfur (S)-containing amino acid cysteine and thus is critical for cellular metabolism, growth and development and response to various abiotic and biotic stresses. A recent genome-wide expression study suggested that several auxin-inducible genes were up-regulated by S deficiency in Arabidopsis. Here, we examined the relationship between auxin signaling and S deficiency. Investigation of *DR5::GUS* expression patterns indicates that auxin accumulation and/or response is suppressed by S deficiency. Consistently, S deficiency resulted in the suppression of lateral root development, but the *axr1-3* mutant was insensitive to this response. Furthermore, the activation of the promoter for the putative thioglucosidase gene (At2g44460) by S deficiency was suppressed by auxin, cytokinin and abscisic acid (ABA). Interestingly, the activation of At2g44460 by S deficiency is regulated by the availability of carbon and nitrogen nutrients in a tissue-specific manner. These results demonstrate that auxin plays a negative role in signaling to S deficiency. Given that activation of the genes encoding the sulfate transporter SULTR1;2 and 5'-adenylylsulfate reductase APR2 are suppressed by cytokinin only, we hypothesize that while cytokinin may play an important role in general S deficiency response, auxin might be only involved in a subset of S deficiency responses such as the release of thiol groups from the S storage sources.

[1] This chapter is a revision of Hanbin Dan, Guohua Yang, Zhi-liang Zheng, *Plant Molecular Biology*, 63:221-235 (2007) with kind permission of Springer Science and Business Media.

2.1 Materials and methods

2.1.1 Promoter::β-glucuronidase (*GUS*) construction and GUS assay

For the putative thioglucosidase gene At2g44460 promoter::*GUS* construct, a 2.7 kb promoter fragment including 9 bp downstream of ATG was PCR amplified from genomic DNA using the high fidelity DNA polymerase Elongase (Invitrogen) and gene-specific primers, with the underlying bases indicating the introduced restriction enzyme sites for cloning: sense (DZP11: 5'- ATCCTGCAGCACAACGAAACCCGATTGATG-3') and antisense (DZP12: 5'-ACAACCATGGTGAAAAAATGCATCTTCATATTCCT-3'). The amplified DNA fragment was digested by *Pst*I and *Nco*I, and then cloned into *Pst*I and *Nco*I sites of the binary vector pCAMBIA1301 (B4) that contains GUS and the CaMV35S terminator.

For the *APR2* (At1g62180) promoter::*GUS* construct, a 3.5 kb fragment including 11 bp downstream of ATG was similarly amplified using the two primers: DZP1, sense: 5'-CAT

CTGCAGAGATAGATGAAGCGATCACGA-3' incorporating a *Pst*I site; DZP2, antisense: 5'-CGAAGATCTACAGCTAAAGCCATTTCTAATC-3'incorporating a *Bgl*II site. This fragment digested by *Pst*I and *Bgl*II was then cloned into the *Pst*I and *Bgl*II sites of pCAMBIA1301.

The replacement of the CaMV35S promoter with At2g44460 and *APR2* promoters resulted in the transcriptional and translational fusion of these promoters with *GUS*, giving rise to the DZ9 and

DZ5 vectors, respectively. The identity of the PCR-amplified promoter fragments for both vectors was verified by DNA sequencing. The DZ9 and DZ5 vectors were respectively introduced into *Agrobacterium tumefaciens* strain GV3101and transformed into *Arabidopsis* Columbia (Col) plants by the floral dip method (Clough & Bent, 1998). Homozygous transgenic lines that had a single T-DNA insertion were obtained by hygromycin selection. Histochemical GUS activity assays were performed on homozygous transgenic seedlings as described (Jefferson et al., 1987), except that the substrate X-Gluc concentration was diluted by four fold.

2.1.2 Plant materials and nutrient and hormone treatments

Plant materials used in this chapter are: *Arabidopsis thaliana* Col, the WT. Two types of transgenic plant lines developed in this experiment, which harbor the reporter construct, At2g44460 promoter::*GUS* and *APR2* (At1g62180) promoter::*GUS,* respectively. Transgenic lines from other labs are: *DR5::GUS* obtained from Dr. Zhenbiao Yang (University of California at Riverside) with the permission of use from Dr. Thomas Guifoyle (University of Missouri-Columbia), and P_{RD29B}*::GUS* from Dr. Erwin Grill (Tubingen University). An auxin responsive mutant, *aux1-3*, which is from Dr. Haiyang Wang (Boyce Thompson institute), is also used in this experiment.

For nutrient and hormone treatments, seeds were sterilized and sowed on the half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and cold treated for 4 days.

Cold-treated plates were vertically incubated in the growth chamber at 22 °C with 16 hour (h) light and 8 h dark. After 5 days of vertical growth, seedlings were transferred to 24-well plates that contain 1.5 ml liquid nutrient solutions with or without hormones, and cultured for 2 days with gentle shaking (150 rpm). For treatments with hormones, all applied hormones were adjusted to a final concentration of 1μM for seedling treatments. These hormones include ABA, GA3, BA, ACC, BR (epibrassinolide), IAA, SA (salicylic acid), JA (methyl jasmonate). All chemicals were purchased from Sigma-Aldrich. Treated seedlings were used for GUS staining, root length measuring and lateral root counting.

The C, N and S nutrient solutions, which were applied for different treatments, were prepared by adjusting the original minimal MS-C-N-S medium (contained 0mM C, 0mM N and 0.0001mM S, which was from $CuSO_4$ only, ingredients are listed in table 2.1). Briefly, sucrose was used as the C source unless specified, and 60 mM sucrose was designated 60 mM C. For the N sources, KNO_3 and NH_4NO_3 were used based on the 1:1 molar ratio; for example, 60 mM N represents 20 mM KNO_3 and 20 mM NH_4NO_3. When lower amounts of KNO_3 were prepared, KCl was added to maintain the same final molar concentration of K^+ as in 1XMS. For the medium involving various S levels, the minor salts $MnSO_4 \cdot 7\ H_2O$ and $MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$ and $FeCl_2 \cdot 4H_2O$ were applied to adjust the S difference but maintain the same molar concentrations of Mn^{2+} and Fe^{2+}as in 1XMS. For example, to prepare the full-strength SO_4^{2-} (+S) that contained 1.6001 mM SO_4^{2-} (instead of 1.7301 mM S in 1XMS), $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ were applied the same as in 1XMS. To prepare for medium containing 0.0001 mM S (designated –S) that was from CuSO4 only, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ were respectively replaced by the same molar concentrations of $MgCl_2 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ as in 1XMS. For the 0.0301 mM

sulfate medium, $ZnSO_4 \cdot H_2O$ (0.03 mM S) was used to replace the original $ZnCl_2$ in the MS-C-N-S medium, $MgCl_2 \cdot 6H_2O$ and $FeCl_2 \cdot {}_4H_2O$ were used at the same molar concentrations as in MS.

In one experiment, *DR5::GUS*, Col and *axr1-3* seedlings after 5 days of germination and vertical growth on the solidified medium as above were also transferred to agar (Phytoblend)-solidified medium of either +S or –S for 10 days before determining GUS expression patterns or root development. For anthocyanin and chlorophyll measurement and RT-PCR analysis, seeds were directly placed in 50 ml of liquid medium of either +S or –S prepared as described above.

2.1.3 Root growth and development assay

The lateral root primordia (LRP) that included Stage I to Stage VII and the lateral roots (LR) were counted under the microscope, according to Malamy and Benfey (1997).

2.1.4 Anthocyanin and chlorophyll assays

Anthocyanins and chlorophylls were extracted from young seedlings germinated and grown in liquid medium of either +S or –S for 5 days after cold treatment. Briefly, for chlorophyll extraction and measurement, about 20 mg of seedling tissue were placed in 700 µl N,N-dimethylformamide, wrapped with foil and shaken gently at 4°C, The extracts were measured twice for the absorbance at 664 and 647 nm. Total chlorophyll levels were determined using the

method of Moran (1982). For anthocyanins, seedlings of about 20 mg were placed in the extraction buffer (99% methanol and 1% concentrated HCl) and shaken as described for chlorophyll extraction, according to the procedure of Rabino and Mancinelli (1986). The extracts were then measured twice for the absorbance at 530 and 657 nm, and anthocyanin levels were determined based on the formula, $A_{530} - 0.25 \times A_{657}$ (Rabino and Mancinelli, 1986). Three replicates for each genotype/treatment were performed for both chlorophyll and anthocyanin measurements.

2.1.5 Reverse transcription (RT)-PCR analysis

Total RNA was extracted with TRIzol (Invitrogen) from 5 to 7 days old seedlings treated by nutrients as above, and then reverse transcribed by Superscript III reverse transcriptase (Invitrogen). PCR analysis was performed using the *Taq* DNA polymerase (Gen-Script), with gene specific primers and *ACT2* as the internal control as described (Xin et al., 2005). Gene specific primers were designed. For *SULTR4;2*, the sense primer DZP50 was 5'-TCCACCGCTTCATCCTCTTCATCT-3', and antisense primer DZP51 was 5'-AGAGCCGATGTTGGAAGCAGTAA-3'. For the putative thioglucosidase gene/At2g44460, the sense primer YZP63 was 5'-AACGAGCTCTTGCCACTGAACT-3' and the antisense primer YZP64 was: 5'-GAGATGGTCCTCATGGTAGCTT-3'.

2.2 Results

2.2.1 Sulfate deficiency reduces the activity of the auxin response marker *DR5::GUS*

To test whether auxin is involved in –S response, we first analyzed the auxin response using the well characterized *DR5::GUS* line (Ulmasov et al., 1997). Treatments of young seedlings by –S (extremely low sulfate, 0.0001 mM) and intermediate low (0.0301 mM) concentrations of sulfate for 48 h resulted in different GUS staining patterns compared to the normal S concentration (1.6001 mM, designated +S). Decreasing S concentrations gradually reduced GUS staining in the cotyledons, primary root tips and LR. In the cotyledons, the strong expression of DR5::GUS at 1.6001 mM S in the tip and surrounding edges, where auxin is synthesized, was dramatically reduced under lower sulfate concentrations (Fig. 2.1). In primary root tips, emerged LR and LRP, the intensity and distribution were also reduced under extremely low and intermediate sulfate concentrations. These results suggest that auxin accumulation and/or sensitivity are suppressed by –S.

2.2.2 Sulfate deficiency suppresses the lateral root development

Auxin is critical for root growth and development, and therefore we investigated the lateral root development from the –S treated *DR5::GUS* plants. As shown in Fig. 2.2, –S only very weakly stimulated primary root elongation but strongly suppressed lateral root development. Because of the weak effect in the primary root growth (Fig. 2.2A), we focused our analysis on lateral root development. When the S concentration decreased, the total number of LRP on primary root for each seedling also decreased, although the number of emerged or elongated LR did not change (Fig. 2.2B). It is possible that these elongated LR already emerged before the –S treatments, or that –S was not sufficient to block the later stages of LRP from emerging. Nevertheless, when the densities of LR or LRP on the primary root were compared, both LR and LRP densities were

reduced by decreasing sulfate to 0.0301 mM, although the LRP density was reduced slightly more than the LR density (Fig. 2.2C). The further reduction of LR or LRP density by extremely low S concentration (0.0001 mM) was not statistically significant (P=0.07). Therefore, our results clearly show that –S suppresses lateral root development, consistent with the decreased *DR5::GUS* activity under –S (Fig. 2.1).

2.2.3 Sulfate deficiency-suppressed lateral root development is altered in the auxin signaling mutant *axr1-3*

To substantiate the involvement of auxin signaling in –S response, we subjected the auxin signaling mutant, *axr1-3*, to two different growth conditions. The *axr1-3* weak allele has a mutation in *AXR1* encoding an enzymatic unit of the E1 RUB1-activating enzyme that is important for ubiquitin-mediated protein degradation (Leyser et al., 1993). When five-day-old seedlings grown vertically on agar plates of +S were transferred to agar plates of either +S or –S, although there was no difference in LR density after 10 days of vertical growth, a difference in LRP density was observed (Fig. 2.3A). For Columbia (Col) wild-type seedlings grown under –S, the LRP density was reduced by 40% compared to that at +S. The *axr1-3* seedlings had a lower density of LRP than Col under +S (the normal S condition), but importantly, they did not show further reduction under –S. When the liquid culture condition was tested, a similar pattern was observed (Fig. 2.3B). Most of the young seedlings after 5 days of germination and growth in the liquid medium of either +S or –S did not develop LR (Fig. 2.3B). However, the density of LRP on the primary roots of Col seedlings under –S was reduced to 87% of that under +S (Fig. 2.3A). Although this difference was not very dramatic, it was statistically significant. The lack of

difference in the LR density and the less dramatic difference in the LRP density compared to that in *DR5::GUS* seedlings might be due to different genotypes because *DR5::GUS* exhibited an almost identical GUS expression pattern under three growth and treatment conditions tested (data not shown). Importantly, we showed that under two different growth conditions, *axr1-3* seedlings exhibited a similar insensitive pattern to changes in sulfate concentrations. Therefore, these results demonstrate that AXR1-mediated auxin signaling plays a negative role of –S response at least in the lateral root development.

2.2.4 Differential responses of *axr1-3* to sulfate deficiency-affected accumulation of anthocyanins and chlorophylls

Another physiological aspect of –S response could potentially be the accumulation of anthocyanins, given that –S caused the strong activation of several MYB transcription factor genes including MYB75 and MYB90 (Nikiforova et al., 2003). MYB75 and MYB90 have been demonstrated to function in the regulation of anthocyanin biosynthesis (Borevitz et al., 2000). Therefore, we measured the contents of anthocyanins under –S and +S. Col seedlings clearly doubled the accumulation of anthocyanins when they were subjected to –S compared to +S (Fig. 2.4A). However, *axr1-3* accumulated similar amounts of anthocyanins under +S as Col, and also had a similar increase by –S. This indicates that *axr1-3* does not alter the anthocyanin accumulation response to –S.

Surprisingly, in response to –S, both Col and *axr1-3* seedlings accumulated more chlorophyll around 5 days after cold treatment and then declined to the same level as +S at Day 7 before a significant decrease at Day 9 (Fig. 2.4B). Interestingly, at Day 3, *axr1-3* accumulated more chlorophyll than Col under both +S and –S conditions, and its chlorophyll levels were similar to that in Col at Day 5. At Day 5, chlorophylls in *axr1-3* under +S was similar to that in Col under –S and also increased by –S. Although at Day 7, there was no difference among all genotype/treatments, chlorophylls decreased dramatically in *axr1-3* under –S at Day 9. Therefore, while *axr1-3* still responded to –S by increasing chlorophyll accumulation transiently, it accumulated more chlorophyll during 3–5 days after cold treatment. These observations implicate that AXR1-mediated auxin signaling is at least part of the suppression mechanism involved in the transient chlorophyll accumulation in response to –S stress. This transient increase has not been reported before and its role needs to be further investigated. Given that excessive chlorophyll could lead to the generation of singlet oxygen as observed in high light or drought stresses (Krieger-Liszkay, 2005), it is possible that over-accumulation of chlorophylls at the early stage of –S might either be toxic to the –S-stressed plants or serve as a signal for adaptation to –S through singlet oxygen.

2.2.5 Auxin suppresses the sulfate deficiency-activated, putative thioglucosidase gene (At2g44460)

The insensitivity to –S in lateral root development and the altered chlorophyll accumulation observed in *axr1-3* led us to test whether auxin can directly suppress the activation of genes by –S. However, RT-PCR analysis using young seedlings grown in liquid medium failed to show the

suppression by IAA of expression of *APR2, SULTR4;2*,and At2g44460, the putative thioglucosidase gene that is most strongly induced by –S in a DNA microarray study (Maruyama-Nakashita et al., 2003). As RT-PCR might not reveal any tissue-specific patterns of gene expression, we decided to investigate the GUS expression patterns using the At2g44460 promoter::*GUS* and APR2 promoter::*GUS* reporter systems. GUS staining in several independent lines showed that the promoters of both genes were strongly activated by –S with a preference in the roots (Fig. 2.5 and data not shown). The consistency between the promoter activity and the gene expression studies (Maruyama-Nakashita et al., 2003; Hira et al., 2003, 2004) thus allows us to use these promoter::*GUS* lines to investigate the effects of auxin and other hormones in the transcriptional regulation of At2g44460 and *APR2* genes. Consistently, treatments of 1 μM IAA for 2 days did suppress the activity of the At2g44460 gene promoter in the primary root but not in the shoots under –S (Fig. 2.5A). This tissue-specific suppression may explain why we did not detect the obvious difference of transcript levels using RNA extracted from the whole seedlings (data not shown). The suppression of At2g44460 is probably triggered by –S because IAA did not suppress the GUS activities under the +S condition. Actually, IAA seemed to slightly activate the promoter activity in the emerging LR and the cotyledons under +S. In contrast, the *APR2* promoter activity was not affected by auxin treatment under –S (Fig. 2.5B). Therefore, the auxin-mediated suppression of gene expression is probably specific to certain genes and/or certain tissue types. Surprisingly, the At2g44460 promoter was also suppressed by ABA and cytokinin (BA), but not by other hormones except brassinosteroids (BR) that seemed to slightly activate the promoter activity (Fig. 2.5A). The slight activation of At2g44460 by BR is not surprising given by the known antagonistic interactions between BR and ABA/auxin (Ephritikhine et al., 1999). In contrast to the At2g44460 promoter, APR2 was only suppressed by

cytokinin (Fig. 2.5B), similar to *SULTR1;2* (Maruyama-Nakashita et al., 2004b). The differential regulation by IAA, ABA and BA for At2g44460 and APR2 was similarly observed by independently transformed promoter::GUS reporter lines (Fig. 2.6). Therefore, on the one hand, cytokinin has a similar inhibitory effect on these three genes likely involved in different aspects of sulfate transport and S metabolism. On the other hand, auxin and ABA seem to have more specific effects on certain genes such as At2g44460.

If ABA suppresses the activity of the At2g44460 promoter like auxin, we would expect that –S might also suppress ABA accumulation or response. To gain some supporting evidence for the possible involvement of ABA in –S response, we treated the P_{RD29B}*::GUS* reporter line under various sulfate levels as in the case of *DR5::GUS* (Fig. 2.1). P_{RD29B}*::GUS* has been demonstrated to reflect the physiologically active pools of ABA (Christmann et al., 2005). Not surprisingly, decreasing sulfate amount from the normal concentration (1.6001 mM) to the intermediate sulfate concentration (0.0301 mM) caused an obvious reduction of GUS staining in the cotyledons (Fig. 2.7). When sulfate was further reduced to 0.0001 mM, GUS staining in the hypocotyl-root junction almost disappeared, resulting in a very weak GUS staining in the seedlings. We did not observe any GUS staining difference in the primary root tips of the seedlings (data not shown). Together with the ABAmediated suppression of the putative thioglucosidase gene At2g44460 promoter under –S, this result indicates that ABA might also participate in modulating –S responses.

2.2.6 The putative thioglucosidase gene (At2g44460) exhibits a carbon and nitrogen nutrient-dependent regulatory pattern in activation by sulfate deficiency

To test whether the auxin- and ABA-suppressed putative thioglucosidase gene (At2g44460) is specifically involved in –S response, we decided to determine whether this gene is also activated by the deficiency of carbon (C) and nitrogen (N) nutrients. Furthermore, it has been shown C and N nutrients regulate the expression of certain genes under –S (Koprivova et al., 2000; Hesse et al., 2003, 2004; Kopriva and Rennenberg, 2004; Maruyama-Nakashita et al., 2004a), but it remains unknown whether C and N nutrients have differential or overlapping effects on –S activation of gene expression. Therefore, we used a combinatorial design to assess the promoter activities of At2g44460 under various combinations of C, N and S nutrients (Fig. 2.8A, B).

Similar to the observation shown in Fig. 2.5A, GUS activity in the seedlings grown at +C +N –S increased dramatically in the roots, while it did not change in the cotyledons, compared to the normal condition +C+N+S (Fig. 2.8A). This indicates that the –S activation of the promoter mainly occurs in the root system. In strong contrast to +C +N –S, deficiencies of C (–C +N +S) or N (+C –N +S) alone did not lead to obvious changes in the GUS staining patterns compared to +C+N+S (Fig. 2.8A). The lack of activation by –C or –N alone indicates that the activation of the At2g44460 promoter is relatively specific to –S. Interestingly, the strong –S activation of At2g44460 promoter activity observed at +C +N –S disappeared if both N and C were also deficient (comparing –C –N –S with +C +N –S or –C –N +S; Fig. 2.8A). This indicates an absolute requirement for C and N in the activation of At2g44460 by –S. However, while the presence of N (–C +N –S) only slightly activated the induction by –S, the presence of C (+C –N

–S) dramatically activated the –S activation, compared to –C–N–S. This indicates that under the conditions tested, N alone only has a minor effect for the –S activation, while C plays a predominant role in activating gene expression under – S. Nevertheless, +C +N –S exhibited a stronger induction than +C –N –S, suggesting a synergistic interaction between C and N on the –S activation of these two genes.

To assess where the observed changes of At2g44460 promoter activity reflected endogenous gene expression patterns in response to various C, N and S combinations, RT-PCR analysis using young seedlings was performed. The –S-activated sulfate transporter gene *SULTR4;2* (Kataoka et al., 2004) was used as a control. As shown in Fig. 2.8B, both *SULTR4;2* and At2g44460 transcripts exhibited a very similar pattern in response to C, N and S nutrients. Interestingly, there was even a very slight decrease of *SULTR4;2* and At2g44460 expression by the deficiency of either C or N alone compared to that of S deficiency alone. In most cases, both RT-PCR and the At2g44460 promoter-driven *GUS* expression showed very similar patterns, demonstrating that the 2.7 kb promoter fragment with five SURE motifs responsible for –S response (Maruyama-Nakashita et al., 2005) contains all of necessary *cis*-regulatory elements in directing gene expression in response to various nutrients. Therefore, the At2g44460 promoter::*GUS* line was used to further reveal their spatial expression patterns in particular in response to C, N and S nutrient interactions.

The putative thioglucosidase gene At2g44460 clearly exhibited a tissue-specific expression pattern (Fig. 2.8C). First, –S activated its expression in the vascular tissues and epidermal cells but not in the root hairs (Fig. 2.8, C1 and C2). Second, GUS was very strongly activated by –S in the root regions close to the tip but not in the tip (Fig. 2.8, C3 and C4). Third, in adult plants grown in the soil where sulfate supply was normal, GUS activity was absent in young leaves (Fig. 2.8, C5) but strong in both male and female reproductive organs (Fig. 2.8, C6).

To gain further insights into how C and N nutrients regulate the expression of the putative thioglucosidase gene expression in response to –S, we performed dose–response studies for the At2g44460 promoter::*GUS* line. We first studied the effects of various sucrose levels in the induction of the promoter. In the +N –S background, the promoter activity increased with sucrose levels (Fig. 2.8D). Interestingly, when 60 mM glucose was used, the –S activation was slightly stronger than 60 mM sucrose, indicating that glucose is more potent than sucrose in activating the –S response. The effect of sucrose or glucose on the –S activation is unlikely due to osmosis because 59.8 mM mannitol plus 0.2 mM sucrose did not affect the putative thioglucosidase gene promoter activity, compared to 60 mM sucrose. Careful observations of the GUS staining patterns revealed that the –S activation appeared to occur only in the roots but not in the cotyledons (Fig. 2.8D). Interestingly, decreasing C levels caused the –S activation to be limited to the regions closer and closer to the root tip.

The indication of the synergism between C and N observed above (Fig. 2.8A, B) led us to further investigate the effects of N under both low C and high C conditions. In the –C–S background

(0.2 mM Suc, 0.0001 mM SO_4^{2-}), the presence of N from 10 to 120 mM increased the promoter activity only very slightly (Fig. 2.8E), consistent with our previous studies (Fig. 2.8A, B). At very high N level, 120 mM, which is twice that of the full strength of the MS medium, the At2g44460 promoter activity increased only slightly in both the cotyledons and the root tip region. However, when C was increased to 30 mM, a strong activation was clearly observed even at the minimal N level (0.2 mM). This activation seemed to be restricted to the tip of primary or lateral root. Furthermore, 10 mM N already strongly activated the promoter throughout the whole root system, and 60 mM N seemed to have already saturated the response. These results clearly support that while C plays a predominant role in –S activation of the putative thioglucosidase gene expression, N has a synergistic effect with C. It is not known why at the low C level, the –S activation modulated by N also occurred in the cotyledons, while it happened exclusively to the roots at the higher C level. Very importantly, as observed for the C effect (Fig. 2.8D), decreasing N from 60 to 0.2 mM under 30 mM C resulted in the suppression of the activation by –S in the root regions to be expanded away from the root tip (Fig. 2.8E).

2.3 Discussion

S nutrition is essential for various cellular activities and, therefore, sulfate uptake and S metabolism must be tightly coordinated in order to optimize cellular metabolism, growth and development. However, the mechanism by which plants constantly and robustly monitor the dynamic changes of S nutrients remains largely unknown. In this report, using a combination of genetic, molecular and physiological approaches, we have shown that auxin plays a negative

regulatory role in –S response. Furthermore, the auxin-suppressed putative thioglucosidase gene (At2g44460) exhibits a C and N nutrient-dependent activation by –S in a tissue-specific manner.

2.3.1 A negative regulatory role of auxin in sulfate deficiency responses

Several lines of molecular and genetic evidence presented here convincingly show that auxin plays a negative regulatory role in –S response. First, using *DR5::GUS* as an auxin response marker, we have revealed that –S likely suppresses auxin level or sensitivity. Second and most importantly, the suppression of lateral root development is not observed in the auxin signaling mutant, *axr1*. The transient activation of chlorophyll accumulation by –S is also altered in *axr1-3*. Third, the activation by –S of the At2g44460 gene promoter is strongly suppressed by auxin in the roots.

The role of hormones in nutrient signaling has received increasing attention. Cytokinin is the major hormone that has been shown to be involved in or demonstrated to participate in signaling to a number of nutrients, including nitrogen, phosphorus and sulfur (Takei et al., 2002; Ohkama et al., 2002; Franco-Zorrilla et al., 2005; Maruyama-Nakashita et al., 2004b). In this study, we show that cytokinin seems to have a similar effect in suppressing the activation by –S of the two genes that encode 5'-adenylylsulfate reductase (*APR2*) and the putative thioglucosidase (At2g44460), respectively. Together with the demonstration of cytokinin suppression of the sulfate transporter (SULTR1;2) gene and sulfate transporter activity (Maruyama-Nakashita et al., 2004b), these results suggest that cytokinin likely plays an important role in mediating or modulating the general –S responses ranging from sulfate uptake to S metabolism and possibly

thiol release from the intracellular S stores. How cytokinin perception and signaling are regulated by –S and subsequently trigger the physiological responses remains to be revealed.

The involvement of auxin in –S response seems to be more restricted to certain genes and/or specific tissue types. Availability of various nutrients differentially modifies root development and architecture (Lopez-Bucio et al., 2003). Part of our physiological observations seems to differ from the general perception that –S stimulates root branching (Lopez-Bucio et al., 2003) that is derived from relatively recent observations (Kutz et al., 2002; Nikiforova et al., 2003). While there were no quantitative analyses on the LR and LRP densities in these studies to support the promotion of lateral root development by a relatively long term (from 10 days to 4 or 6 weeks) –S stress, we did not observe a dramatic difference in the LR density in *DR5::GUS* and Col. Instead, we found a significant difference in the LRP density after 2–10 days of –S stress (Figs. 2.2–2.3). Similar discrepancies have also been observed in several studies that addressed the relationship between auxin and phosphate-starvation (Hardtke, 2006). These include the opposite effects of phosphate-starvation on lateral root density, and the contrasting results of *DR5::GUS* expression patterns in response to phosphate starvation (such as Linkohr et al., 2002; Lopez-Bucio et al., 2002; Al-Ghazi et al., 2003; Lopez-Bucio et al., 2005; Nacry et al., 2005). It is likely that these are caused by experimental variations given that plants are very sensitive to their surrounding environments and different developmental stages may show different responses (Nacry et al., 2005). In our study, we have found that the LRP density is suppressed by –S under all of the three different growth conditions (Figs. 2.2, 2. 3). The consistency in the suppression of the LRP formation with the *DR5::GUS* staining pattern and the axr1 phenotypes allows us to conclude that the suppression of lateral root development is a physiological response to –S

stresses at least in our experimental conditions. Together with the observed transient chlorophyll accumulation, the suppression of the LRP formation is an early response to the –S stress so that plants would optimize cellular metabolism in other parts of the plant by reducing cell division in the roots. If the –S stress persists, plants might sense this as a sustained stress and therefore increase root branching and growth in order to compete for the uptake of S nutrients.

What is the role of auxin in regulating the –S response? The involvement of auxin in –S response has been implicated by earlier studies, although there has not been convincing evidence to support it. First, the *NIT3* gene has been shown to be activated by –S in a study using the promoter::*GUS* reporter system (Kutz et al., 2002), and several genes likely involved in tryptophan-IAA pathway (such as a putative myrosinase and *NIT3* genes) have been revealed to be up-regulated by –S in a DNA microarray study (Nikiforova et al., 2003). The *NIT3*-encoded nitrilase isoform 3 has been proposed to convert the IAA precursor indole-3-actetonitrile, which results from the degradation of glucobrassicin (one of major types of glucosinolates), into IAA (Vorwerk et al., 2001). There seemed to be a correlation between the *NIT3* promoter activation and the decline in total inorganic sulfate and thiols in root tissues of –S stressed plants (Kutz et al., 2002). However, the measurement of root IAA levels did not show a clear difference between plants treated with –S and +S (Kutz et al., 2002). Furthermore, the DNA microarray study also revealed the activation of several auxin-inducible genes such as *IAA9, IAA17, IAA18* and *IAA28* (Nikiforova et al., 2003). Although the precise roles for most of the auxin-induced genes in auxin signaling remain to be determined, they are generally transcriptional repressors (Weijers and Jurgens, 2004), indicating a negative role for auxin signaling in –S response. More recently, an auxin signaling circuit was proposed to explain the –S effect in root development

(Nikiforova et al., 2005a) based on the integrative network analysis of both transcriptome (Nikiforova et al., 2003) and metabolome data (Nikiforova et al., 2005b). In this proposed circuit, IAA28 is induced to suppress auxin-induced genes, ultimately leading to the tight and balanced regulation of auxin response. Our results show that the *DR5::GUS* activity is suppressed by decreasing sulfate levels. Furthermore, the *axr1* mutant did not show further suppression of lateral root development. Together with the observation that –S does not change the auxin levels (Kutz et al., 2002), we conclude that it is more likely that auxin sensitivity, instead of auxin level, is suppressed by –S.

2.3.2 The role of ABA in response to –S is intriguing.

ABA is critical for plant response to a wide array of abiotic and biotic stresses, yet it seems to inhibit the activation of the putative thioglucosidase gene At2g44460 in response to the –S stress. Future work using various ABA signaling mutants will help to determine which branch of ABA signaling is actually involved in–S response. Promoter scan of At2g44460 revealed many *cis*-regulatory elements (such as ABRE and MYB/MYC recognition sites) that are involved in ABA response. This gene also contains five SURE motifs that are important for –S activation (Maruyama-Nakashita et al., 2005). Therefore, it will be interesting to determine how the –S signal leads to the suppression of ABA accumulation or response so that At2g44460 is preferentially activated by transcriptional regulators recognizing the SURE motifs. The possibility that ABA and auxin signaling pathways interact with each other in response to –S cannot be ruled out. It has been shown that ABA stimulates lateral root primordium formation through auxin and inhibits lateral root growth independent of auxin (De Smet et al., 2003). In our

study, we show that ABA and auxin affect the expression of the putative thioglucosidase gene in the roots with a similar pattern of retaining the expression in the primary or lateral root tips (Fig. 2.5A). However, decreasing sulfate levels lead to distinct tissue-specific patterns of *DR5::GUS* and P_{RD29B}*::GUS* although both are suppressed (Figs. 2.1, 2.8). Therefore, if the cross-talk between auxin and ABA signaling exists in –S response, it is likely a complex interaction.

2.3.3 Role of the auxin-suppressed putative thioglucosidase gene (At2g44460) in sulfate deficiency response and its regulation by C and N nutrients

In this study using a combinatorial design of C, N and S nutrient treatments, we have revealed novel differential transcription patterns in response to S deficiency for genes encoding the auxin-suppressed, putative thioglucosidase (At2g44460) and a sulfate transporter SULTR4;2. We confirm that the –S activation requires both C and N availability, and also show that the promotive effect of C and N is dependent upon the S availability because the deficiency of either C or N alone did not activate expression of these genes. Furthermore, N alone does not greatly affect gene expression in response to –S if the C level is kept low. However, when the C level increases, N shows a dramatic effect in activating gene expression under –S. Therefore, these results convincingly show that these genes are first responding to the S availability but the realization of the –S activation depends on the C and N nutrients. In this regulatory process, C plays a predominant role and N synergistically interacts with C.

Although the biochemical function of the protein encoded by the putative thioglucosidase gene remains to be characterized, it has been hypothesized that it acts in the release of thiol groups from glucosinolates in response to –S (Maruyama-Nakashita et al., 2003). Glucosinolates are a large group of secondary compounds that contain C, N and S and are important for biotic and abiotic stress responses either as defense compounds or S storage sources (Rausch and Wachter, 2005). In an integrative transcriptome and metabolome study (Hirai et al., 2004, 2005), a regulatory link between glucosinolate metabolism, primary metabolism and S and N nutrition has been revealed. This implicates the importance of glucosinolates as alternative S source when plants are –S stressed. The lack of promoter activity in the root hairs (Fig. 2.8C) implicates that this gene is not involved in sulfate uptake. It is possible that the enzyme encoded by this gene acts to recycle S by hydrolyzing either generally all or specifically some of glucosinolates.

Interestingly, the regions of the roots in which the putative thioglucosidase gene is activated by the –S stress seem to be controlled by C and N nutrients. Decreasing the C or N levels causes the –S activation of the promoter to be limited to the root regions closer and closer to the root tip. This indicates that some sort of signal derived from the root tip is required for –S activation of gene expression and that this signal is likely controlled by the availability of either C or N nutrients. This novel tissue-specific expression pattern by C and N nutrients implies that both C and N may specify the root regions in which gene expression can be activated by –S.

Although the regulatory mechanisms by which C and N nutrients are sensed and the signals are subsequently transduced and integrated to control –S activation remain largely unknown, the

regulatory pattern of C, N and S interactions we observed seems to be a general phenomenon of gene expression in response to S deficiency stress. This is because similar GUS expression patterns were observed for the genes involved in the S storage release (At2g44460), sulfate uptake (*SULTR4;2*) and reduction (*APR2* and *APR3*; see promoter::*GUS* activities in Fig. 2.9). To further test this phenomenon with the Arabidopsis full genome chip using our combinatorial design will help reveal the regulatory network in C, N and S nutrient signaling pathways and their cross-talk.

In summary, our results provide molecular genetic evidence that auxin plays a negative regulatory role in –S response. When the S nutrient status is perceived as being at very low levels, plants then downregulate the auxin sensitivity, which would in turn release the suppression of gene expression and inhibit the lateral root development. Given that auxin suppresses the promoter activity of the putative thioglucosidase gene At2g44460 but not that of *SULTR1;2* and *APR2*, we hypothesize that auxin might participate in only a subset of –S responses such as remobilizing S from the intracellular storage. Future work should test the biochemical and physiological functions of the putative thioglucosidase gene product and investigate which auxin signaling branch is involved in the response to sulfur nutrient status.

Table 2.1

Components of MS-C-N-S medium (mg solute in 1000ml distilled water)

Macronutrients		
	KH_2PO_4	170
	$CaCl_2 \cdot 2H_2O$	440
	Na_2-EDTA$\cdot 2H_2O$	37.2
Micronutrients		
	H_3BO_3	6.2
	$MnCl_2 \cdot 4H_2O$	22.3
	$ZnCl_2$	4.1
	$Na_2MoO_4 \cdot 2H_2O$	0.2
	KI	0.8
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.025

Ingredient with amount of smaller than 20mg will be prepared from master solution.

Prepared MS-C-N-S medium is adjusted to pH 5.7-5.8, then autoclaved for use.

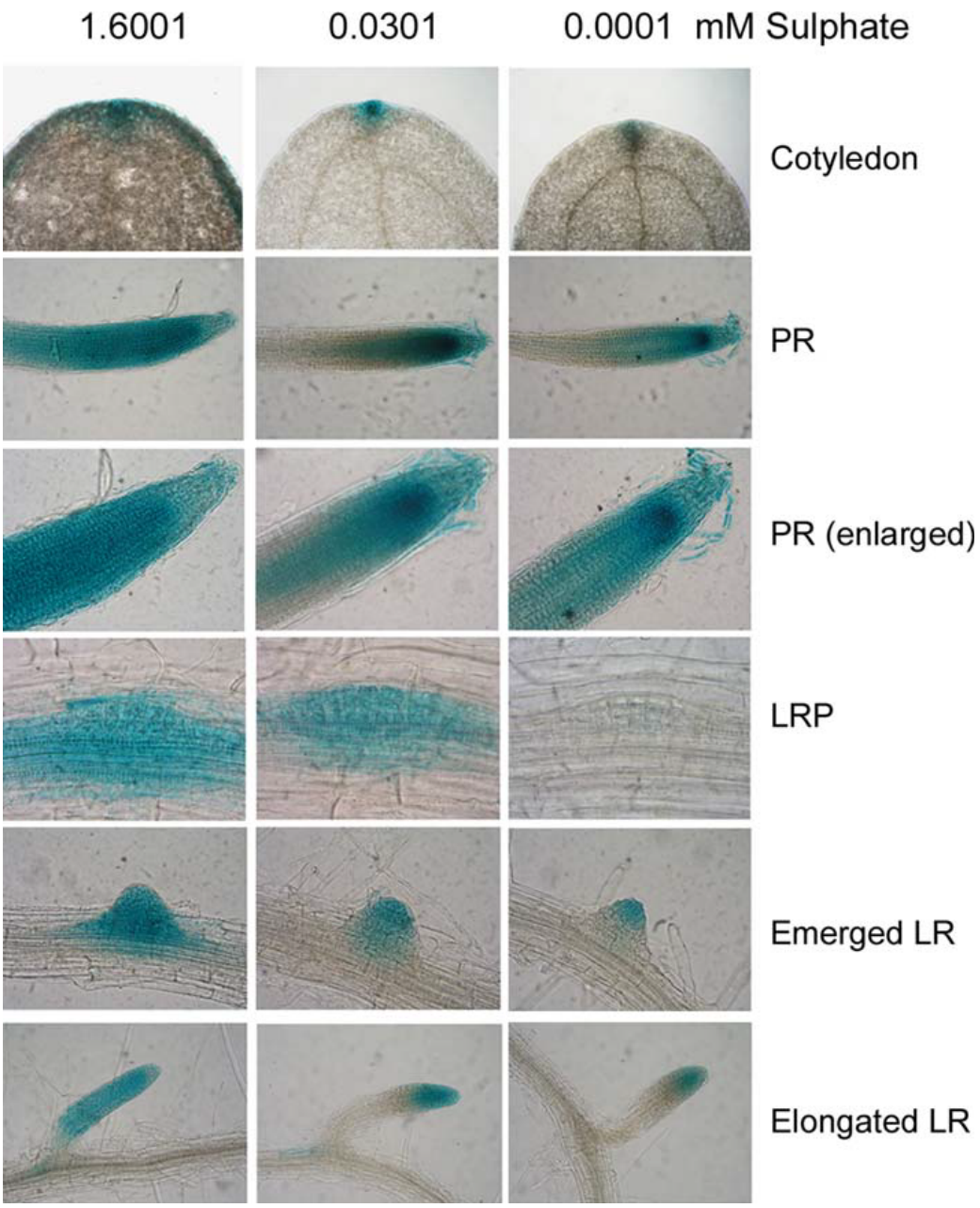

Figure 2.1 *DR5::GUS* expression is suppressed by sulfate deficiency.

Shown are GUS staining patterns in cotyledons, primary roots (PR), lateral root primordia (LRP), and emerged or elongated long lateral roots (LR)

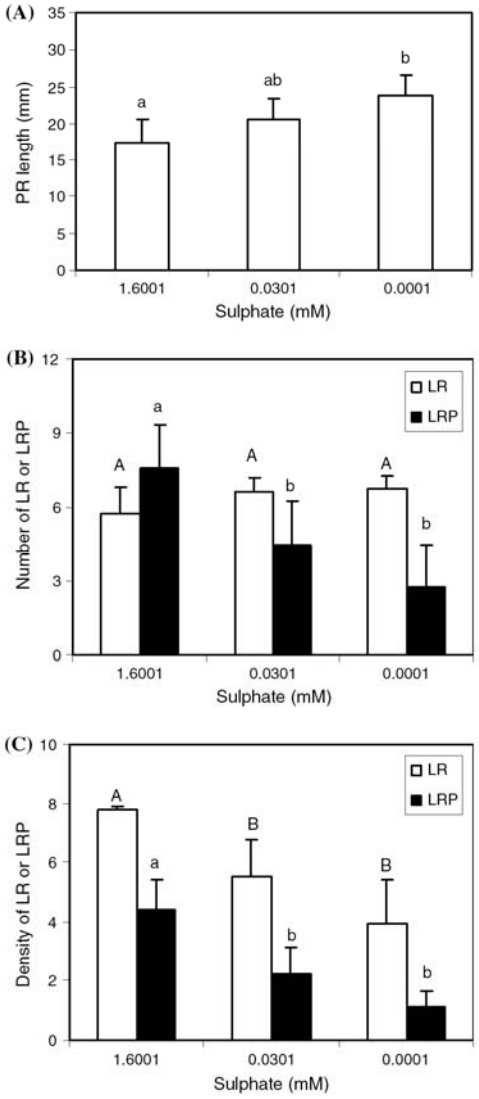

Figure 2.2 Sulfate deficiency suppresses lateral root development.

DR5::GUS seedlings treated identically in Fig. 2.1 were used to measure the primary root (PR) length and count under microscope the number of the emerged or elongated lateral roots (LR) and the lateral root primordia (LRP). Various stages of LRP were determined according to Malamy and Benfey (1997). The average of 4–7 seedlings measured or counted were shown with the bar representing the standard deviations. (A) Primary root length. (B) Total number of lateral roots and LRP for each seedling. (C) Density of lateral roots or LRP on the primary roots. Density is expressed as the average number of LR or LRP on per cm primary root (PR). Different letters (in B and C, uppercase letters for the LR, and lowercase letters for the LRP) above the columns indicate statistically significant difference (P=0.05)

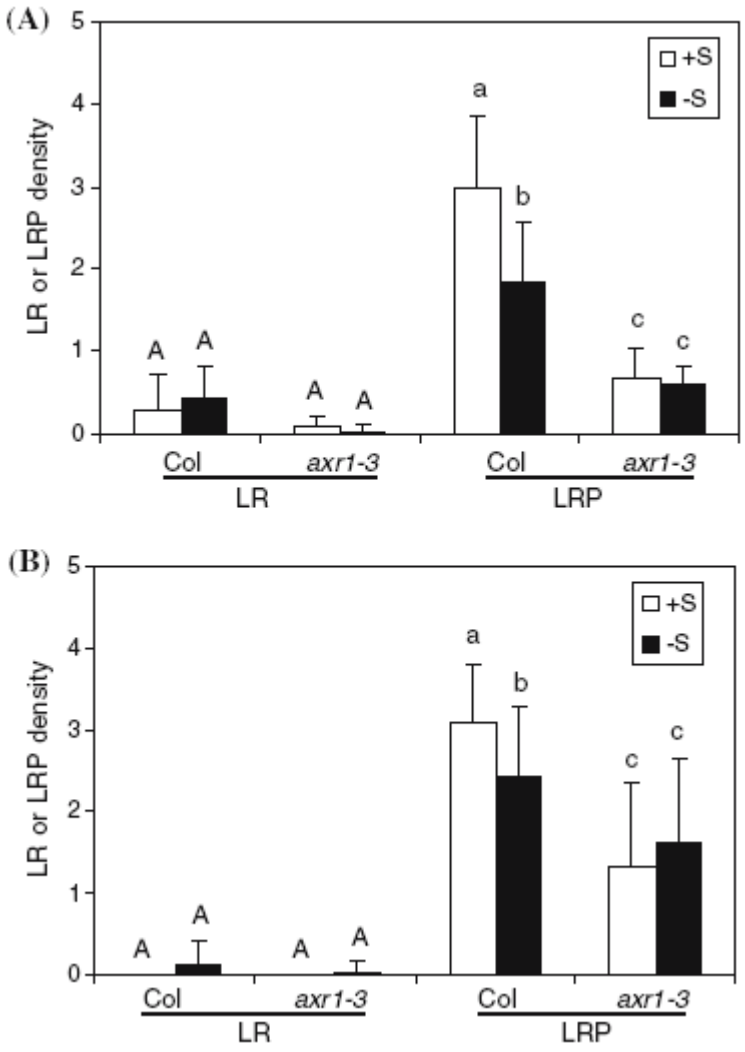

Figure 2.3 Altered sulfate deficiency stress response in lateral root development in *axr1*.

LR, lateral roots; LRP, lateral root primordia.

Density is expressed as the average number of LR or LRP on per cm primary root (PR), with the SD bars shown. (A) Seedlings were transferred to and grown vertically on the agar-solidified medium for 10 d after 5-d vertical growth on the normal, solidified medium (n=4–5). (B) Seedlings were continuously grown for 5 d in liquid medium (n=16). Different letters (uppercase letters for the LR density, and lowercase letters for the LRP density) above the columns indicate statistically significant difference (*P*=0.05)

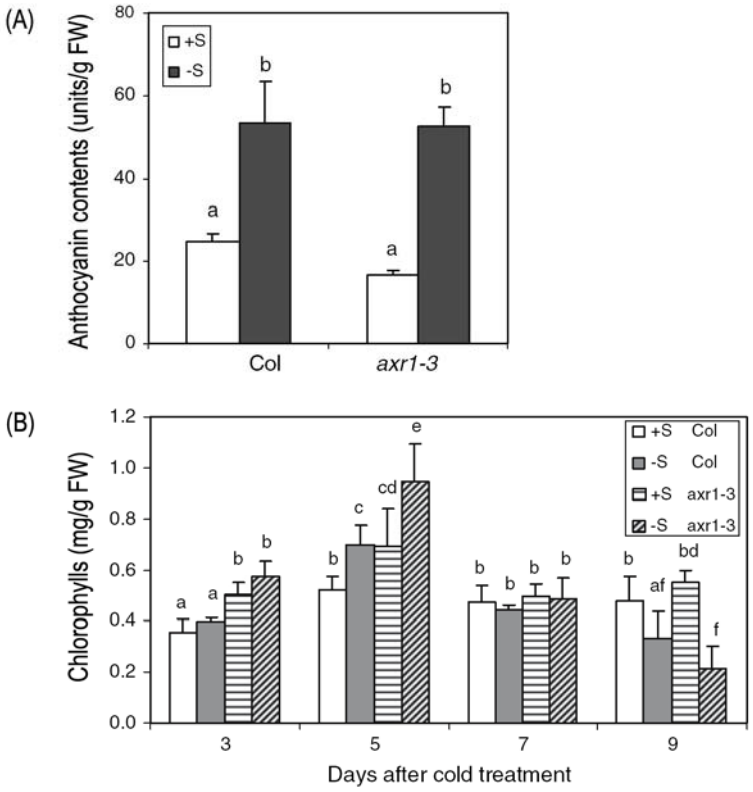

Figure 2.4 Altered sulfate deficiency stress response in anthocyanin and chlorophyll contents in *axr1*.

Anthocyanin (A) and chlorophyll (B) levels were measured for Col wild-type and axr1 after 5 days (for anthocyanins) or 3, 5, 7, and 9 days (for chlorophylls) of growth in +S or –S. The average of at least three replicates were shown, with the bar representing the SD. Different letters above the columns indicate a statistical significant difference (P=0.05) FW, fresh weight

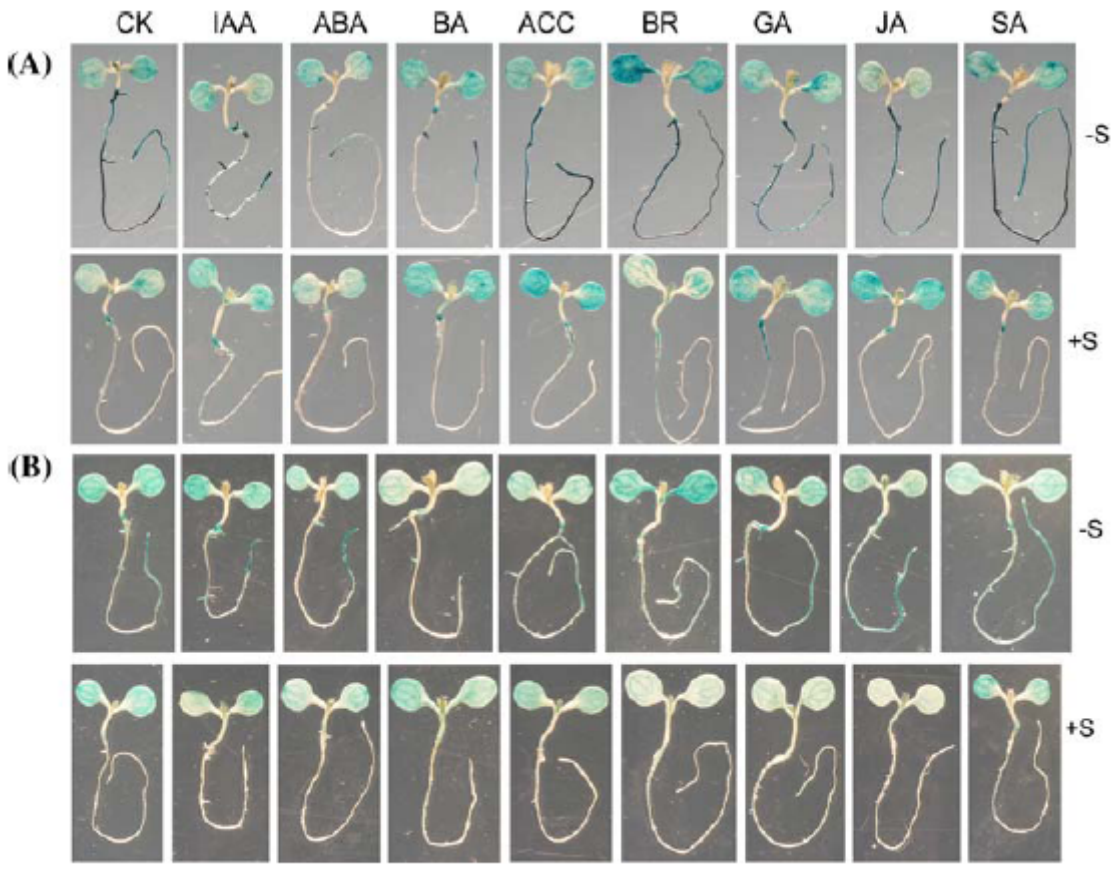

Figure 2.5 Promoter activities of the putative thioglucosidase gene At2g44460 and *APR2* are differentially suppressed by hormones.

Five-day old seedlings grown identically as in Fig. 2.1 were treated for 48 h under either –S or +S supplemented with 1 μM hormones. CK, no hormone control; IAA, auxin; BA, Cytokinin; ACC, ethylene precursor; BR, brassinosteroids; JA, methyl jasmonic acid; SA, salicylic acid. (A) GUS staining pattern of the putative thioglucosidase gene At2g44460 promoter. (B) GUS staining pattern of the APR2 gene promoter

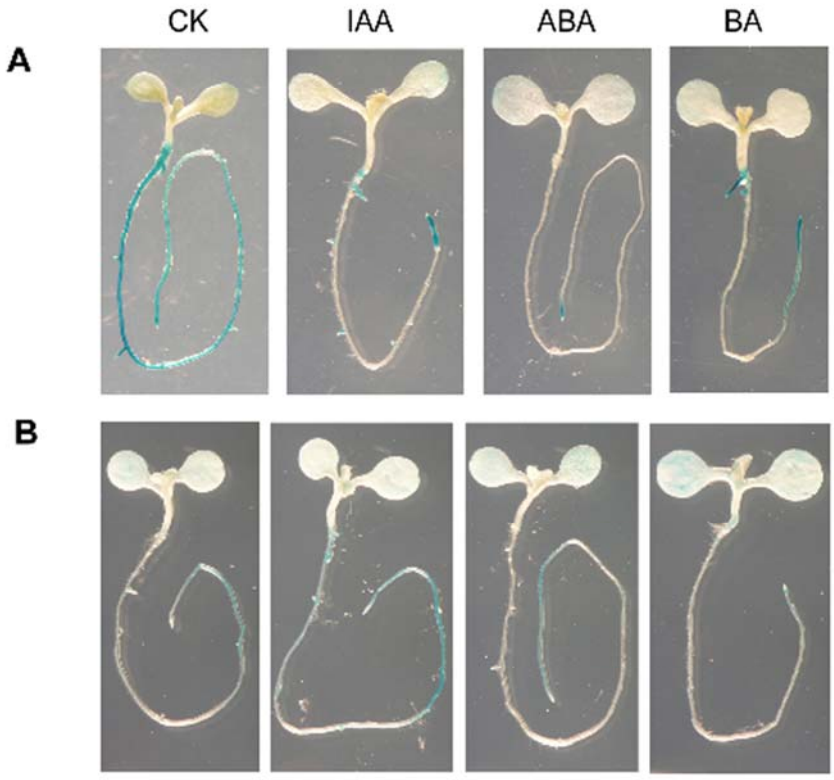

Figure 2.6 Similar hormonal regulation of promoter activities for the putative thioglucosidase (At2g44460) and *APR2* genes under sulfate deficiency stress, as shown by independently transformed lines.

(A) A homozygous line expressing At2g44460 promoter::*GUS* transformed independently of the line shown in Fig. 2.5A exhibited similar suppression by IAA, ABA and BA. CK, no hormone control. (B) A homozygous line expressing *APR2* promoter::*GUS* that is independently transformed as that in Fig. 2.5B showed similar suppression by BA, but not by IAA and ABA. CK, no hormone control. Five-day-old seedlings were grown and treated identically as in Fig. 2.5 before GUS staining.

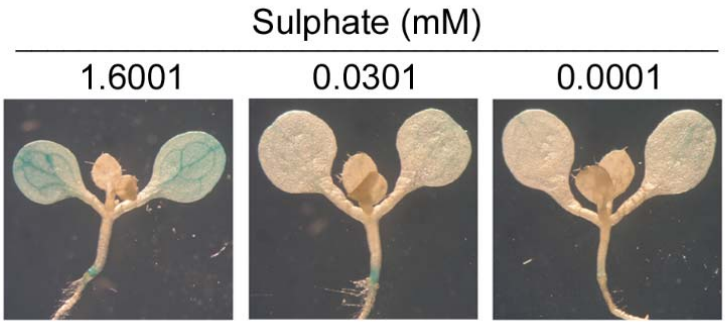

Figure 2.7 P_{RD29B}*::GUS* expression is suppressed by S deficiency

Note that the GUS staining in the cotyledons and the hypocotyl-root junctions is most affected. Five day old seedlings grown identically as in figure 2.1 were treated for two days before GUS staining.

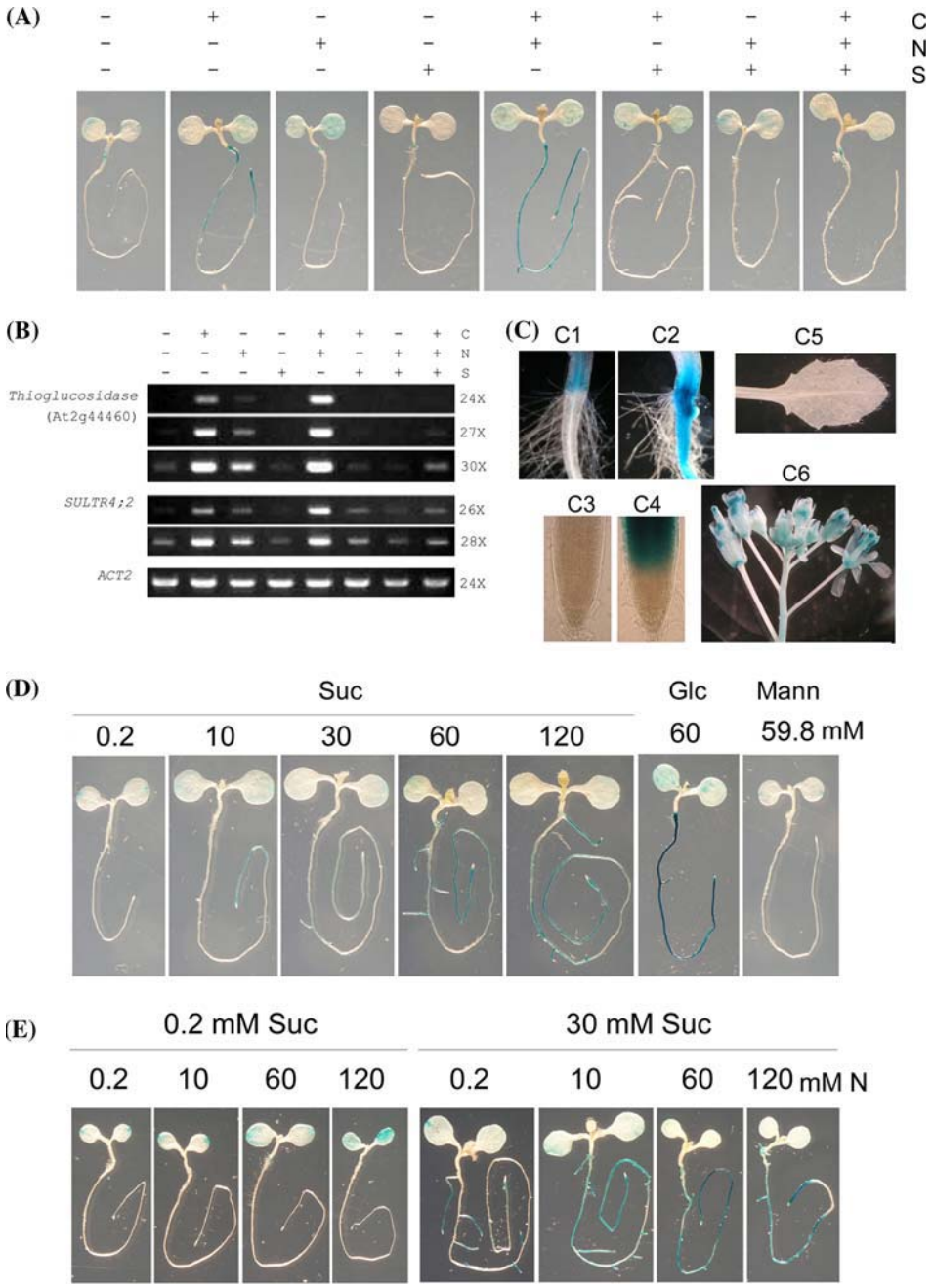

Figure 2.8 The putative thioglucosidase gene (At2g44460) promoterdriven GUS expression is differentially regulated by C, N and S nutrients in a tissue-specific manner.

(A) GUS staining patterns under various C, N and S nutrient conditions. Five-day old seedlings were grown under half-strength MS basal salts supplemented with 1% sucrose and 1% agar were treated with various combinations of C, N and S liquid medium for 48 h and then stained for GUS activity for 3–5 h. +C, +N and +S indicate the presence of 60 mM sucrose, 60 mM total N (20 mM KNO_3 and 20 mM NH_4NO_3), and 1.6001 mM SO_4^{2-} respectively. –C, –N and –S represent the deficiencies of C (0.2 mM sucrose), N (0.2 mM total N), and 0.0001 mM SO_4^{2-} (from $CuSO_4$), respectively. (B) RT-PCR analysis of SULTR4;2 and the putative thioglucosidase gene expression under various C, N and S nutrient

conditions. Five-day old seedlings were similarly treated by various combinations of C, N and S for 48 h (see legend in Fig. 2.8A) before RNA extraction. *ACT2* was used as the internal control. PCR using 24, 27 and 30 cycles for the putative thioglucosidase gene and 26 and 28 cycles for *SULTR4;2* were performed to differentiate the transcript abundance for every treatment. (C) Tissue-specific GUS expression patterns. Both the root-hypocotyl junctions (C1 and C2) and the root tips (C3 and C4) were shown to compare the effects of +S (C1 and C3) and –S (C2 and C4) treatments. A true leaf (C5) and flowers (C6) from a 30–day-old plant grown in the normal soil in the growth chamber were also shown. (D) GUS staining patterns under –S with various C levels. (E) GUS staining patterns under –S with various N levels at both low C and high C conditions. For (D) and (E), five-day old seedlings grown identically as in Fig. 2.8A were treated for 48 h before GUS staining. Suc, sucrose; Glc, glucose; Mann, mannitol

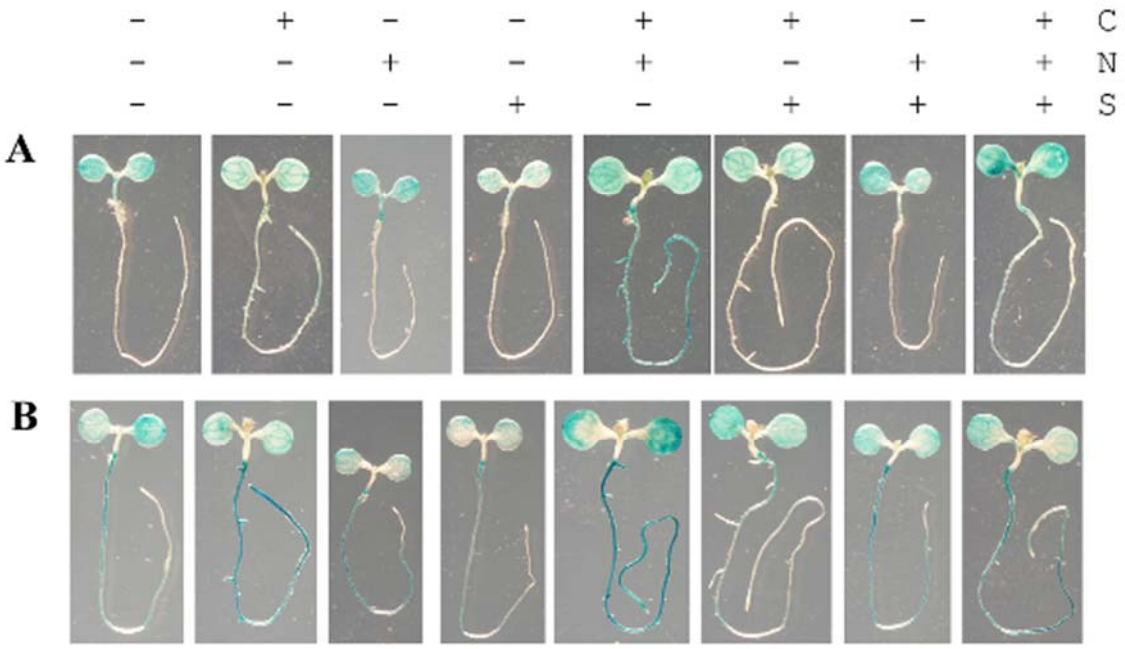

Figure 2.9 *APR2* and *APR3* promoters are activated by S deficiency but the activation is dependent on C and N nutrients

(A) *APR2::GUS* expression. (B) *APR3::GUS* expression. Five-day-old seedlings were grown and treated identically as in Figure 2.8A for two days before GUS staining. For the *APR3::GUS* construct, a 0.9 kb promoter fragment of *APR3*/At4g21990 gene was PCR amplified using two primers (DZP3, sense: 5'-CATCTGCAGTTGATTTGTTGAGATCATGATTAC-3'; DZP4, antisense: 5'-AGTGCCATGGTCACTCCAAACCAAATCTCTCG-3') and then cloned into the binary vector pCAMBIA 1301 (B4) that contains GUS and the CaMV35S terminator as described in Materials and methods.

Chapter 3

Mutant isolation and gene cloning

The screened reporter line GHF1 was characterized in the work of Chapter 2. The response of C-N-S in GHF1 can be easily detected in the mutant screen. For mutagenesis, ethane methyl sulfonate (EMS) was applied to the seeds carrying the reporter construct. After that an effective mutant screen strategy was established. For the final screen of the mutant, a C and S minimal medium (–C-S medium, which contains 2mM C and 0.0001mM S) was applied to obtain three inheritable mutants, 11p34h, 11p101h and 11p201h. The mutants were characterized based on morphology and disruption in nutritional response to C and S status. Genetic characterization demonstrated that all three mutants were monogenically mutagenized, and allelic to one another. The mutations were molecularly cloned using map-based cloning, and were shown to belong to two alleles of a single gene, *SULTR1;2*, which encodes a high-affinity sulfate transporter.

Functional complementation was performed by transforming the Wild type (WT) SULTR1;2 with mutant plants. The GUS phenotype demonstrates that the mutated gene is responsible for the mutant phenotype.

3.1 Materials and methods

3.1.1 Mutagenesis and mutant screening

As described in Chapter 2, the reporter line $P_{At2g44460}$*::GUS* called GHF1, which displayed a strong –S-response on roots, was chosen for mutagenesis. Homozygous seeds were selected and named M0 generation prior to mutagenesis treatments. Approximately18,000 GHF1 M0 seeds were chemically mutagenized by treatment with 0.3% ethyl-methane sulfonate (EMS) for 16 hours (hr) as described (Lightner and Caspar, 1998). The mutagenized M0 seeds were named the M1 generation and were sown on 11 soil trays to generate M2 seeds. About 400,000 M2 seeds from 11 pools were selected for mutant screening in –C-S medium.

At least three categories of methodology concerning non-destructive GUS assay have been tested for their screening effectiveness and seedling rescue ratio either from reference (Martin et al., 1992) or designed by this lab. Briefly, 18-20-day old seedlings were grown on soil were rinsed thoroughly with distilled water, and treated with a carbon (C) minimum medium (MS medium modified to contain 2 mM C, 60 mM N, and 0.0001 mM S) for 24 hr. Part of the root system was excised prior to staining with GUS for up to 3hr. If the GUS staining is stronger than WT (GHF1), the seedling will be considered as a putative mutant of C hypersensitive and transplanted to soil to obtain M3 seeds. The isolated putative M3 mutants were labeled as XpYh, where Xp stands for the number of pool and Yh for the number of treated seedling, which expressed hypersensitivity to C. For example, 2p108h indicates that the putative mutant was

screened from the 2nd pool and was the 108th seedlings treated in that experiment; also 2p108h was C hypersensitive. The process of mutant screening is illustrated in Figure 3.1A.

A ranking system was used to quantitatively record GUS phenotype in the mutant screen result. 0 was assigned for unstained roots, 10 for 100% stained roots, and number of 1-9 for increasing GUS staining roots (Fig. 3.1B).

The putative M3 mutants were then rescued by transplanting into soil in order to harvest the M3 seeds, which were further verified by screening using C minimum medium. Only the lines that showed very strong GUS staining in this test without phenotypic segregation were retained as homozygous mutants for the morphological and genetic characterization.

3.1.2 Morphological characterization of screened mutants.

The seeds used for morphological, physiological and genetic studies were obtained from F3 generation which resulted from a backcross with the WT in attempt to remove undesired mutations from the mutant.

For morphological analysis, both mutants and WT (GHF) seeds on MS +1% Sucrose plates. After 2 day (d) cold-treatments at 4°C, plates were incubated vertically in growth chamber at 22 °C with 16 hour light and 8 hr dark diurnal program. The root length was measured at the 3rd, 5th, 7th, and 10th day after incubation. Both mutants and WT seeds were also sown on soil for growth phonotypical observation. The incubating temperature and diurnal program was the same as for plate incubation.

Four C and S nutrient combinations were applied to determine the nutritional responses of the mutants. The four applied media are: -C-S, -C+S, +C-S, and +C+S. -C represents a 2 mM C source; +C represents a 60 mM C source as in the standard MS medium. –S represents 0.0001 mM S source, and + S represents 1.6 mM S source. The media were prepared on the basis of MS minimal medium (MS-C-N-S) as shown in Table 2.1, autoclaved and stored at -4°C. The seedlings of 5 d growth on MS plates were transferred to a 24-well microplate containing the above four liquid nutrients, and treated for 24 hr on a shaker at 150 rpm. The Incubating temperature and light/dark cycle were programmed as for all other treatment and plant growth as shown above. The treated seedlings were GUS stained for up to 3 hr, followed by decolorization with 75% ethyl alcohol. The decolorized samples were used to collect data on GUS strength, and stored at room temperature.

In order to remove undesired mutations, M3 mutants were backcrossed to their WT (GHF1). The F1 seeds were obtained and were grown on MS plates. The root lengths of the plants were measured and compared to the WT. The F1 seeds were also grown and treated with the four C, S combinatory media, -C-S, -C+S, +C-S, and +C+S, data of GUS staining and root length were collected for analysis.

3.1.3 Genetic characterization of screened mutants

Three mutants, 11p34h, 11p101h and 11p201h, were crossed to the WT (GHF1) and F1 and F2 seeds were obtained for genetic characterization. For F1, seeds were sowed on soil cups and allowed to grow for 20 d. After that, seedlings were rinsed and treated with the C minimum medium for 24 hr. Half of the 1 inch root system was removed for GUS staining for 3 hr. Comparison with the WT will indicate whether the mutant is dominant or recessive. The F1 seedling tested can be rescued by transplanting back to soil cups to generate F2 seeds. The seeds of F2 generations from the cross between WT (GHF1) and mutant were sown on MS plates for 5 d growth, and then treated with the C minimum media for 24 hr. They were GUS stained and the data were analyzed statistically.

An allelic test was performed between 11p34h, 11p101h and 11p201h by crossing the mutants with each other. The F1 seeds were sown on MS plates and seedlings were either treated with the

C minimum medium only or treated with the four nutritional statuses, -C-S, -C+S, +C-S, and +C+S. Following that, treated seedlings were GUS stained for GUS strength data collection.

3.1.4 Verification of endogenous At2g44460 expression in the mutants.

The expression of At2g44460 was investigated by reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (q RT-PCR). The seeds of mutants (11p34h, 11p101h and 11p201h) and WT were sown on MS plates and harvested after 5 d growth at the standard growth chamber. The seedlings were rinsed with –S medium (with C 60 mM, N 60 mM, and S 0.0001 mM) for three times before subjected to the four –C-S nutritional treatments. After 24 hr, the treated seedlings were harvested and frozen with liquid nitrogen immediately, and stored at -80°C or directly used for RNA isolation.

For RNA isolation, the first strand cDNA synthesis, and RT-PCR were conducted as described in Chapter 2. The same set of primers for regular RT-PCR as in Chapter 2. In qRT-PCR, the primers were YZP75 (sense): 5'- CGCGTTACGTTGCTCATATT-3', and YZP76 (antisense): 5'- GAGCTGATGATCGGTTACGA-3'). Quantitative PCR was performed in SmartCycler II (Cypheid, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, USA), according to the protocols provided by the manufacturer. *ACT2* was used as reference gene, and the gene-specific primers were: ActSDS (sense): 5'-GCACCAAGCAGCATGAAGATT-3'; ActSDA (antisense):

5'-GGAACCACCGATCCAGACACT-3'. The key parameters for qRT-PCR reaction parameters are: annealing temperature 60°C, and number of cycles 45. The qRT-PCR results were firstly normalized by using the internal control *ACT2*, then normalized by WT (GHF1) under the condition of –S, which was transformed into a gene expression power of 100% , and all other genes expression levels are relative to this condition.

3.1.5 Map-based cloning of the mutant genes.

Molecular cloning of mutant genes started from the crossing of the mutant (♂) with *A. thaliana* Landsberg erecta (L*er*, ♀) ecotype. The F2 population of 3 mutants, 11p34h, 11p101h, and 11p201h were obtained and stored for molecular cloning. The first mutant isolated, 11p34h, was the first clone, and it was revealed to be allelic to both other two mutant, 11p101h and 11p201h.

A population of about 4,000 F2 seeds was collected for the following map-based cloning. 387 F2 seedlings with strong GUS staining phenotype in treatment of C minimum medium were identified, and genomic DNA was extracted for PCR identification. The F2 mutant gene was first PCR rough-mapped with the set of 22 SSLP (Single Sequence Length Polymorphism) markers as described (Lukowitz, 2000; Jander et al., 2002). After narrowing down the mutant locus to the lower arm of Chromosome I with the ciw1, nga280, and nga111 SSLP markers, 12 more SSLP markers were carefully designed to fine-map the mutant gene into an 81kb BAC

clone F28K19, which contains 25 putative genes. As the designed GUS reporter line GHF1 has a strong –S response, and the mutant was also screened from the –S responsiveness, we firstly sequenced the two S transporter gene At1g77990 (*SULTR2;2*) and At1g78000 (*SULTR1;2*) within the F28K19 region by designing 12 primers that covered the whole sequence of them. A point mutation in the 4th exon of the putative gene *sultr1;2* was identified. The base replacement from G to C led to a missense mutation of G208D on the transmembrane S transporter SULTR1;2. Primers for map-cloning are listed in table 3.1.

For the mapping of another mutant 11p201h, many lines of evidence showed the similarity between 11p201h and the mapped mutant 11p34h. These two mutants cannot complement in the complementation test, which further support the hypothesis that they may be allele to each other. 106 F2 samples of Ler x 11p201h were used for a preliminary rough-mapping test (Table 3.4B), which confirmed the original hypothesis.

We sequenced the hypothesized locus At1g78000 of mutant 11p201h by using the same set of primers as 11p34h. The point mutation was found at the 2nd exon of *SULTR1;2*, with a base replacement from G to A, which led to a missense mutation from D108 to N108 on the transmembrane S transporter SULTR1;2.

For the mapping of 11p101h, we directly sequenced the two loci, At1g77990 and Ag1g78000 and identified the point mutation of 11p101h at the same locus as 11p201h, which means they are the same mutant of different plant (Figure 3.7). After the process of map-cloning, we renamed the mutation of 11p101h, 11p201h as *sel1-15*, and mutation of 11p34h as *sel1-16*.

3.1.6 Functional complementation

Plasmid constructs were made by amplifying the coding sequence of wild-type *SULT1;2* with the primers DZP190 and DZP193 using PCR (Table 3.1). The gene was cloned into the vector GZ6 that contains the CaMV 35S promoter and GFP and terminator. The newly made construct, DZ18, was identified and verified by restriction digest and sequencing, and transformed into mutant plants, *sel1-15*and *sel1-16* by the floral dip method (Clough and Bent, 1998). The seeds were harvested and called T1, and were sown on soil cups and tested with –C-S medium. T1 seedlings with phenotype of WT were rescued to generateT2 seeds, which were subjected for further complementation test as described in Fig. 3.8.

3.2 Results

3.2.1 Three mutant plants, 11p34h, 11p101h, and 11p201h, were isolated in this project

By testing and comparing different methods, we developed a practical procedure for mutants screening and rescuing the tested plants (Figure 3.1A). This process is effective in screening mutants with response both to C and S nutritional availability. The mutants that were isolated had an altered –S response in a very low C source (2mM in comparing to the normal C level of 60mM), whereas the wild type, GHF1 has a very weak GUS staining (Fig. 3A, as shown in –CK). The putative mutant with the elevated GUS staining under C minimum medium (Fig. 3A arrow indicated) was rescued for F3generation. The screened mutant as indicated has a GUS staining almost as strong as WT at +C-S condition (Fig. 3A, +CK). From the screening, the mutants were called XpXh, where the "h" stands for C-'hypersensitive'. Approximately 1 putative mutant can be screened out of each 100 M2 seedlings. Even with the death of plants in rescuing and the numbers of non-inheritable mutations, the screening processes allow the recovery of mutants.

The GUS measuring system was simple and efficient (Fig. 3.1B). To simplify, GUS strength was artificially assigned as 0 if the sample has no GUS staining, and 10 if it is 100% stained. Between 0 and 10, we have number 1 to 9; represent the increasing GUS staining level from very low to very high. The error in the numbering was minimized by increasing the test population. In the practical experiment, two genotypes will have an average GUS difference larger than 7 levels. So under this condition, it is very confident to apply the quantified GUS data to draw the conclusions (see Fig. 3.3B). The advantage of this method is the quick, simple, and convenient in recording lab results quantitatively without the need of GUS buffer and fluorometer. The evaluation system was applied not only in mutants screening, but also more often in GUS characterization of the screened mutant (Fig. 3.3B). The system was also used to screen and

collect sample for the mapping cloning. If the tested population is homozygous, the data variation (standard derivation) in a tested group is very low.

Multiple putative mutants with altered –S responsiveness were isolated by the GUS measuring system and M3 seeds were germinated on soil cups. M3 seedlings were subjected to the same treatment (with the –C-S liquid media) as they were originally isolated. The heritable mutants were expected to have a unified elevated GUS staining in the whole tested population without segregation (see Fig. 3.1C). It is important to have both –CK and +CK of GHF1in the screening process (see Fig. 3.1C). 11p34h was the first mutant we obtained and it was proved to be inheritable and genetically stable for several generations (Fig. 3.1C).

After that, two other mutants, 11p101h and 11p201h were isolated from the same M2 pool and both passed the following M3 generation tests on their genetic stability. After completing the GUS screening of about 13,000 (7X11X170=13,090) M2 seedlings, three genetically confirmed mutants were obtained and used for the project.

3.2.2 Phenotypic characterization of the mutants

In comparison to WT, the screened mutants initially displayed retarded growth in soil cups (Fig. 3.2A) with shorter shoots and fewer branches. Some other mutants, such as 11p201h displayed a typical chlorosis phenotype on young leaves (pictures are not shown here). However, after one backcross, the pleiotropic phenotypes disappeared, indicating that the phenotypes are unrelated to the mutations. The 20 day old seedlings on soil cups were tested on the GUS staining for both cotyledons (Fig. 3.2B) and whole seedlings (Fig. 3.2C). Mutant 11p34h and 11p101h had a much stronger GUS strength on their cotyledons. The averaged GUS strength for 11p34h was 7.7±2.4 from 171 samples, for 11p101h was 8.9±1.5 from 201 samples, while for WT was 0.7±0.8 from 179 samples. The data was collected by using the GUS evaluation system we developed in this project (see material and methods). The quantified data was much more accurate than simply describing as weak or strong, stained or non-stained. The experiments also displayed the different staining pattern between mutants and WT (Fig. 3.2B), where mutant 11p34h and 11p101h stained on the whole cotyledon, while WT stained only on the cotyledon tip or tip margin. Whole seedling stain showed stronger GUS staining of mutants not only on the cotyledons, but also on the true leaves and root system (Fig. 3.2C). Time course staining showed that cotyledons were stained earlier than true leaves. The cotyledons stained stronger than true leaves. While in WT, only cotyledons are weakly stained, which is consistent with observation in Fig. 3.2B, no staining is identified from true leaves or roots. Staining on the junction of hypocotyls and roots can be observed if staining extended long enough, such as to 24 hr (Fig. 3.2B). The tissue-specific GUS expression pattern of WT is highly consistent with our previous published experiments (Dan, et al., 2007).

In order to characterize the mutants, the root growth was examined of all genotypes on MS plates. The genotypes that were checked include not only the WT and the three mutants, but also the three F1 generations from the backcrossing of mutants (♂) to their WT, GHF1 (♀). The seeds were sown on MS plates for 2 d cold-treatment before vertically incubated in the programmed growth chamber. The root length was measured in millimeters (mm) as shown in Fig. 3.2D, and data could be collected from the 3rd, the 5th, the 7th and the 10th day of incubation. The difference between WT and mutant 11p34h, 11p101h and 11p201h, is not clear in the early observations, especially on the 3rd day. The difference became more and more obvious after the 5th day. On day 10, all three mutants show an obvious stunted root extension in comparison to the WT. The root shortening of the three mutants was finally restored by their WT in the F1 generation (each with an average of 18-35 seedlings). This result shows that the root shortening in mutants is possibly caused by the mutated gene in each mutant, and that all the mutated genes, which are responsible for the root shortness, are recessive.

3.2.3 GUS patterns of the mutants in various C-N-S treatments

Three mutants, 11p34h, 11p101h and 11p201h, were subjected to the eight nutrient conditions, with a combination of C, N, and S, as described in Chapter 2. The WT (GHF1) displayed identical responsive pattern as discussed in the previous chapter, the –S responsive At2g44460 promoter activity is highly regulated by C presence, and synergistically regulated by N presence (Fig. 2.8A and Fig. 3.3A). All the three mutants seem to have a stronger GUS response under all

tested nutrient conditions as shown in Fig. 3.3A. The conclusion from this experiment is that the mutants respond to the nutritional conditions are as if in a sulfur-starving condition.

A careful examination of the results showed some differences between nutrient statuses. The most observable difference is that the generally indicated –S status (+C+N-S) gives rise to the highest GUS staining among all the nutrient states. Another difference is the mildly stronger GUS staining when C is present, no matter what combination is from the other two nutrients (N and S) (Fig. 3.3A). These results showed that the mutants retained the response to C and S. But further experiments are needed to confirm to what extent the mutations affect the C and –S sensing, or whether the mutants lose the ability of sensing the C and S status completely. More experiments are also necessary to confirm the N responses in the mutants, because it's hard to identify the effect of N on mutants solely by the GUS staining evidence, although some mild synergistic up-regulation can be observed from the addition of N. If all these observations are true, then the conclusion from the mutation will be the loss of S and C (or include N?) sensing ability partly or totally, which needs further experimental tests.

In order to further study the GUS modification by the mutation, GUS strength was quantified by using the evaluation system developed in this project (Fig. 3.3B). GUS strength under –C-S treatment was 1.4±1.9 for WT; 9.1±0.6, 9.9±0.3, and 9.0±1.6 for the three mutants, 11p34h, 11p101h, and 11p201h, respectively. The quantified GUS strength from 18-34 seedlings of each

genotype displays four times stronger GUS staining in the mutant, and shows relatively small variations (the small standard deviation) between different treated seedlings, especially in mutant seedlings. In the experiment shown in Fig. 3.3B, the three F1 generations from the backcrossing of the three mutants (♂) to their WT were introduced to the GUS tests. The results show the decline of GUS activity in the presence of WT genome in the F1 generation. This result also proves that the GUS alteration by the mutation has a genetic base, and is not due to environmental changes.

Quantification of GUS strength shows the similar C and S response when all the genotypes shown above are tested under +C+S liquid medium (Fig. 3.3B). This result can be easily explained on the aspect of S status, because the mutant will lack of or have a decline in S sensing ability even when present in S-sufficient condition. From the results shown in Fig. 3.3A, we observed the retention of the C response when gene is mutated, but here we can't find the expected up-regulation when experimental condition changes from –C (left in Fig. 3.3B) to +C (Fig. 3.3B). A possible explanation for this observation may be that in +C up-regulation is balanced, or neutralized by the down-regulation from +S such as the mutants still maintain some extent of C and S sensing ability, but these two signaling systems are working antagonistically.

3.2.3 Genetic characterization of mutants

As shown in Fig. 3.2D and Fig. 3.3B, all three mutants lost their phenotypic characteristics in the F1 backcross, and F1 showed the WT phenotype (the root length as in Fig. 3.2D and GUS pattern as in Fig. 3.3B). These two results both proved that all the three mutants harbor recessive mutations.

To further genetically characterize the mutants, the three mutants were backcrossed to the WT (GHF1), and F1 were self-pollinated for F2 generations. 21 F2 seedlings of F2: GHF1 X 11p34h from soil cups were rinsed and treated with the –C-S medium for 2d prior to GUS staining (Table 3.2A). The F2 GUS phenotype segregated in a 3:1 monogenic ratio, which indicated that the mutant phenotype was due to a single-gene mutation. More seedlings were tested from soil cultivation of F2: GHF1 X 11p101h, which were sowed on soil and treated as the previous F2 seedlings. Out of 184 F2 seedlings tested, 43 seedlings exhibited the mutants' GUS pattern (strengthened GUS staining), where the estimated number for this phenotype was 46 for a monogenic mutation (Table 3.3). The seeds of F2: GHF1 X 11p201h were sown on MS plates and grown for 5d followed by 2d treatment with –C-S. 66 treated F2 seedlings were subjected to GUS staining, and the 3:1 segregation ratio was also observed as expected for a monogenic mutation (Table 3.3).

The mutants were also crossed to another ecotype *Arabidopsis thaliana* Ler, for the preparation of gene mapping. F2 generation of Ler x 11p34h was tested for the phenotypic segregation as

shown in Table 3.2B. In a population of 125 F2 seedlings from 20 d soil growth, 27 F2 seedlings had the mutants' GUS pattern (strong at –C-S medium), while the expected number for one-gene-mutation was 23.4. It is noticeable that the segregation of F2 Ler x 11p34h is different from that of F2 GHF1 X 11p34h on the ratio of homozygous mutants, the former is 3/16 while the latter is ¼ or 4/16. The difference made from F2 Ler x 11p34h is due to that inside the ¼ part of homozygous population there is also another ¼ population that are homozygous from Ler without GUS staining. The final ratio for homozygous mutants with a strengthened GUS staining is ¼ - ¼ * ¼ = 3/16. This process was applied to screen F2 homozygous population from F2 Ler x 11p34h for gene mapping and it was proved to be very efficient in identifying the correct samples. The segregation of another two F2 from the crossing of mutants with Ler was also tested and proved (data not shown) in this project. The results from the F1 and F2 generation suggest that all the three isolated mutants, 11p34h, 11p101h, and 11p201h are recessive and monogenic mutations.

The morphological, physiological and genetic characterization of the three mutants showed a high similarity among them. Complementation tests were conducted to see whether they are allelic to each other or not. F1 seeds of 11p34h x 11p201h, 11p101h x 11p201h, as well as all the control genotypes include GHF1, the three mutants, were sowed on MS plates and grown for 5 d before 2 d treatments as indicated (Fig. 3.4A, B). The GUS results of the two F1 generations did not show any complementation such as the F1 did not restore the GUS pattern to WT (GHF1). We performed another complementary test on the F1: 11p34h x 11p101h, which was conducted at 01/15/08, and failed too to find the complementation between the two tested mutants (data not

shown). These results suggest that the three isolated mutants are allelic, or, possibly they may represent different plants seed from a single parental mutagenized M1 plant. Anyway, the tiny growth difference between 11p34h and 11p201h may partly ensure us to identify more than one mutant from the three isolated ones.

As displayed in Chapter 2, the endogenous reporter gene, At2g44460, responds to all the three nutrients, C, N, S, coordinately (Fig. 2.8B). To determine whether this GUS patterns reflects the changes in the expression of endogenous gene, qRT-PCR was performed to check the gene expression under four nutrient conditions (-C-S, +C-S, -C+S, +C+S), and two of which are shown in Figure 3.4C. It is not surprising to see the up-regulation of the gene express of mutants (11p34h, 11p101h, and 11p201h) in comparing to the WT under the two nutritional conditions (-C-S, +C+S), which is consistent with the GUS pattern.

3.2.4 Map-based cloning of the mutants genes

The first mutant to be cloned was 11p34h, which was identified first, revealed that the three mutants are alleles to each other. The DNA samples used for mapping were screened by the treatment of soil grown F2 seedlings with –C-S liquid medium following by a quantification of GUS strength as shown in Table 3.2B.

At the first step, following the methodology reported (Lukowitz, 2000; Jander et al., 2002), we roughly mapped the mutation to the lower arm of chromosome I by a bulked segregant analysis of 62 DNA samples of homozygous F2: Ler x 11p34h (Fig. 3.5). After this, more Arabidopsis markers were carefully picked to narrow down the mutation to an 890 kb region downstream of marker nga 111. This region was flanked by the marker NGA 692, which was located inside the BAC clone F28O16, and another marker YUP8H12RM2, which was inside the clone YUP8H12R (Fig. 3.6A).

The fine mapping was carried out by both increasing the population of test samples and the genetic markers. All markers used and their corresponding primers are listed in Table 3.1. The mapping process was recorded briefly in Table 3.4A. Notice that the recorded numbers are recombination frequency (Rf%), which was calculated by the formula: Rf= [(number of heterozygous sample + 2X number of Ler sample) / 2X total number of sample]*100%. 387 DNA samples screened from 1959 F2 plants of LerX11p34h, the mutation inside the mutant 11p34h was finally mapped to an 81kb BAC clone F28K19 (Fig. 3.6A, and Table 3.4A).

Information from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/) indicated the existence of 25 putative genes in this region of F28K19. Two loci, At1g77900 and

At1g78000, which encodes the two sulfate transporters, *SULTR2;2* and *SULTR1;2* respectively, are among these 25 genes. The two sulfate reported genes were sequenced the because of the involvement with [what is S and C?] metabolism and balancing, and previous results that reveal a direct relationship with the reporter construct P_{GHF1}*::GUS* (Figure 2.8B). Primers used in this sequencing were listed in Table 3.1. The results revealed a point mutation on the 4th exon of the putative gene *SULTR1;2* (Figure 3.6B). The base replacement from G to A leads to a missense mutation of G208 to D208 on the transmembrane sulfate transporter SULTR1;2 (Fig. 3.7). The identified mutation 11p34h was renamed after the previously reported mutation on SULTR1;2 (Rose, 1997: Shibagaki et al, 2002) as *sel1-16* for mutation inside 11p34h. The mutation inside 11p101h and 11p201h was renamed as *sel1-15*.

In addition, a preliminary rough mapping was conducted for 11p201h, as shown in Table 3.4B. 106 DNA samples screened from F2 population of L*er* x 11p201h, for a direct test on the major markers applied in *sel1-16* (in 11p34h) gene cloning. The mutated locus was narrowed down to the same marker of F28K19M4 inside the BAC clone F28K19 as the *sel1-16*.

The another mutant, 11p101h, showed similar GUS patterns and are allelic to 11p201h and 11p34h, we decided to directly sequence *SULTR1;2* and *SULT 2;2* genes for these mutants. The results supported that both the two mutants harbored the allelic mutation with 11p34h, and more interestingly, both mutants contained the same point mutation at the 2nd exon of *SULTR1;2*, with

a base replacement from G to A, which led to a miss sense mutation from D108 to N108 on the transmembrane S transporter SULTR1;2 (Figure 3.7).

3.2.5 Functional complementation using transgenic lines

The sel1-15 and sel1-16 mutations typically strengthened the GUS staining under both +S and –S states (Fig. 3.3A and 3.8A). The endogenous reporter gene At2g44460 was detected to be up-regulated on the mRNA level, under both +S and –S conditions by qRT-PCR (Fig. 3.4C) and RT-PCR (Fig. 3.8B) tests. The mutants 11p101h and 11p201h were renamed as sel1-15, and 11p34h as sel1-16, after the map-cloning process. In order to genetically prove the GUS alteration by SULTR1;2 mutation, we transformed the DZ18 construct (Table3.1), which was originally used for GFP observation of WT SULTR1;2, into one of the cloned mutants, sel1-16 to check whether there might be a complementation between the WT and mutated SULTR1;2. Transformed T1 seeds were sowed on soil cups, and subjected to –C-S treatment with the method of half-cut root system. The seedlings with complemented GUS phenotype such as the weakened GUS staining like the WT, were transplanted back to soil cups and rescued for T2 seeds. Obtained T2 seeds were sowed on MS + 1% Suc plates, which were equivalent to +C+S medium and grew for 5 d before directly subjection for GUS staining. The four tested lines as shown in Fig. 3.8C displayed typical GUS staining as the WT. This complement test provided evidence to show that the mutations in SULTR1;2 are responsible for the enhanced At2g44460 expression phenotype in this mutant.

3.3 Discussion

The fact that the two point mutations are on the major sulfate transporter gene, *SULTR1;2,* led us to investigate more information about the transporter and transporter gene. It is easy to understand that functionally disrupted sulfate transporter will lead to the stunting growth (Fig. 3.2A), and many other symptoms similar to –S stress such as the induction of some –S-responsive genes (e.g. At2g44460) even under the S-sufficient conditions (Fig. 3.4C).

3.3.1 *sel1-15, sel1-16* point mutations lead to diverse alteration physiologically and genetically

As shown in Figure 3.2, *sel1-5* and *sel1-16* missense mutation lead to a typical –S stressed growth on soil cups, and also increase the GUS staining on cotyledons and true leaves mimicking a –S condition (Fig. 3.2B and C). The combinatory treatment with different conditions of C, N, and S statuses even showed a constitutive activation of the promoter of At2g44460 in the mutants (Fig. 3.3A). All these observations lead to the conclusion that these two missense mutations disrupted the sulfate transporting function of the high affinity sulfate transporter SULTR1;2. Or, more clearly, these two mutations lead to the malfunction of the transporter. The activation of the promoter of the –S-responsive reporter line (GHF1) gives us the strongest evidence for this conclusion. Not only this, the mRNA level of the endogenous gene of At2g44460 was also observed to be strongly up-regulated in the mutants under various nutritional conditions (Fig. 3.4). As shown by other reporters (Rose, 1997; Shibagaki et al, 2002; Maruyama et al, 2003; El Kassis et al., 2007), the sulfate transporter SULTR1;2's transporting function can be altered or totally wiped off by multiple point mutations and T-DNA insertions.

So what is the relationship between the two mutations identified in our lab with other reported mutation? The measurement of root length with several repeats and several batches of seeds available, displayed a complicated map for the effect of SULT1;2 mutation. As observed in Fig. 3.2D, all three mutants, or both the two mutations show the serious inhibition on the primary root extension in comparing to WT. This phenotype is just the opposite of the observed –S response (Nikiforova et al., 2003; Dan et al., 2007). This phenomenon can be explained from two aspects: The first explanation is that the mutation on *SULTR1;2*, or the malfunction of SULTR1;2, did not actually change the intra-cellular S level seriously, or even increased the S level by some mechanisms when gene mutated, so that the SULTR1;2 mutants displayed a +S phenotype. The 'constitutively' increased activity of At2g44460 is in favor of the hypothesis, because it putatively encodes a β-glucosidase which is responsible for decomposing the S-reservoir gulcosinolates. If this is the case, then even under the disruption of the SULTR1;2 transporting function, mutant cell may not be necessary in short of S source, sometimes there may be more provided, if the hydrolase activity increased very high. The second possibility is that the high affinity SULTR1;2 lost its sensing function after the mutations of *sel1-15* and *sel1-16*, so even the intra-cellular S level is very low, the sensor could find out that. So plants still take the phenotype of +S. In the latter case, SULTR1;2 serves not only as a transporter, but also as a sensor. There may exist other possibilities for this phenotype. More experiments will be needed to address this question that SULTR1;2 is only a sulfate transporter, or a dual-functional protein with the function of both transporter and sensor.

3.3.2 Various mutations on the same *SULTR1;2* gene

Before our experiment, there are already a few reported work on screening and characterizing mutants on *SULTR1;2*. Some of the previously reported *sultr1;2* mutants are identified through a medium containing selenate, which is a sulfate analogue but toxic to normal plants if the feeding concentration is too high as to 10-20μM. Arabidopsis with the mutation on *SULTR1;2* displayed a highly resistance to slenate, so they are designated as *sel1* mutants. There are 14 *sel1* mutants reported before our work. Among which, some are point missense mutations like the two isolated in our lab, such as *sel1-1* (S96F), *sel1-3* (G509E) (Rose, 1997), and *sel1-8* (I511T) (Shibagaki et al, 2002); some are T-DNA insertions, such as *sel1-9* with a T-DNA insertion in the promoter regions (Shibagaki et al, 2002), *sel1-10* with a T-DNA insertion at 10 bp upstream the junction of the 9th exon and intron (Maruyama et al, 2003). Other *sel1* mutants with different deletions on the *SULTR1;2* gene, such as *sel1-11*, *sel1-13*, which deleted 7 bp at the beginning of the 2nd exon; *sel1-12, sel1-14* processed a 65 kb chromosomal deletion, among which was the deletion of the whole *SULTR1;2* gene (El Kassis et al., 2007).

Except the most obvious characteristic of growing well at the selenate high concentration medium, the previously isolated *sel1* mutants generally disrupted their sulfate uptake capacity (Shibagaki et al, 2002; El Kassis et al., 2007), retartedly grew as observed in our experiments, reduced the abundance of sulfate and S-containing compounds such as Cys, Met and GSH, and activated the alternative sulfate transporters, such as the SULTR1;1 (Maruyama et al, 2003; El Kassis et al., 2007). We rename our newly identified mutant from11p101h and 11p201h as *sel1-15,* and the mutant from 11p34h as sel1-16 based on previously established nomenclature. As for the study of structural versus functional mechanism on SULTR1;2, more interests are focused on the C-terminus of SULTR1;2 polypeptide, the STAS (sulfate transporter and anti-sigma factor

antagonist) domain (Shibagaki and Grossman, 2004; Rouached et al., 2005; Shibagaki and Grossman, 2006; 2010), which was identified to be important in protein-protein interaction, S-metabolism regulation and sulfate transportation. But what is the role of the N-terminus? The presently accumulated 16 *sel1* mutants may provide a very good tool in understanding the function of the N-terminus of this important transporter.

3.3.3 More information about the reporter gene, At2g44460, and its putatively encoded protein, a β-glucosidase

Preliminary results from searching The Arabidopsis Information Resource (TIAR: http://www.arabidopsis.org) database indicates that the locus At2g44460 is at chromosome 2 from the location 18,346,439 to 18,349,867, with a length of 3.528kb (last updated at 04/22/2008). Tissue-specific expression profile from GENEVESTIGATOR(Zimmermann et. al., 2004) shows that this gene can be expressed in almost every organ of Arabidopsis. Among which callus expressed the highest level, followed by cell culture, seed coat, lateral root, and root cortex (last updated at 07/20/11).

A few microarray reports can be found about At2g44460, where it was strongly responsive to –S stress, i.e., it was highly induced by –S (Maruyama-Nakashita et al., 2003; Hirai and Saito 2004; Zimmermann et. al., 2004). At2g44460 is also the most strongly induced among all –S responsive genes with an induction of 5.8×10^4 fold in a –S treatment reported by Maruyama-Nakashita et al., 2003. Another interesting report is that At2g44460 was also strongly responsive

to the floral regulatory gene *LFY* in angiosperms, where it was highly induced by LFY and its angiosperm homologs UNI and ALF, respectively (Maizel et al., 2005).

The putative protein encoded by At2g44460 is β-glucosidase 28 (BGLU28) (Xu et al., 2004), which belongs to glygoside hydrolase, family 1. This putative protein has a length of 582 amino acid residues. Some other synonyms are thioglucosidase, myrosinase (TAIR), among which thioglucosidase was a more commonly used term concerning gene At2g44460. The study of SLIM 1 transcription factor linked At2g44460 strongly with the –S-responses, where the putative protein thioglucosidase (myrosinase) expression level was largely elevated so as to decompose more glucosinolates (GLSs), the important sulfate sink inside Capparale cells (Maruyama-Nakashita et al., 2006). Thioglucosidase and its catalized substrate, GLS serve in many Capparale plants in the defensive mechanism against pathogens and herbivores. This mechanism is termed as the 'glucosinolate-myrosinase system', or sometimes called the 'mustard oil bomb' (Wittstock and Halkier 2002; Grubb and Abel, 2006), because it induces a rapid releasing of sulfate from glucosinolate during –S. This rapid release of sulfate during –S can also serve as an alternative sulfate source for S assimilation when –S (Maruyama-Nakashita 2003, Hirai and Saito 2004). However, the exact biochemical function of At2g44460-encoded protein remains to be investigated.

Table 3.1 Primers used in the cloning of the *sultr1;2* mutant gene and related experiments.

Mapping	BAC clones	Name of markers	Name of primers [b]	Primer sequences (5'→3')
	F14J22	ciw1	ciw1S	ACATTTTCTCAATCCTTACTC
			ciw1A	GAGAGCTTCTTTATTTGTGAT
	F14J16	NGA280	NGA280S	CTGATCTCACGGACAATAGTGC
			NGA280A	GGCTCCATAAAAAGTGCACC
	F5I14	NF5I14	NF5I14S[c]	GGCATCACAGTTCTGATTCC
			NF5I14A	CTGCCTGAAATTGTCGAAAC
	F28P22	NGA111	NGA111S[c]	TGTTTTTTAGGACAAATGGCG
			NGA111A	CTCCAGTTGGAAGCTAAAGGG
	F28O16	NGA692 (14) [a]	NGA692S[c]	AGCGTTTAGCTCAACCCTAGG
			NGA692A	TTTAGAGAGAGAGAGCGCGG
	T14N5	T14N5 (7)[a]	T14N5M1S	GCTGCAGGTAGATAAACATTTC
			T14N5M1A	AATTTGAACTGTCACCTGGCTC
	T5M16	T5M16(5)[a]	T5M16M1S	GACGCGGCTAATTTAGGAATCA
			T5M16M1A	CCCTCGAGAGCAATGTAATAGT
	T32E8	T32E8(2)[a]	T32E8M1S	GAGTAGTTTAAGTCTCATCCTG
			T32E8M1A	GCGTGGCCAAAGAAGGATAATG
	F28K19	CER451736 (2)[a]	F28K19M1S[c]	GTTCTCTCTCAGATTCATCTTC
			F28K19M1A	CAGAGGAAGTAGAATATATTGGC
		CER451737(2)[a]	F28K19M3S	TTACTGAGTTTGAACAAGACTCC
			F28K19M3A	TTCCTCCTCTATAGGAAACAACC
		CER451738(0)[a]	F28K19M5S	TGGCTTTTTCTAGTGTCTTCTCT
			F28K19M5A	AGCGCCGAATTCATATTTGGATA
		CER451739(0)[a]	F28K19M4S	GAAAAGAGAACGTATTATTTTTCA
			F28K19M4A	TTTAACTTGTGCAAGTTGATGG
		CER451742(2)[a]	F28K19M2S	CATAAGCTGGTCATAACCGCTTAG
			F28K19M2A	TAATAATAATAGACCACGAGG
	T11I11	470559 (6) [a]	T11I11M1S[c]	GCGTTAAATGATGTACGATCAGGT
			T11I11M1A	GTCTGCCGGAAAAACATACTATAG
	F3F9	470249 (10)[a]	F3F9M4S[c]	CCTCCTCATCCAAAACTATACCAA
			F3F9M4A	CCTTGTGCTGGATAAGGTATGCAC
	YUP8H12R	CER451530	YUP8H12RM2S[c]	TGAATCATACTAATACAGAAGGAA
			YUP8H12RM2A	ATTACGAGTTGTCTAATTTCTCCG
Sequencing	**Gene ID**	**Positions on Chromosome 1**	**Name of primers**	**Primer sequences (5'→3')**
	AT1G77990.1	29322718	**DZP121**	5'GAA TAT TTT GTA GCC TTA TGC AT
			DZP122	5'TAC CTT TGA AAA ACT GGC TGT G
			DZP123	5'TAT TTC TAA TGG CAT ACC TAT GT
			DZP151	5'GTT TAT AAA AAA TGG ACA TGC CT
			DZP152	5'AAA GTT CAG AGG TTG CCA CTG
			DZP153(A)	5'ACC AAT TGT AAT CGG TAT ATG TC
			DZP124(A)	5'TGG GAC ATA ATT AGG AGT ATC AT

	AT1G78000.1	29334602	**DZP125**	5'AAG CTT CAT CAA TGT TGC TCC A
			DZP154	5'TTT GAT TTT GAC ATG CTC ACA CT
			DZP155	5'CCT TCT CAC GTC TAA GAT CAT T
			DZP156	5'CGT AGT TGG ATC GAT GTC TTC
			DZP126 (A)	5'AGA ATC ATA AAG AAA GGG TCC A
Construct	Inserted Gene name	Name of primers	Sense (S)/ Antisense (A)	Primer sequences (5'→3')
DZ18	*SULTR1;2* CDS	DZP190	S	GAAGATCTATATGTCGTCAAGAGCTCACCCTG (*Bgl* II)
		DZP193	A	TGCTCTAGAGACCTCGTTGGAGAGTTTTGGA (*Xba* I)

Notes: [a] The numbers after the marker names indicate the number of recombinants. The *sultr1;2* mutant gene is mapped to a region between the markers, CER451738 and CER451739 on the BAC clone F28K19. [b] In the mapping category, primer name ending with "S" denotes "sense", while "A" denotes "antisense". In the sequencing category, antisense sequences are labeled following the primer names as (A). [c] Primers are from previously reported experiments (Gao, 2008).

(Table 3.1 continued)

Table 3.2 F2 segregation of 11p34h

A. F2: GHF1 X 11p34h GUS strength on roots of 21 seedlings (08/08/07)
(The expected homozygous mutants will be 1/4 of the tested population based on the ratio of 3:1)

GUS level	0	1	2	3	4	5	6	7	8	9	10
Number of GHF1	1	1	4	0	0	0	0	0	0	0	0
Number of F2	6	5	1	1	2	1	2	1	1	0	1
Sum up to show the ratio of WT: Mutants phenotype in F2	16 (Number of seedlings like WT)						5* (Number of seedlings like mutant)				
Expected if monogenic mutation	15.8						5.2				

* chi-sqaure value based on an expectation of 3:1 segregation is : 0.06

B. F2: L*er* X 11p34h GUS strength on roots of 125 seedlings (01/28/08)
(The expected homozygous mutants will be 3/16 of the tested population based on the ratio of 9:3:3:1)

GUS level	0	1	2	3	4	5	6	7	8	9	10
Number of GHF1	0	2	1	2	0	1	0	0	0	0	0
Number of F2	32	23	16	9	12	6	3	9	8	5	2
Sum up to show the ratio of WT: Mutants phenotype in F2	98 (Number of seedlings like WT)						27 ** (Number of seedlings like mutant)				
Expected if monogenic mutation	101.6						23.4				

** chi-sqaure value based on an expectation of 9:3:3:1 segregation is: 0.54

Table 3.3 F2 segregation of 11p101h and 11p201h

F2 type	Test medium	Test date	Phenotypic ratio WT/mutants	Expected ratio*	Chi-square value**
F2: GHF1x11p101h	-C-S	10/17/09	141/43	138/46	0.20
F2: GHF1x11p201h	-C-S	10/23/08	51/15	49.5/16.5	0.14

*Expected ratio if mutation happens monogenetically, i.e. 3:1. ** chi-sqaure value was calculated based on an expectation of 3:1 segregation.

Table 3.4 Map-based cloning of 11p34h mutant gene (A), and a preliminary mapping of 11p201h gene (B)

- Data of recombination frequency (Rf%), which is calculated by the equation of: Rf= [(number of heterozygous sample + 2X number of L*er* sample) / 2X total number of sample]*100%.

A

Marker	ciw1	NGA280	NF5I14	NGA111	NGA692	T14N5M1	T5M16M1	T32E8M1	F28K19M1	F28K19M4	F28K19M2	T11I11M1	F3F9M4	YUP8H12RM2
62 DNA samples	37.7*	25.4	13.9	4.9	3.3	3.3	3.3	3.3	3.3			3.3	3.3	3.3
103 DNA samples				2.4	1.9	1.9	1.9	1.9	1.9			3.4	3.9	3.9
158 DNA samples				3.2	1.9	1.9	1.6	1.6	1.6			2.2	2.8	3.7
185 DNA samples					2.7	1.9	1.6	1.4	1.4			2.4	3.0	
307 DNA samples					1.8	1.0	0.8	0.3	0.3			0.8	1.3	
367 DNA samples					1.9	1.0	0.7	0.3	0.3	0	0.3	0.8	1.4	

B

Markers	NGA111	NGA692	T14N5M1	T5M16M1	T32E8M1	F28K19M1	F28K19M4	F28K19M2	T11I11M1	F3F9M4
51 DNA samples	4.9	3.9	4.9	4.9	3.9	2.9	2.9	2.9	3.9	4.9
106 DNA samples		1.9					1.4			2.4

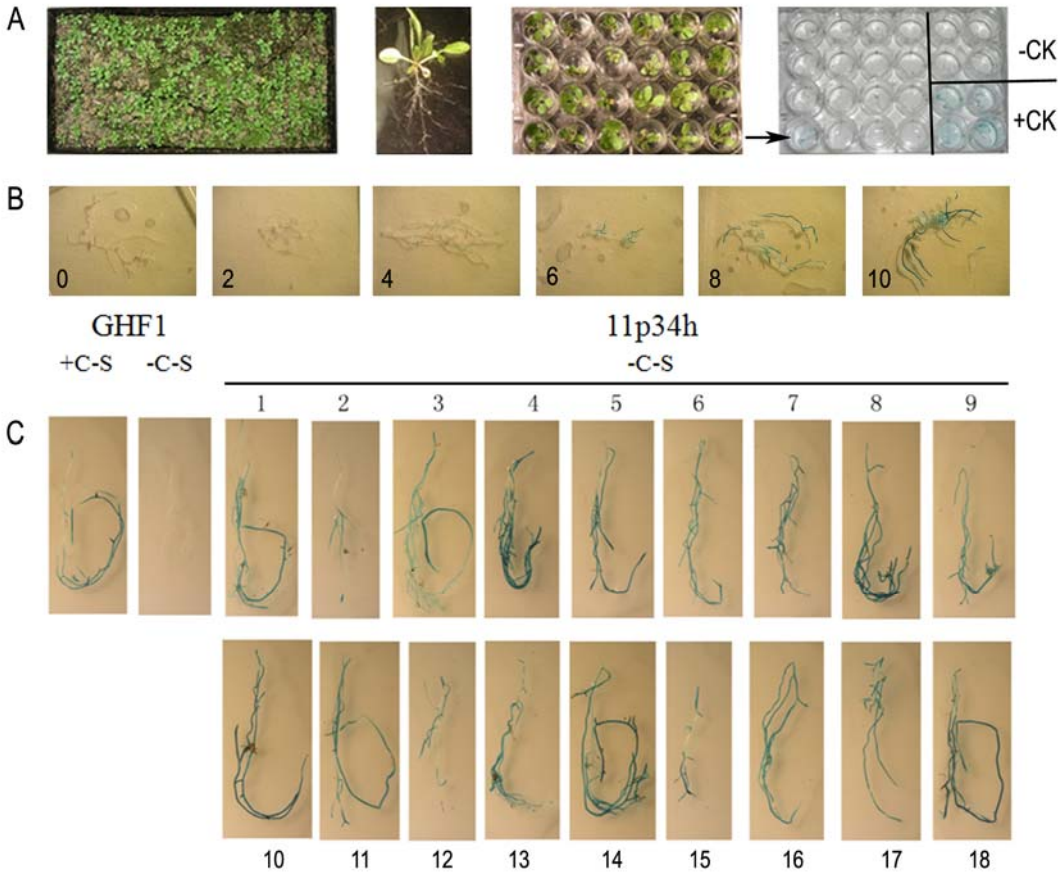

Figure 3.1 Screen mutants from M2 population

A. M2 mutagenized seeds were sowed on soil tray for 20d growth (shown as the first picture). Seedlings were rinsed (2nd picture) and treated with the C minimum medium (-C-S: of 2mM C, 0.0001mM S) for 24h (third picture), following by GUS staining of half cut root system from the treated seedlings (the last one). The WT, GHF1 was used both as –CK (treated with the same C minimum medium) and +CK (treated with +C-S medium: of 60mM C, 0.0001mM S). The well with arrow indicated shows a possible mutant with altered –C-S response. Any mutants screened by this method can be rescued by transplanting the treated seedlings back to soil.

B. Standard developed for rapid GUS strength quantification in this project. 11 levels of GUS strength are designated from 0, which represents no GUS signal, to 10 of 100% stained. Number 1-9 represent the increasing level from low to high GUS signals. Shown here are 6 representatives. Number inside picture shows the corresponding level of GUS strength.

C. The first isolated mutant, 11p34h shows a typical C-hypersensative response under –S stress. 11p34h was first isolated from M2 population with a strong GUS staining under –C-S medium and was rescued to obtain the M3 generation. M3 seeds were sowed on soil cups and seedlings were treated as in Fig. 3.1A. 18 treated seedlings display an inheritable C-hypersensitive –S response as comparing to –CK.

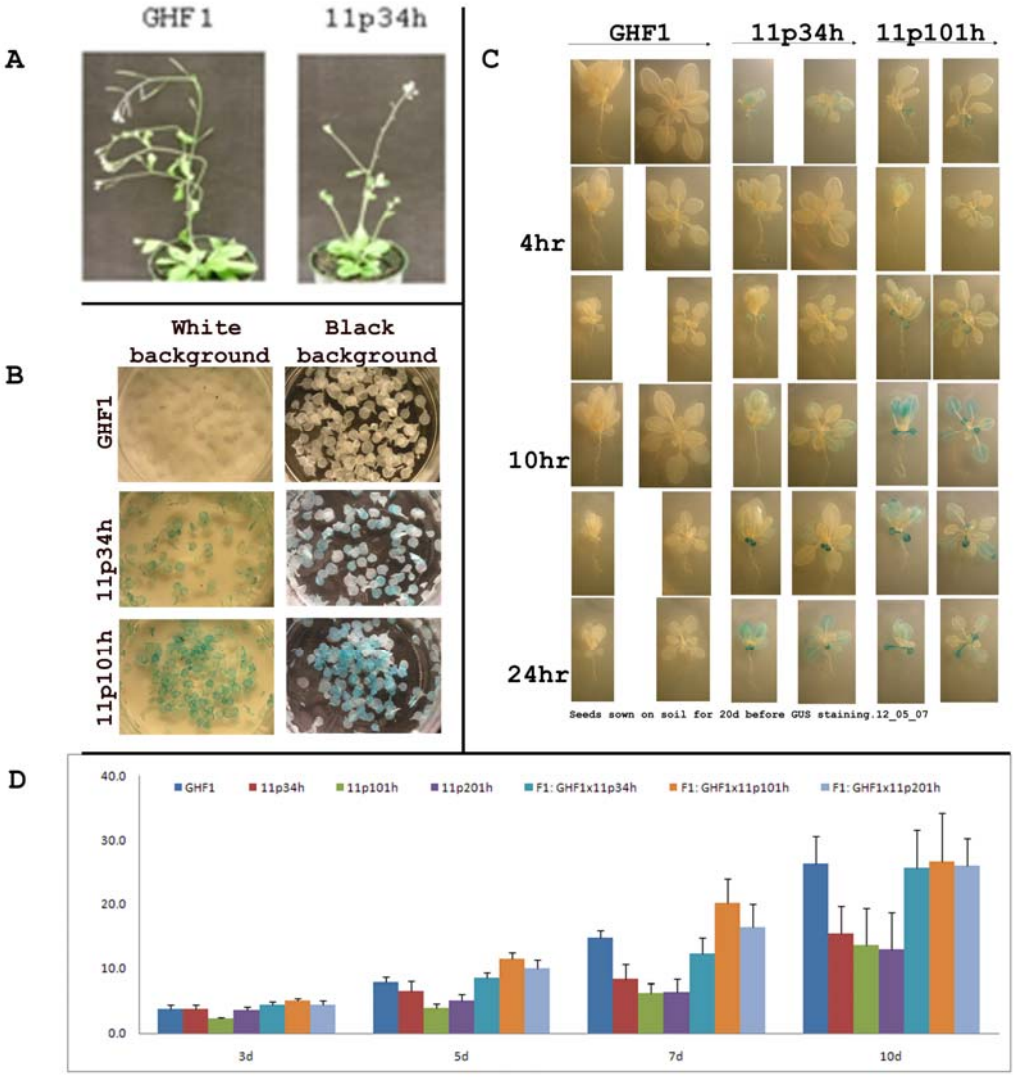

Figure 3.2 Morphological characterization of screened mutants

A. Plants with 38d growth, showing the difference between WT and mutant.

B. Cotyledons from seedlings of 20d growth on soil cups, showing the GUS staining difference between WT and mutants. Number of seedlings used for this test is 179 for GHF1, 171 for 11p34h, and 201 for 11p101h. The left panel is pictured with a white background, while the right panel is on a black background of the same samples as left.

C. Whole seedling of 20d growth on soil cups, showing the GUS staining difference between WT and mutants. The labeled time indicates the GUS staining time. It is obvious that the longer the staining, the stronger the true leaves were stained in mutants. But true leaves of WT will not be stained even after 24h staining.

D. Root length (in mm) of seedlings on MS plates, showing the difference between WT and mutants. The repression of root growth in mutants was complemented by WT in F1 generation. Genotypes used here are (in order as shown in the graph): WT (GHF1); Mutants (11p34h, 11p101h, 11p201h); and F1 (GHF1x 1134h, GHF1x11p101h, GHF1x11p201h). Data shown here are for 3d, 5d, 7d and 10d respectively.

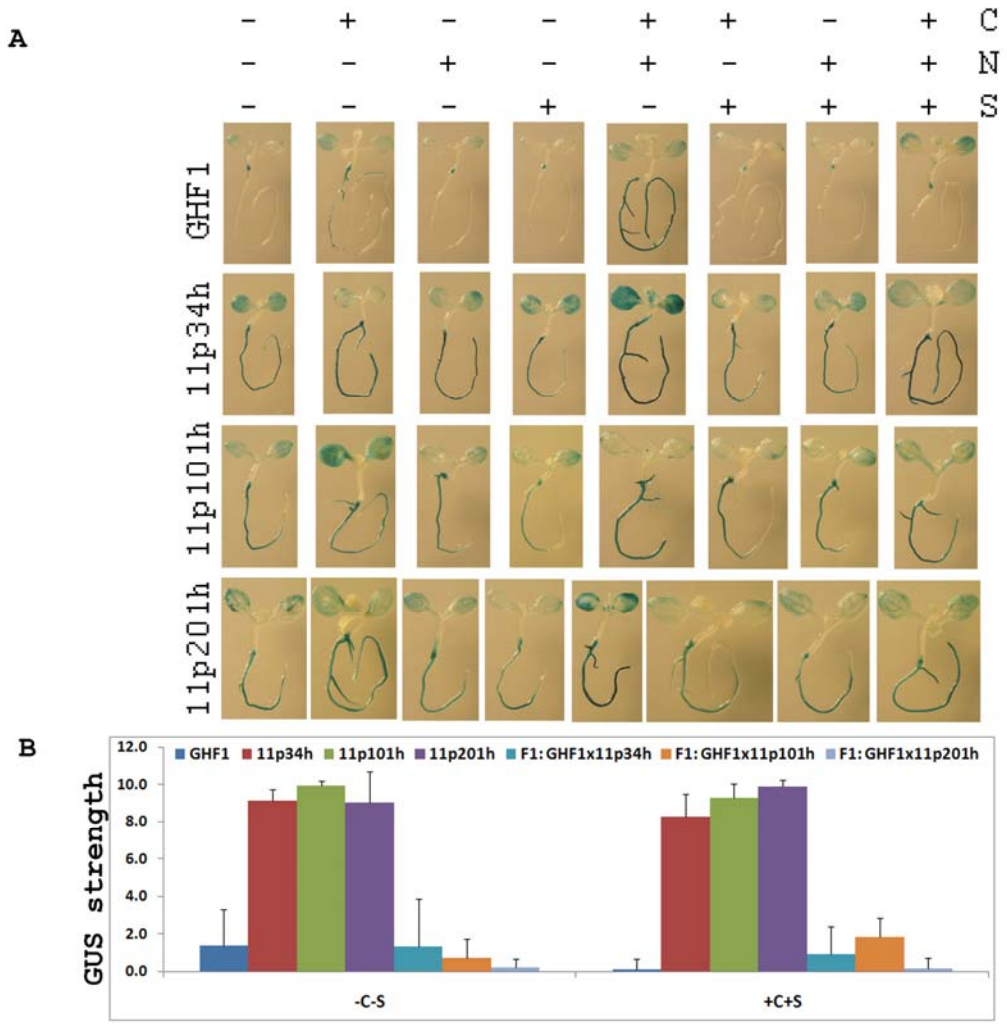

Figure 3.3 GUS responsive patterns under different nutritional conditions

A. Three mutants responded to the eight combinatory nutrients with different C, N, and S status. Seeds were sowed on MS plates for 2d cold-treatments before vertically incubated in programmed growth chamber. Seedlings after 5d growth were treated with the indicated 8 liquid nutrients for 1d, and then GUS stained. –C, -N, -S represent the deficiencies of C (0.2mM sucrose), N (0.2 mM total N), and 0.0001 mM SO_4^{2-} (from $CuSO_4$), respectively. +C, +N and +S indicate the presence of 60 mM sucrose, 60 mM total N (20 mM KNO_3 and 20 mM NH_4NO_3), and 1.6001 mM SO_4^{2-} respectively.

B. Results of another experiment, showing the three mutants responded to the two commonly used nutrient statuses: -C-S and +C+S. And more importantly, F1 generation of the three mutants were used to show the genetic complementation. Seeds were sowed on MS plates and vertically incubated as in Fig. 3.3A, but seedlings were incubated for 10d before the 1d treatment. GUS strength was evaluated as described in Fig. 3.1B. Number of seedlings used for GUS strength evaluation was between18-34.

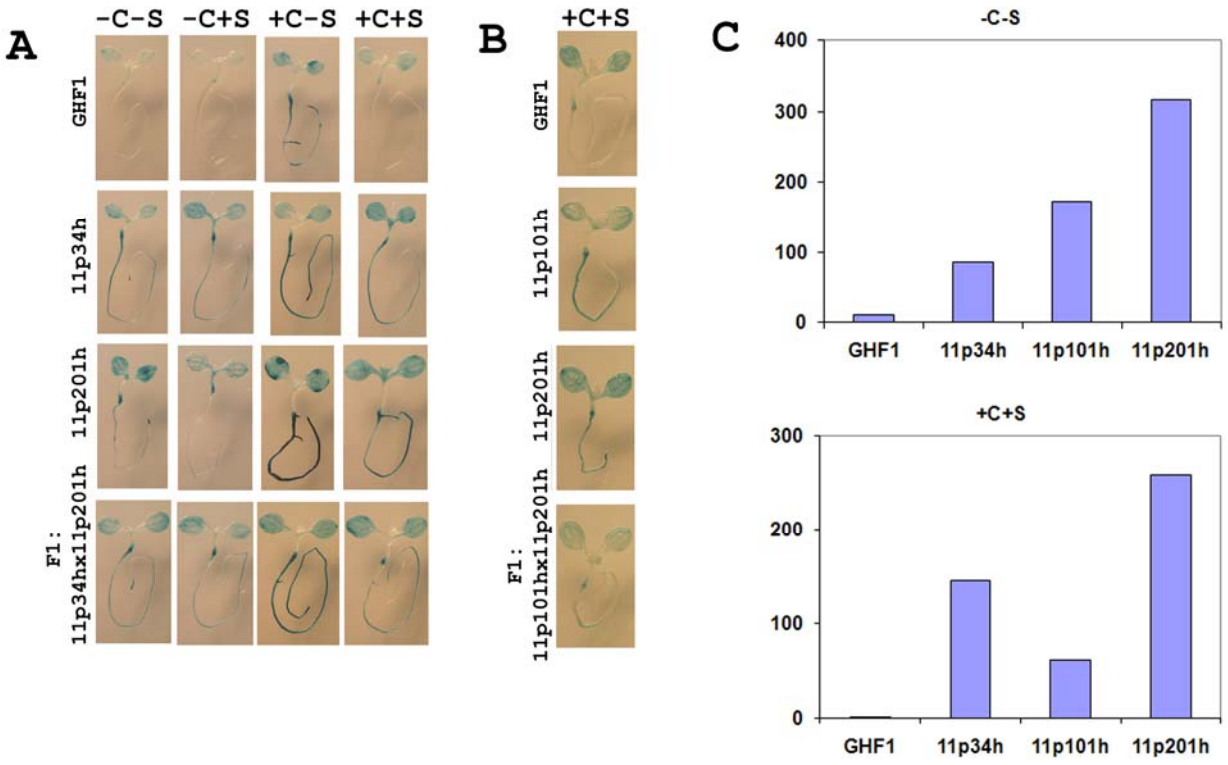

Figure 3.4 Complement tests and qRT-PCR test on the endogenous gene, At2g44460

A. Complementary test of 6/18/08: WT, the two mutants and their F1 generation were sowed on MS plates. After 5d growth, seedlings were treated by the four indicated nutrients for 2d. Then GUS stained.

B. Another complementary test between 11p101h and 11p201h conducted at 12/10/07. Seeds were germinated and grew, treated and GUS stained the same as above.

C. qRT-PCR tested the endogenous reporter gene, At2g44460 under two nutritional conditions. Experiment was conducted at 11/30/07. Seeds of WT and mutants were sowed on MS plates and incubated for 5d as above. After that, seedlings were subjected to liquid treatment as indicated for 2d. Method of RNA extraction, first-strand cDNA synthesis, and qRT-PCR is described in the text.

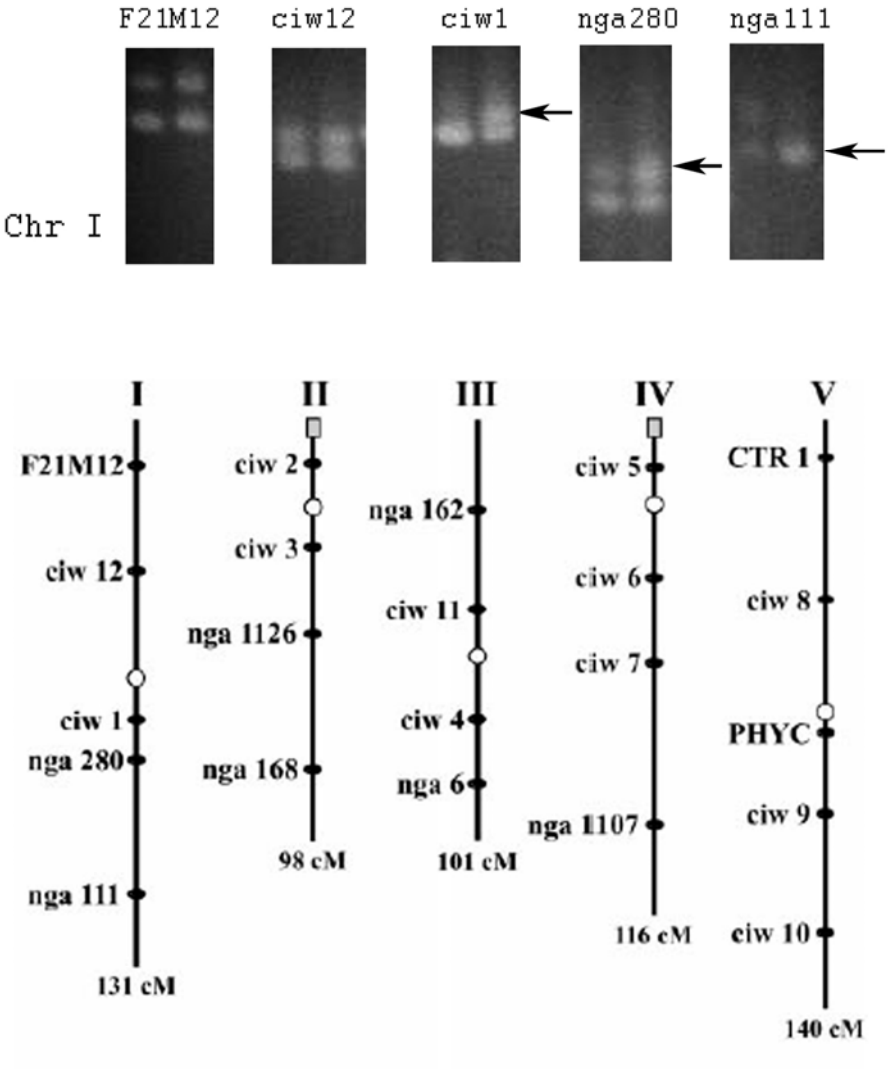

Figure 3.5 Bulked segregant analysis of 11p34h mutation.
The upper panel displays the experimental results. The three arrows indicate the strengthened Col-specific bands, showing the linkage of mutation with these three markers on Arabidopsis chromosome I. The lower panel shows the schematic diagram of the 22 markers used in this experiment, the mutation-linked markers, ciw 1, nga 280 and nga 111, are on the lower arm of chromosome I (picture copied from Lukowitz et al., 2000).

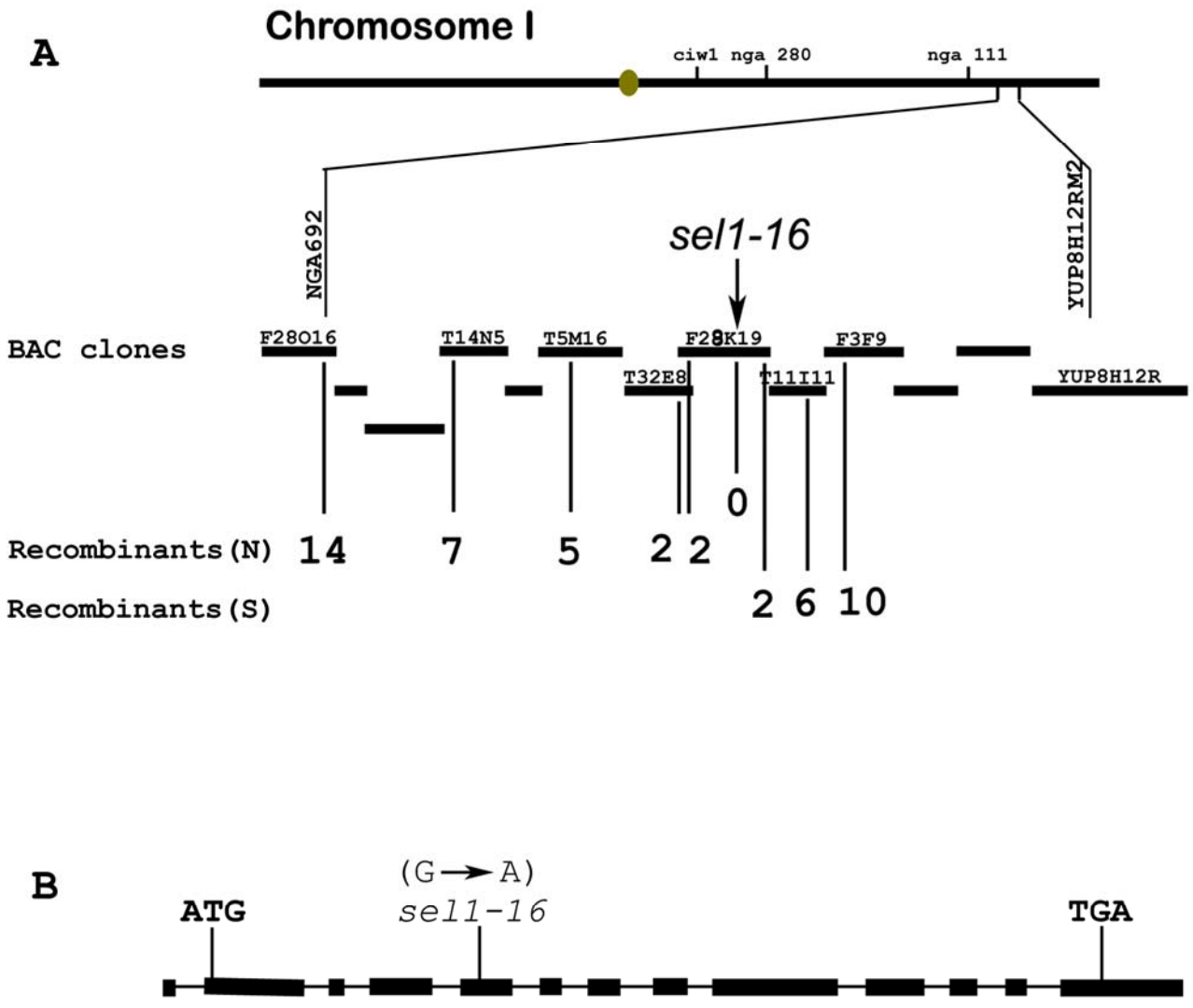

Figure 3.6 Molecular cloning of the mutant gene in 11p34h.

A. A diagram showing the mapping of mutated locus *sel1-16*. The mutation was first narrowed down to a 890 kb region in the down-stream of nga 111 on the lower arm of Chromosome I. This region was flanked by the two markers, NGA 692 and YUP8H12RM2, locating in BAC clone F28O16 and YUP8H12R, respectively. All primers used here and their corresponding clones are listed in Table 3.1. The number of recombinants north (N), or south (S) of the mutation is indicated under each marker tested. This number is the recombinant number out of the totally tested population of 387.

B. A schematic representation of the *SULTR1;2* gene structure. The mutation *sel1-16* and the start codon (ATG) and stop codon (TGA) are indicated.

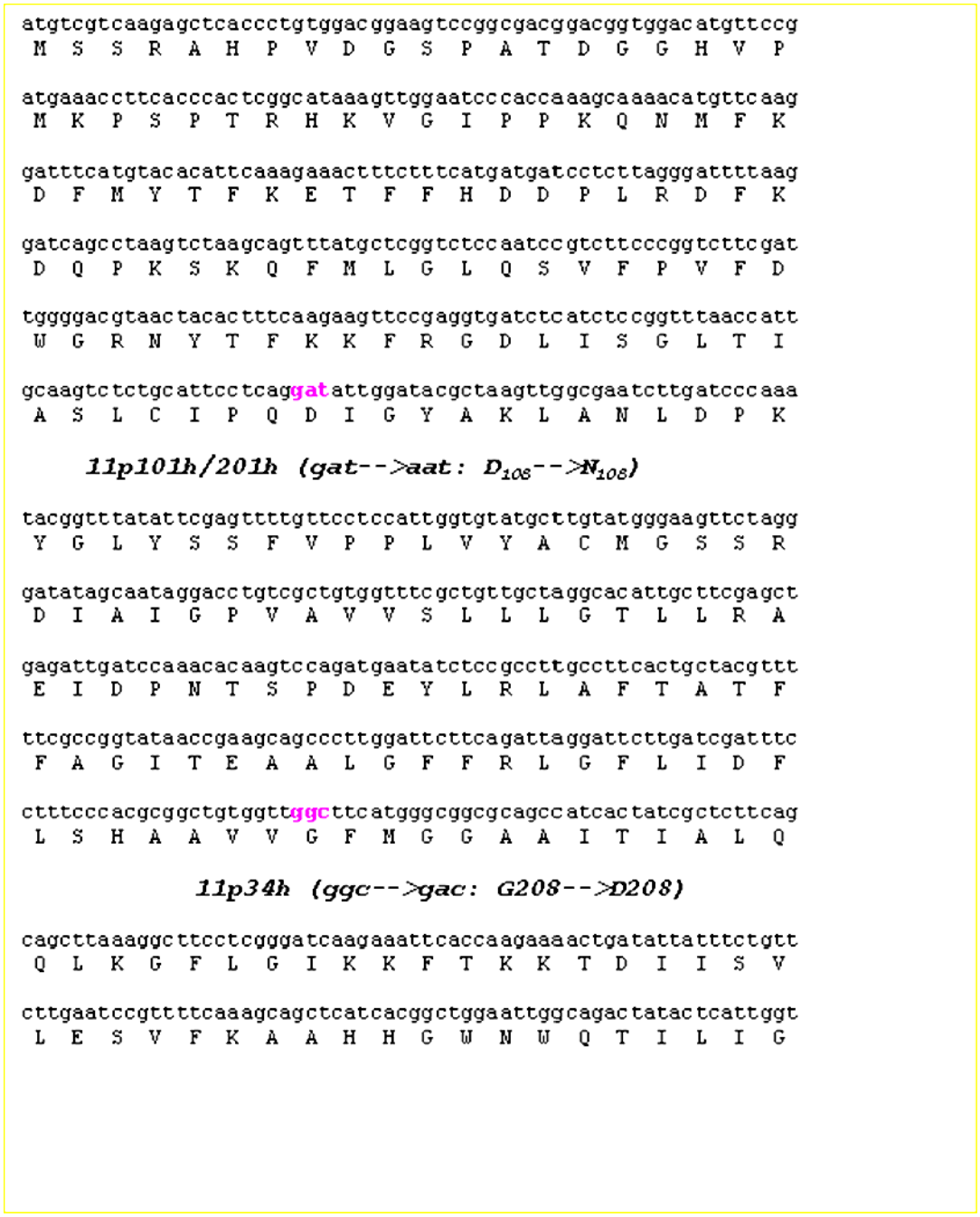

Figure 3.7 Map Cloning of the mutant gene in 11p34h, and two three related mutants

Displayed in this picture is the first half part of the 2 kb cDNA of *SULT1;2* with its protein sequence aligned. The two mutated loci, *sel1-15* (in 11p101h and 11p201h) and *sel1-16* (in 11p34h), are indicated.

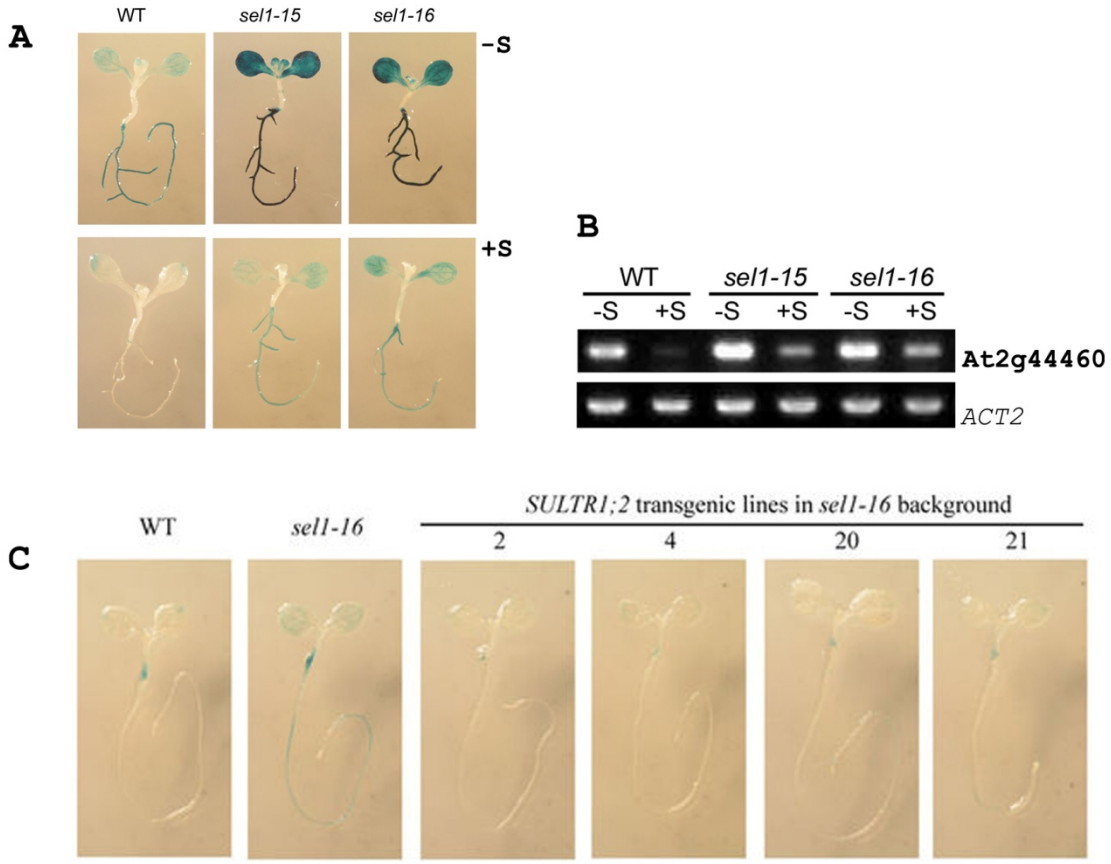

Figure 3.8 *SULTR1;2* transgenic mutant lines complemented the GUS and gene alteration of *sel1-16* mutation.

sel1-15 and *sel1-16* was characterized in altering the GUS strength and the reporter gene At2g44460 as shown in A and B. Figure C shows a complement test by using 4 transgenic lines that harboring the WT *SULTR1;2* CDS, and display a typical GUS staining as WT, instead of their parental line, *sel1-16*. The CDS cloning and plant transformation were described in the text. The four lines, #2, #4, #20 and #21 shown here are T2 transgenic lines of *sel1-16*. T2 seeds were sowed on MS+1%Suc (equivalent to +C+S medium) plates and incubated for 5d, then directly subjected to GUS staining.

Chapter 4

Evidence for SULTR1;2 to function as a sulfur sensor

Our proposed work is to screen some putative signaling molecules, such as some transcription factors, to analyze the molecular mechanism that involved in the carbon, nitrogen and sulfur (C-N-S) nutritional interactions. The starting WT, GHF1 was characterized to be responsive differentially to C, N and S status. The mutant screening strategy was designed to isolate those mutants with disrupted nutritional responses, especially with hypersensitive C response. Interestingly the three isolated mutants (11p34h, 11p101h and 11p201h) in this project were finally mapped into a single gene, *SULTR1;2*, which is known to be functioned in sulfate transport. It is unclear if SULTR1;2 has any signaling function in the cell. By using some genetic strategies, the goal was identify its transporter capability by using the classic yeast complementation strategy, and to identify some of the signaling possibilities of this transporter. Finally, confocal imaging GFP-trangenic mutants were use to determine and analyze the SULTRA1; 2 protein.

4.1 Materials and methods

4.1.1 Plant materials and nutrient treatment

Unless noted, all experiments concerning plants growth and seedling treatments follow this protocol: *Arabidopsis thaliana* ecotype Columbia (Col), a transgenic line designated GHF1 which has the At2g44460 promoter:*GUS* construct (Dan et al., 2007), and the various mutants derived from this transgenic line were grown in greenhouse for seed collection. For seedling growth in the petri-dish, seeds were sown on agar-solidified half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose, cold-treated at 4°C for 2 days and then vertically incubated at 25°C for 5-7 days under 16 hours light and 8 hours dark. After rinsing with distilled water 3 times, seedlings were then transferred to medium containing different concentration of sulfate. As described previously (Dan et al., 2007), the –S medium contained 0.0001 mM sulfate from $CuSO_4$, and +S medium included 1.6 mM sulfate.

4.1.2 Yeast complementation.

The *sul1 sul2* double mutant (Cherest et la., 1997) of *Saccharomyces cerevisiae*, cp154-7A , (MATa{*leu2, trp1, his3, ura3, ade2, sul1::LEU2, sul2::URA3*}), and its wild-type strain W303-1B (MATa{*leu2-3, 112trp1-1, his3-11, 15ura3-1,can1-100,ade2-3*}) were used. Coding Sequence (CDS) of wild-type *SULTR1;2* and mutant forms of *SULTR1;2* (*sel1-15, sel1-16*, and *sel1-8*) were amplified with primers DZP190 and DZP191 (Table 4.1) using PCR, and cloned into the galactose-inducible expression vector pYES2, which was modified to contain a *TRP1* gene as a selective marker. The constructs made from this process were named: DZ38, DZ39, DZ41, and DZ42, which contained CDS from *sel1-16, sel1-15*, Col, and *sel1-8*, respectively. *sel1-8* is a *SULTR1;2* allelic point mutant from previously reported source (Shibagaki et al, 2002), which was used here as a control. The modified pYES2, which contained *TRP1*, was transformed into W303-1B and CP154-7A through electroporation, as an empty vector control.

Other expression constructs (DZ38, DZ39, DZ41, and DZ42) were transformed into CP154-7A following procedures described previously (Becker and Guarente, 1991). For yeast growth on agar plates, transformants were streaked on Trp deficient, minimal sulfate (0.05 mM) agar with or without 0.4 mM Met and the presence of 2% glucose or galactose. For quantitative analysis of yeast growth, liquid culture was used, following the procedure described in (Shibagaki and Brossman, 2004). Briefly three independent transformants for each genotype, which were first grown in the Met-containing medium, were recovered and diluted into 2 ml of 0.05 mM sulfate containing medium in the presence or absence of 1 mM Met, with a starting A600 of 0.04. A600 was monitored every 8 hr and doubling time of yeast growth was derived.

4.1.3 Construction of GFP fusuion proteins and observation of protein subcellular localization

The full length CDS of *SULTR1;2* from wild type *Arabidopsis thaliana* Col and the two mutants *sel1-15, sel1-16* were amplified by PCR using primers, DZP190, DZP191, and DZP193, with specific restriction sites (Table 4.1 and 4.4). The PCR products were digested with the specific restriction enzymes and ligated to the two vectors, GZ0 for GFP-SULTR1;2 (N-terminal GFP fusion) construct, and GZ6 for SULTR1;2-GFP construct (C-terminal GFP fusion) with CaMV 35S promoter and terminator. The two vectors used were modified constructs from pCAMBIA B8 with Kan^R in *E. coli* and *BAR* in plants (Yang et al., 2007).

The six GFP-constructs were introduced into *E. coli* DH5α strain (Table 4.4). The mutations of *sel1-15*, *sel1-16* in different constructs were further identified by sequencing (data not shown). The plasmid constructs were identified, verified, and introduced introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis thaliana* Col by the floral dip method as described (Clough and Bent, 1998). The first generation seeds harvested were defined as T1, which were sown on soil trays and screened with herbicide (Finale ®) for three days' spraying treatment. Screened T1 seedlings were transplanted to soil cups for the next generation (T2) seeds, which were applied for protein localization.

The procedures for plant material preparation are: F2 seeds were sown on MS +1%Sucrose (Suc) +25μg/mL Finale® plates and vertically incubated in the programmed growth chamber at 22 °C with 16 hr light and 8 hr dark. 5d seedlings were transformants with selective marker, *BAR* gene and ready for GFP observation. GFP signal observation was performed on a Leica TCS SP5 confocal laser scanning microscope system, which was driven by a LAS AF software. The excitation laser line was Argon 488 with an intensity of 20%, and corresponding to an emission peak at 509 nm. The GFP fluorescence signal was collected between 500-530nm. The micrograph of fluorescent, visible and the overlap of both were stored. A lambda (λ) scan of each collected micrograph was performed to guarantee the collected fluorescence signal was from GFP, which has an emission peak at 509 nm, instead of other sources. For each observation, at least 10 cells with uniformed localization were obtained and relative pictures and videos are filed.

4.1.4 Real-time RT-PCR analysis.

The RNA was extracted using TRIzol and reverse transcribed using SuperScript III (Invitrogen). Quantitative PCR was performed in SmartCycler II (Cepheid, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, USA), according to the protocols provided by the manufacturers. *ACT2* was used as reference gene (Xin et al., 2009), and all of the gene-specific primers were listed in Table 4.1.

4.2 Results

4.2.1 Structural prediction of SULTR1;2

From the identification of this gene in1997, *SULTR1;2* has been the most studied among several known sulfate transporter genes because it is the predominant protein in Arabidopsis root tissue that encodes a sulfate transporter absorbing more than 80% of sulfur source from environment (Yoshimato et al., 2002; Buchner et al., 2006; Rouached et al., 2009). The phylogenic analysis of protein sequences for the 12 sulfate transporters in Arabidopsis reveals the closest similarity between SULTR1;2 and SULTR1;3, which is another high-affinity sulfate transporter (HAST) proposed to be localized in phloem and functioning in source-to-sink S transport (Fig. 4.1A)(Yoshimoto et al., 2003; Davidian and Kopriva, 2010). The third group I sulfate transporter, SULTR1;1, which was proposed to be co-localized with SULTR1;2, shared only 72.6% sequence similarity with SULTR1;2 (Yoshimato et al., 2002), which is obviously less

than that between SULTR1;3 and SULTR1;2 (83.8%). The relationship may to some extent explain why SULTR1;1 and SULTR1;2 work together at the same location but contribute differently to sulfate absorption in the cells, SULTR1;1 works synergistically whereas SULTR1;2 predominantly.

SULTR1;2 encodes a 653 amino acid, high-affinity sulfate transporter. The two alleles cloned in this project change the amino acid sequences close to the N-terminus (Fig. 4.1B), *sel1-15* (D108N), *sel1-16* (G208D). The comparisons of these two mutation sites (D108N, G208D)with the corresponding positions of the 11 other Arabidopsis SULTR proteins (Fig. 4.1B) revealed that D108 residue is conserved in the group I transporters, while G208 is conserved in all of the SULTR proteins. SULTR1;2 has been described as a transmembrane (TM) protein with 12 membrane-spanning domains (MSDs), which was based entirely on transmembrane prediction algorithms, not on any direct experimental evidence. Multiple online algorithms are available for prediction of membrane protein topology. Demonstrated on Fig. 4.1C is one of those calculated model (Jones et al., 1994; Jones, 2007; Nugent and Jones, 2009). Some modeling programs do not predict the existence of MSD 1 and MSD2, but all other 10 MSDs are consensus. The N- and C-termini are both predicted to be cytoplasmic, whereas the TM5-TM6 and TM7-TM8 domains are predicted to be extracellular. A similar topology was proposed earlier for the tropical forage legume (*Stylosanthes hamata* cv. Verano) high-affinity sulfate transporter by comparison with various sulfate transporters from yeast and animals (Smith et al., 1995). Based on this model, D108N is localized either to putative MSD1 or in the cytoplasm (if MSD1 does not exist), and G208D exists in MSD5 (Fig. 4.1C).

4.2.2 Effects of the two *SULTR1;2* Mutations on sulfate transport and accumulation.

A functional complementation test was performed using a Saccharomyces cerevisiae sulfate transport mutant to determine whether the two missense mutations had an effect on sulfate transport. The yeast strain CP154-7A carrying deletions of two sulfate transporter genes *sul1*and *sul2* is defective in sulfate transport resulting in an inability to grow on low sulfate concentration unless provided with Methionine (Met) as an S source (Cherest et al., 1997). The coding sequences for WT and each of the newly isolated mutant alleles of *SULTR1;2* were prepared in a galactose-inducible expression plasmid. As shown in Fig. 4.2, while the parental WT yeast strain W303-1B grew in the absence of Met and the presence of galactose, CP154-7A grew poorly. As reported by others (Shibagaki et al., 2002; Shibagaki and Grossmann, 2004), *SULTR1;2* complemented growth of CP154-7A in the absence of Met. Similar to *sel1-8* (Shibagaki et al., 2002), *sel1-15* and *sel1-16* complemented CP154-7A weakly compared to WT *SULTR1;2*. The replacement of galactose with glucose in the growth medium did not lead to complementation (Fig. 4.2A), showing that growth was dependent on expression of *SULTR1;2*. In the presence of Met the strains grew regardless of the carbon source (Fig. 4.2A, B, C), indicating that poor growth of the *sel1-15* and *sel1-16* transformants on medium with 50μM sulfate as the sole sulfur source is due specifically to an inability of the mutant proteins to carry out high affinity sulfate transport compared with WT SULTR1;2. Quantitative analysis of the yeast growth in liquid culture confirmed this observation (Fig. 4.2B, C and Table 4.2). Based on the yeast complementation test it is concluded that *sel1-15* and *sel1-16* are defective in sulfate uptake similar to the allele *sel1-8* (Shibagaki et al., 2002).

The sulfate uptake capacity of Arabidopsis WT and *sel1* mutant plants was measured under conditions that selectively measure high affinity transport (20 μM [^{35}S]Na_2SO_4) and both high and low affinity transport (500 μM [^{35}S]Na_2SO_4). Similar to the yeast complementation experiment (Fig. 4.2), WT plants were capable of sulfate uptake under 20 μM [^{35}S]Na_2SO_4, but *sel1-15* and *sel1-16* plants are defective in sulfate uptake (Fig. 4.3A). The fact that sulfate uptake capacity was induced for all of the *SULTR1;2* alleles by S deficiency (–S) indicates that the –S condition normally induces expression of sulfate transporters, including *SULTR1;2*. Furthermore, the analysis showed that *sel1-15* and *sel1-16* were defective in sulfate uptake similar to a T-DNA null mutant of *SULTR1;2*, *sel1-10* (Maruyama-Nakashita et al., 2003). The data suggest that D108 and G208 are critical for sulfate transport activity.

Sulfate accumulation inside a cell was also tested (Fig. 4.3B). Six-day-old seedlings grown on +S medium were transferred for 24 hr to medium containing various doses of sulfate. Prior to the treatment, there was no statistically significant difference among the genotypes. The *sel1-15* and *sel1-16* seedlings showed a defect in internal sulfate accumulation at 1.6 mM and 5 mM treatment concentrations. The result is consistent with the finding that sel1-15 and sel1-16 are defective in sulfate transport. On the 10 mM treatment the genotypes showed similar internal sulfate concentrations, with the exception that *sel1-16* was slightly lower than the others. At such a high external sulfate concentration low affinity transporters are able to maintain the internal sulfate pool. but at low external sulfate concentrations the expression of high affinity

sulfate transporters is insufficient to maintain the internal sulfate pool and/or in the *sel1-15* and *sel1-16* mutants defective *sultr1;2* is unable to maintain the internal sulfate pool. In summary, the results show that *sel1-15* and *sel1-16* alleles are defective in high affinity sulfate uptake, and in the maintaining of an internal sulfate pool.

4.2.3 *sel1-15* and *sel1-16* have a general defect in the sulfate deficiency response.

To gain further insights into *the* response of the three novel *sultr1;2* alleles to sulfate, The expression of S-responsive genes was measured by real-time PCR analysis in plants treated with a range of sulfate concentrations to gain further insights into the response of the three novel *sultr1;2* alleles to sulfate. The treatment conditions were identical to those for the experiments shown in Fig 4.3B so that gene expression could be directly correlated with internal sulfate content. The relative expression levels are diagrammatically presented in Fig. 4.4 and are also given in tabular form in Table 4.3. To compare the sensitivity of gene expression to increasing sulfate concentrations, the expression level was normalized to that at 0 mM, which was set to 100%, for each genetic background (Table 4.3). We first quantified expression of At2g44460, the gene from which the reporter gene system was constructed. After treatment for 24 hr with 0 mM sulfate the three mutant alleles showed 2 to 6 fold higher At2g44460 expression than WT (Fig. 4.4A; Table 4.3). The expression was reduced in all genotypes treated with 1.6 mM sulfate, and was 40 to 90 fold higher in the mutants than in WT. When the data are presented as a sensitivity difference, the expression at 1.6 mM sulfate in WT was reduced to 0.3% compared to 0 mM sulfate, but the *sel1* alleles all showed a less pronounced repression (1.8 to 6.5% of the

expression at 0 mM sulfate). At other sulfate treatments, the expression levels were always higher in the three alleles than in WT, and indeed, the percent change was in all cases reduced compared to WT. The 10 mM sulfate treatment is that under this condition the sel1-15 showed 2 to 7 fold higher At2g44460 expression than WT (Table 4.3). Under the same treatment condition these mutants had an internal sulfate pool that was similar to WT (Fig. 4.3B), indicating that misregulation of At2g44460 is not due to a limitation of internal sulfate content. At 10 mM external sulfate, the internal sulfate concentration in all the mutants was much higher than WT treated with 1.6 mM sulfate, yet At2g44460 expression was higher than it was in WT. This result suggests that that both mutants have an S-sensing defect independent of the sulfate transport defect of *sel1-15* and *sel1-16*.

The expression of another S-responsive gene *SULTR4;2*, which encodes a vacuolar transporter involved in internal sulfate re-mobilization (Kakaoka et al., 2004), was investigated after *SULTRA1:2*. *SULTR4;2* is much less responsive to –S than is At2g44460 (Fig. 4.4B ; Table 4.3) as has been previously reported (Maruyama-Nakashita et al., 2003). Nonetheless, the two mutant alleles showed 0.5 to 2 fold higher *SULTR4;2* expression than WT at 0 mM sulfate, and expression was 2 to 4 fold higher when they were treated with 0.1 to 5 mM sulfate. Both two mutant alleles were similar to WT at 0.1, 1.6, 5 and 10 mM sulfate, and the sensitivity was reduced in response to 0.5 mM sulfate (Table 4.3).

To determine whether other –S responsive genes also exhibit reduced sensitivity to increasing sulfate concentrations in the *sel1* alleles, an interaction network was constructed using the Arabidopsis gene-gene interaction dataset (Ma et al., 2007) as previously described (Xin et al., 2009). Using this method, no functional interactions were found for *SULTR1;2*, but a network of 59 interactors was found for the S-response genes examined above, *SULTR4;2* and At2g44460, with a map of 151 interactions (Fig. 4.5). GENEVESTIGATOR (Zimmermann et al., 2004) analysis indicated that 25% of those genes are sulfur deficiency responsive. Among those, two genes were chosen for a complete gene expression analysis using the experimental conditions defined for At2g44460 and *SULTR4;2* including *LSU1 (RESPONSE TO LOW SULFUR 1*, At3g49580, annotated as an expressed protein without known biological function), and *SDI1 (SULPHUR DEFICIENCY-INDUCED 1*, At5g48850, annotated as a protein similar to male sterility family protein MS5,) (Howarth et al., 2009). These two genes showed higher expression in both *sel1* alleles than that in WT at most of the sulfate concentrations (Fig. 4.4C and 4.6D, and Table 4.3). Similar to At2g44460, the sensitivity of expression of both genes in the two mutants to increasing sulfate concentrations was reduced at 0.1, 0.5 and 1.6 mM treatments (Table 4.3). *SDI1* expression at 5 and 10 mM treatments was also less sensitive in the mutants, but only *sel1-15* showed reduced sensitivity with respect to *LSU1* expression at 10 mM sulfate (Table 4.3). In total, these results show that the *sel1* mutants exhibit a general reduction in their perception of S-deficiency as measured using several known S-responsive genes.

4.2.4 *sel1-15* and *sel1-16* show reduced sensitivity to externally applied sulfur metabolites

The observations that both mutants have defective sulfate deficiency responses suggest that *sel1-15 and sel1-16* affect the response to sulfur metabolites. The endproducts of S-assimilation including cysteine (Cys) and glutathione (GSH) are known to repress expression of S-response genes (Hawkesford et al., 2006; Yi et al., 2010). The five-day-old seedlings grown on S sufficient (+S) medium were treated for 24 h to -S medium or medium containing very high sulfate (20 mM), 1 mM Cys or 1 mM GSH. 1 mM sulfide (Na_2S) was also tested because it is known to bypass the rate limiting APS reductase and sulfite reductase steps in the assimilation pathway. The alternative S sources are known to be utilized by plants and most likely taken up by plant cells via a sulfate transporter-independent mechanism. As shown in Fig. 4.6, At2g44460 promoter activity in the WT root system was undetectable in the presence of 20 mM sulfate or 1 mM of the alternative S metabolites compared with -S, but both two mutant alleles showed a significant level of At2g44460 promoter GUS activity, indicating that in addition to sulfate, the mutants also are defective in sensing the endproducts of S-assimilation.

4.2.5 Protein localization of SULTR1;2

The tissue-specific gene expression reported the promoter activities on root hair, root epidermis, cortical cells and guard cells of leaves, by transgenic plants with the *SULTR1;2* promoter::*GUS* or *SULTR1;2* promoter::*GFP* (Shibagaki et al., 2002; Yoshimoto et al., 2002). The gene expression information can be obtained from the GENEVESTIGATOR website (https://www.genevestigator.com/)(Zimmermann et al., 2004), which indicates a preferential expression in radicals, lateral roots and root tips of the seedlings.

Although SULTR1;2 has been reported to be a plasma membrane transporter, no direct evidence exist. Recently, it has been shown in the yeast system that a large proportion of GFP-fused SULTR1;2 is located in the cytoplasm likely due to protein degradation (Shibagaki and Grossman, 2004). Therefore, the GFP fusion system was applied to determine the subcellular localization pattern of SULTR1;2 in Arabidopsis cells. In addition, we attempted to address whether the newly isolated *sel1* alleles affect the protein localization or not. Transgenic seedlings of T2 generation were used for GFP observation using fluorescence confocal microscope. As shown in Fig. 4.7, the WT, *sel1-15*, and *sel1-16* transgenic lines seemed to be localized in plasma membrane. The GFP signals were observed in the surrounding the nuclei of all three genotypes. Close observation of the recorded videos found that the GFP signal is present outside the nuclei only but never inside. The localization pattern indicates that SULTR1;2 and its various mutant forms are also localized to the cytoplasm, consistent with an earlier report (Shibagaki and Grossman, 2004). Importantly, *sel1-15* (SULTR1;2^{D108N}) and *sel1-16*(SULTR1;2^{G208D}) did not seem to affect the SULTR1;2 protein subcellular location. Therefore, the sulfate transport and response phenotypes observed in our mutants are less likely due to the alteration in the protein location.

4.3 Discussion

Although a number of *sel1* alleles have been isolated and characterized, there has until now not been any evidence that *SULTR1;2* functions as a sensor for S nutrient. Detailed genetic characterization of the two mutation reveal that D108N (*sel1-15*) and G208D (*sel-16*) are not only severely debilitated in high affinity sulfate transport, but also display reduced sensitivity to increasing sulfate concentration by using a sulfate dose response experimental strategy (Table 4.3). The phenotype cannot be solely explained by their inability to transport and accumulate sulfate since at high external sulfate they showed similar internal sulfate content as WT (Fig. 4.3B). For example, *sel1-15* and *sel1-16* treated with 0.5 mM external sulfate showed similar internal sulfate concentration as WT treated with 0.1 mM external sulfate (Fig. 4.3B), and yet they showed higher expression of S-response genes (Fig.4.6 and Table 4.3).

Also, the observation that the two mutants show higherAt2g44460 promoter activity than WT when treated with reduced S-compounds (Fig. 4.6) shows that they all have a reduced ability to perceive the presence of alternative S sources including sulfide, Cys, or GSH; that the perception defect is not restricted to sulfate. Taken together, characterization of the two novel alleles of *SULTR1;2* provides evidence that SULTR1;2 acts as a putative transceptor.

The mechanism by which SULTR1;2 acts as an S nutrient sensor remains to be studied. A key question is what form of S is being sensed by SULTR1;2. One possibility is that SULTR1;2 senses external sulfate, such as the proposed model NRT1.1, which functions as a nitrate sensor,

but sensing defective mutants show reduced sensitivity to ammonium (Munos et al., 2004; Little et al., 2005, Ho et al., 2009). However, it is possible that SULTR1;2 also senses intracellular S-status or both intracellular S-metabolites and external sulfate. Another central question is the location of the S sensing domain in SULTR1;2 and its relationship to sulfate transport. Previous genetic and biochemical studies of *sel1* missense mutations identified several amino acid residues that are necessary for sulfate transport (Shibagaki et al., 2002; El Kassis et al., 2007).The results of the present study indicate that residues D108 and G208 are also important for sulfate transport, yet, the evidence also suggests that these residues are required for the sensing function. Recent work with the yeast amino acid transceptor Gap1 indicates that the same binding site functions both for transport and signaling and that a conformational change after the ligand binding step triggers activation of a signaling pathway (Thevelein and Voordeckers, 2009; Van Zeebroeck et al., 2009).

In a recent study, the STAS domain of SULTR1;2 was shown to form a physical interaction with serine acetyltransferase and that the binding causes reciprocal activation/inactivation of the partners (Shibagaki and Grossman, 2010), a finding that was interpreted to indicate that a direct connection exists between sulfate transport and sulfate assimilation. It is not clear at this time how the present findings impact such a model. Serine acetyltransferase produces O-acetylserine (OAS), a compound that has been shown to regulate the expression S-responsive genes (Leustek et al., 2000). An intriguing possibility is that the SULTR1;2 sensor may directly control the assimilation pathway which indirectly controls the gene expression response through serine acetyltransferase. Another possibility is that the SULTR1;2 sensor may control multiple

pathways one of which is the signaling pathway controlling gene expression and the other being the assimilation pathway.

Studies in yeast and recently in animals and plants have revealed the existence of three types of nutrient sensors: classical receptors (such as G-protein-coupled receptor Gpr1), non-transporting receptors (such as Snf3 and Gcr1), and transporting receptors (such as Gap1) (Thevelein and Voordeckers, 2009). The latter two transporter-related receptors, collectively termed transceptors, are now thought to be evolutionary intermediates between nutrient transporters and classical receptors of chemical signals (Thevelein and Voordeckers, 2009). In plants, the first demonstrated transceptor is the dual affinity nitrate transporter and nitrate receptor CHL1/NRT1.1 (Ho et al., 2009), which provides a useful approach for identifying nutrient sensors from functional membrane proteins, such as in the case of our project. Future work on the sensing mechanism of SULTR1;2 will provide insightful information on the evolution of inorganic ion receptors and sensing in plants.

Table 4.1 Primers used in Chapter 4

Purpose	Gene name	Name of primers	Sense (S)/ Antisense (A)	Primer sequences (5'→3')
Regular PCR	At2g44460	YZP63	S	AACGAGCTCTTGCCACTGAACT
		YZP64	A	GAGATGGTCCTCATGGTAGCTT
CK	*ACT2*	ACT2S	S	CTAGGATCCAAAATGGCCGATGGTGAGG
		ACT2A	A	GAAACTCACCACCACGAACCAG
qRT-PCR	At2g44460	YZP75	S	CGCGTTACGTTGCTCATATT
		YZP76	A	GAGCTGATGATCGGTTACGA
	SULTR4;2	DZP143	S	ATTGTTCGAATCGATGCTCCC
		DZP144	A	CCCTTGCTTGTGTGTTTGTCG
	AT3G49580 (*LSU1*)	ZZP159	S	GGATGAGCTAAGGAGGAGGA
		ZZP160	A	TTCGCTGCCACAACTTAATC
	AT5G48850 (*SDI1*)	ZZP167	S	CAAATCTTTCCGTCCTCGTT
		ZZP168	A	CAACTCAACTTGCTCCTCCA
		ZZP166	A	CGAGTGGAAACATGAGCTTG
CK	*ACT2*	ActSDS	S	GCACCAAGCAGCATGAAGATT
		ActSDA	A	GGAACCACCGATCCAGACACT
Plasmid Constructs				
GFP-SULTR1;2 DZ38,39,40,41,42 (For yeast transformation)	*sultr1;2* CDS	DZP190	S	GAAGATCTATATGTCGTCAAGAGCTCACCCTG (*Bgl* II)
		DZP191	A	GCTCTAGAATTTCAGACCTCGTTGGAGAG (*Xba* I)
SULTR1;2-GFP DZ18 (Transformed to sel1-16 for complementation)	*SULTR1;2* CDS	DZP190	S	GAAGATCTATATGTCGTCAAGAGCTCACCCTG (*Bgl* II)
		DZP193	A	TGCTCTAGAGACCTCGTTGGAGAGTTTTGGA (*Xba* I)

Table 4.2 Doubling time of yeast growth*

The data from Fig. 4.3B and C were used to derive the doubling time (h) of yeast growth for each strain/plasmid, with mean and SD (n=3) shown in the table.

Strain/plasmid	+Met		-Met	
	Mean	SD	Mean	SD
W303-1B/Vector	16.1	1.2	19.4	1.3
CP154-7A/Vector	22.8	2.8	1766.2	498.0
CP154-7A/SULTR1;2	29.1	3.4	34.4	3.0
CP154-7A/sel1-15	28.9	4.4	2310.5	1222.6
CP154-7A/sel1-16	37.2	13.8	2006.8	365.9
CP154-7A/sel1-8	21.7	6.0	3115.9	2127.6

- The data were generated by Dr. *Thomas Leustek at Rutgers University and used with the permission of Dr. Leustek.*

Table 4.3 Quantitative RT-PCR analysis of gene expression in *sel1* mutants

Genes	[Sulfate]	Relative expression levels			Percent of change		
	mM	WT	*Sel1-15*	*Sel1-16*	WT	*Sel1-15*	*Sel1-16*
At2g44460	0	100.0	347.4	740.3	100.0	100.0	100.0
	0.1	14.6	123.1	321.8	14.6	34.2	44.8
	0.5	3.6	55.4	103.2	3.6	16.6	16.1
	1.6	0.3	18.4	28.2	0.3	6.5	4.2
	5	0.2	2.2	3.1	0.2	0.7	0.4
	10	0.2	1.5	1.1	0.2	0.5	0.2
SULTR4;2	0	100.0	150.2	270.9	100.0	100.0	100.0
	0.1	63.1	118.4	192.8	63.1	79.0	80.7
	0.5	23.4	93.6	117.0	23.4	62.4	47.0
	1.6	25.4	77.1	71.4	25.4	59.1	26.1
	5	9.7	33.0	18.7	9.7	23.9	7.5
	10	11.7	18.2	22.2	11.7	12.3	6.9
LSU1	0	100.0	474.9	685.6	100.0	100.0	100.0
	0.1	21.3	154.5	308.5	21.3	48.0	51.6
	0.5	6.2	179.6	134.4	6.2	46.8	24.3
	1.6	4.4	107.7	79.8	4.4	16.2	11.7
	5	1.9	17.2	12.3	1.9	3.4	1.6
	10	2.9	6.9	14.0	2.9	2.0	1.6
SDI1	0	100.0	252.5	498.8	100.0	100.0	100.0
	0.1	13.7	119.1	293.2	13.7	49.1	64.5
	0.5	3.5	70.7	99.1	3.5	27.9	26.4
	1.6	0.7	44.4	42.3	0.7	15.2	8.9
	5	0.2	7.0	3.6	0.2	2.3	0.8
	10	0.2	2.3	3.6	0.2	0.8	0.7

Notes: 1) Relative Expression Levels: For WT at 0 mM control, the relative level is set to 100;
2) Percent of Change: For each genotype at 0 mM control, it is normalized to 100%.

Table 4.4 Constructs for GFP-fused SULTR1;2

construct	Plant For CDS	Primers For CDS	Restriction Enzyme	Vector	Restriction Enzyme	GFP fusion	Selection in *E.coli*	Selection in plant
DZ17	Col	DZP190/ 191	*Bgl* II/*Xba*I	GZ0	*Bgl* II/*Xba*I	N-terminal	Kanamycin resistance	Bialaphos resistance
DZ18		DZP190/ 193	*Bgl* II/*Xba*I	GZ6	*Bam* HI/*Xba* I	C-terminal	Kanamycin resistance	Bialaphos resistance
DZ10	*Sel1-16*	DZP190/ 191	*Bgl* II/*Xba*I	GZ0	*Bgl* II/*Xba*I	N-terminal	Kanamycin resistance	Bialaphos resistance
DZ13		DZP190/ 193	*Bgl* II/*Xba*I	GZ6	*Bam* HI/*Xba* I	C-terminal	Kanamycin resistance	Bialaphos resistance
DZ11	*Sel1-15*	DZP190/ 191	*Bgl* II/*Xba*I	GZ0	*Bgl* II/*Xba*I	N-terminal	Kanamycin resistance	Bialaphos resistance
DZ14		DZP190/ 193	*Bgl* II/*Xba*I	GZ6	*Bam* HI/*Xba* I	C-terminal	Kanamycin resistance	Bialaphos resistance

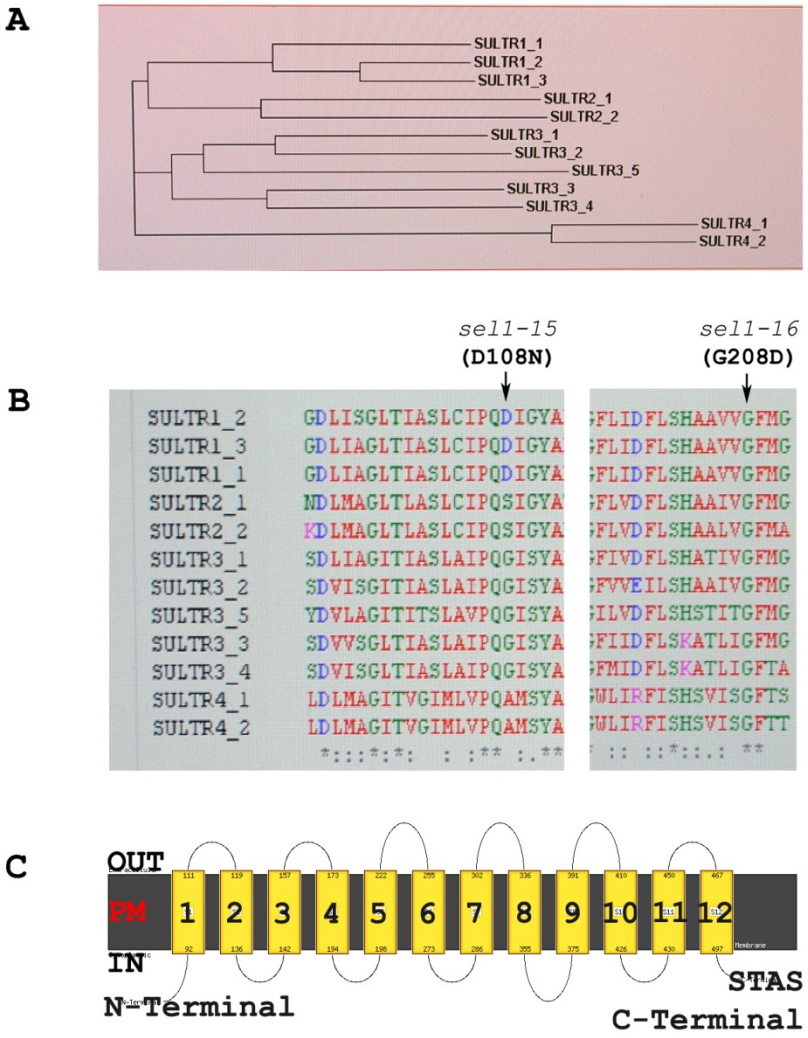

Figure 4.1 Molecular analysis of SULTR1;2

A. Phylogram of 12 SULTRs by their protein sequences developed using ClusteralW2.

B. Schematic representation of the missense mutation sites in SULTR1;2 protein. An alignment of SULTR1;2 with 11 other Arabidopsis SULTR proteins was constructed using ClusteralW2, with only the regions surrounding the mutation sites shown.

C. A computerized MEMSAT-SVM diagram of SULTR1;2 topology developed following Jones, 2007; Nugent and Jones, 2009.

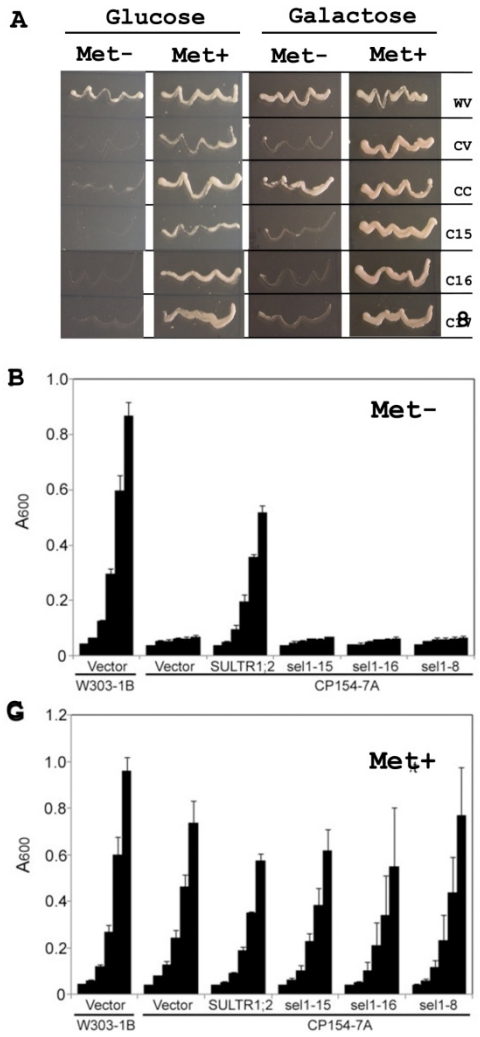

Figure 4.2 The WT, but not *sel1-15* and *sel1-16*, complements the yeast *sul1 sul2* double mutant.

A. Yeast transformants with the empty vector control or various alleles of *SULTR1;2* were grown in agar-solidified medium supplemented with glucose or galactose and in the presence or absence of 0.4 mM Met. Abbreviations: WV, WT with empty vector (CK); CV, CP154 with vector (-CK); CC, CP154 with CDS from *Arabidopsis thaliana* Col.; C15, CP154 with CDS from *sel1-15*; C16, CP154 with CDS from *sel1-16*; C8, CP154 with CDS from *sel1-8*(CK).

B and C. Quantitative measurement of yeast growth in liquid culture. Absorbance at 600 nm wavelength of various yeast transformants at 0 mM Met (B) or 0.4 mM Met (C). The starting culture had A_{600} of 0.04, and A_{600} values were measured after 8, 16, 24, 32 and 40 hr. The bar represents the SD of three independent colonies for each construct.

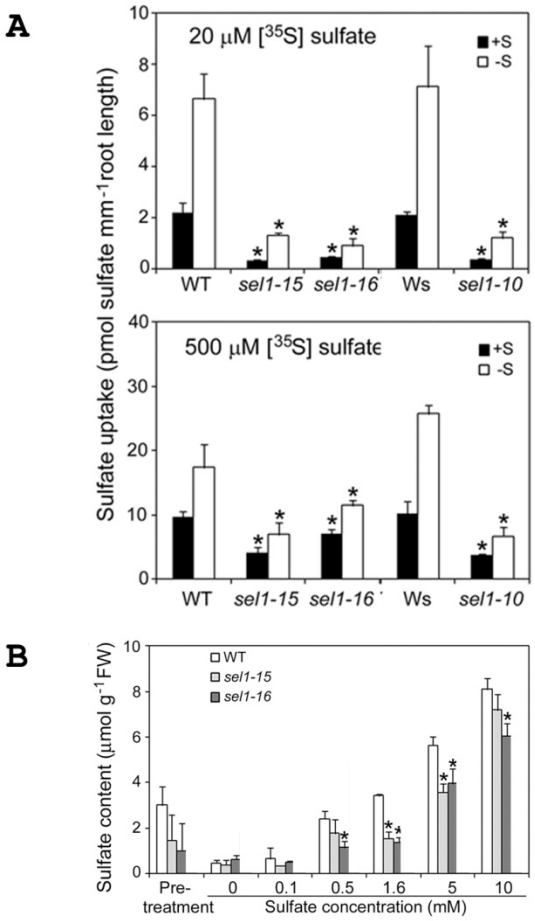

Figure 4.3 Sulfate uptake and assimilation is inhibited in *sel1-15* and *sel1-16* alleles*.

(A) Measurement of [^{35}S]-sulfate transport activity. Six-day old seedlings pretreated with either –S or +S for 24 h were transferred to [^{35}S]-Na_2SO_4 for 30 min and [^{35}S] activity was then measured. The *sel1-10* background is Ws.

(B) Sulfate contents in various *sel1* alleles. Seedlings grown in normal +S medium were transferred to liquid culture with different doses of sulfate for 24 h before total internal sulfate was measured. WT, wild-type for *sel1-15, sel1-16*; Ws, wild-type for *sel1-8*. The bar represents the SD of three measurements. The asterisk (*) above the column indicates a significant difference ($p<0.05$) between *sel11-15, sel1-16* and WT (or between *sel1-10* and Ws) under the same nutrient condition.

- This data were generated by Dr. *Thomas Leustek and his lab at Rutgers University and used with the permission of Dr. Leustek.*

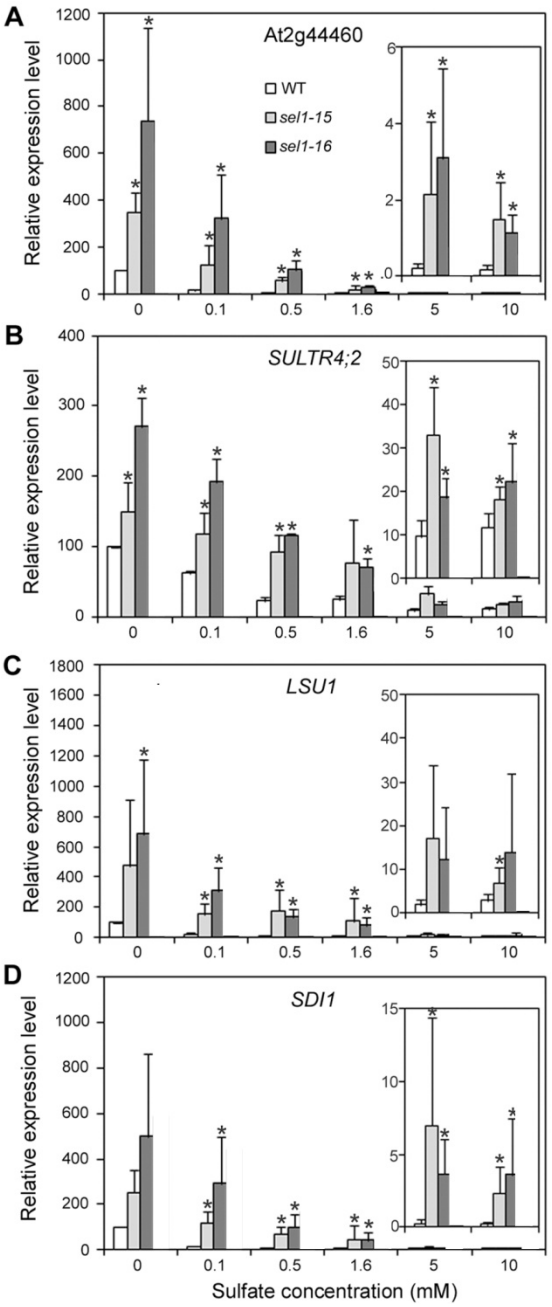

Figure 4.4 The *sel1-15, 16* alleles reduce the sensitivity to sulfate in gene expression.

Expression of four genes, At2g44460 (A), *SULTR4;2* (B), *LSU1*(C) and *SDI1* (D), were quantified using real-time RT-PCR. Relative mRNA levels were determined by first normalizing their PCR threshold cycle numbers with those of the *ACT2* reference gene and setting the relative mRNA levels for each gene in WT at 0 mM sulfate control at 100. Insets in each figure showed expression levels at 5 and 10 mM treatments. WT, wild-type . The bar indicates the SD of three biological replicates. The asterisk (*) above the column indicates a significant difference ($p<0.05$) between *sel1* and WT under the same nutrient condition.

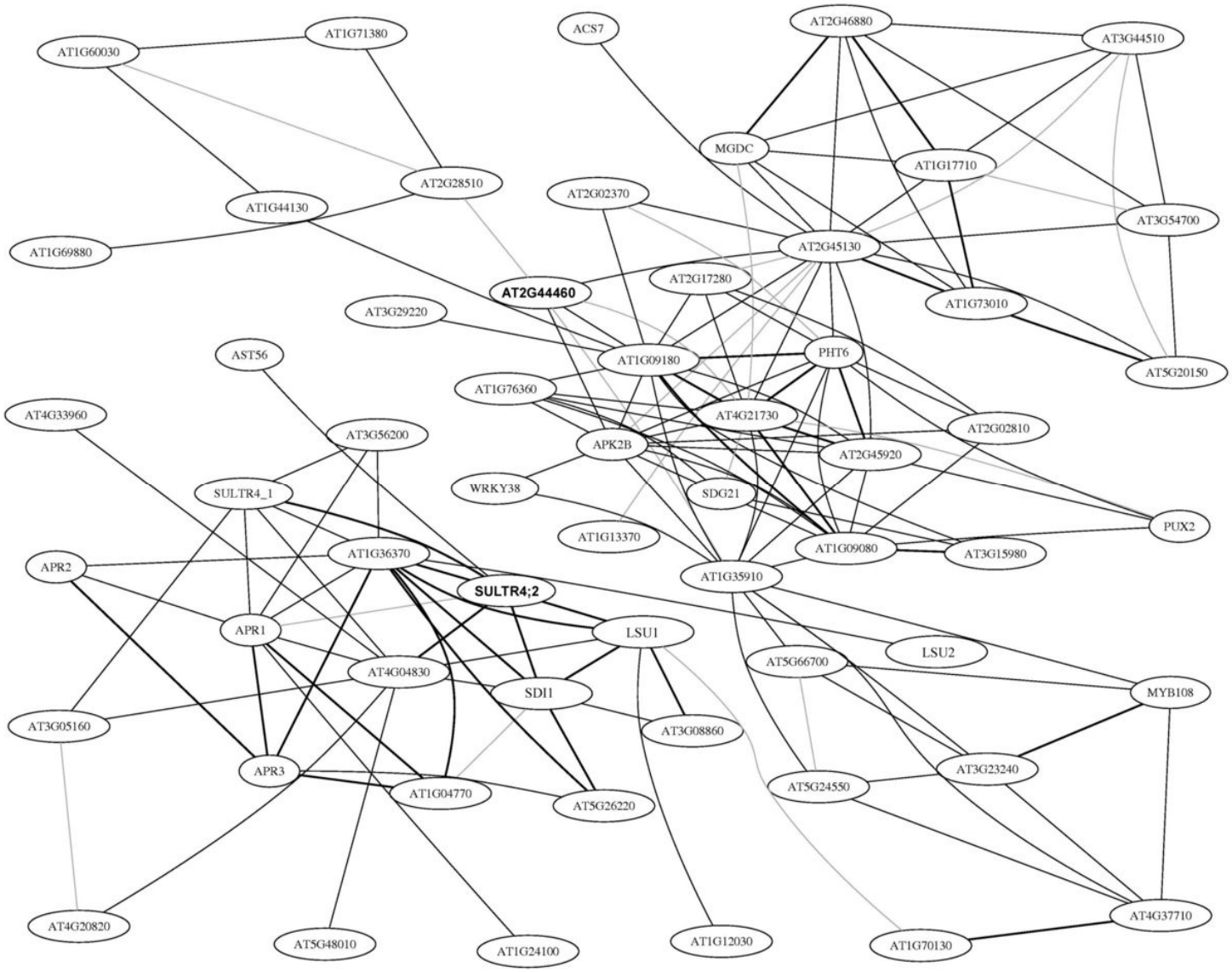

Figure 4.5 A gene-gene interaction network involving At2g44460 and *SULTR4;2.**

SULTR1;2, SULTR4;2 and At2g44460 were mapped to the gene-gene interaction database (Ma et al., 2007), with *SULTR4;2* and At2g44460 present in the database. This analysis revealed a total of 59 unique genes (Dataset 4.1), including *SULTR4;2* and At2g44460, that showed 151 interactions, and a network was then assembled based on these interactions.

- This data were generated by Dr. *Yihong Zhao at Columbia University and used with the permission of Dr. Zhao.*

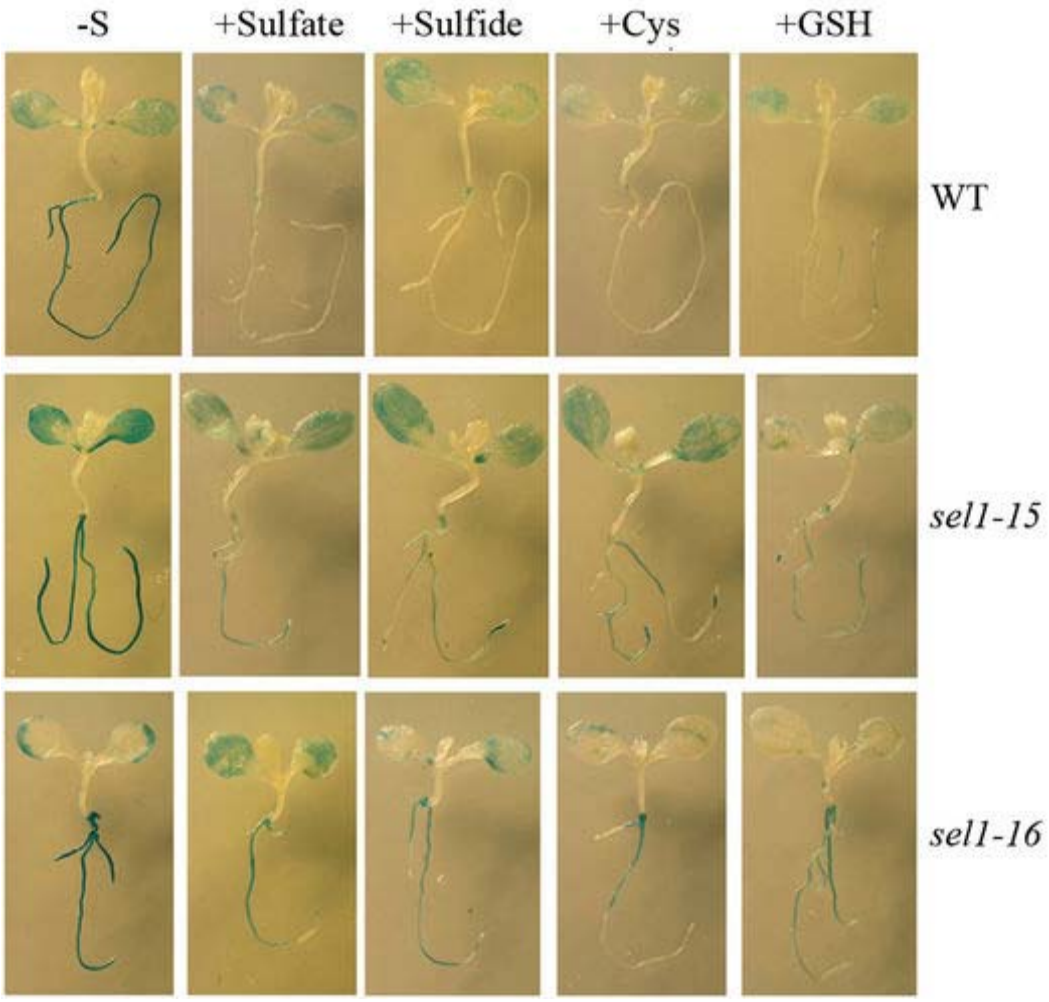

Figure 4.6 At2g44460 promoter::GUS reporter expression in response to various sulfate metabolites.

Five-day-old seedlings grown in normal MS medium were transferred to the –S medium supplemented with 20 mM sulfate or 1 mM of sulfide, Cys or GSH for 24 hr before GUS staining.

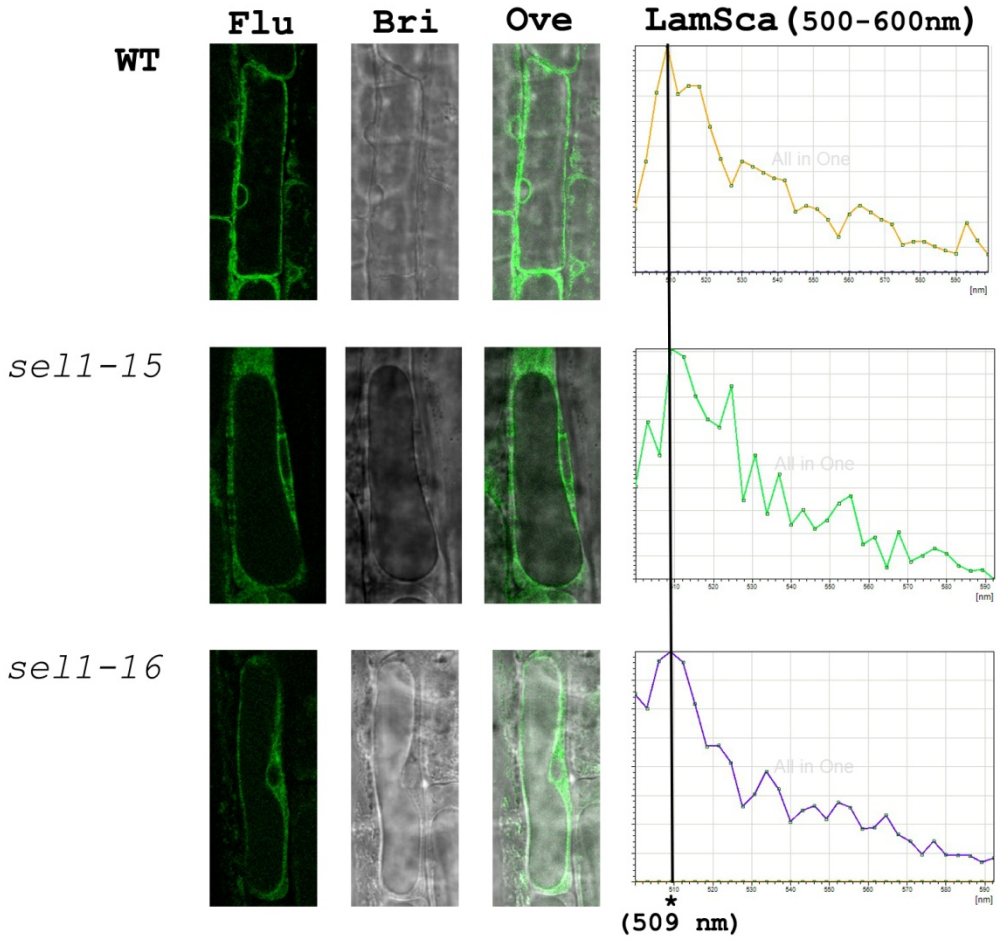

Figure 4.7 Sub-cellular localization of SULTR1;2-GFP fused protein in *Arabidopsis thaliana* Col.

CDS from WT and the two mutants were cloned into vector GZ6 containing GFP and a 35S CaMV promoter to form construct of DZ 18, DZ14 and DZ13, which contain CDS from WT, *sel1-15, sel1-16* respectively. Primers used are listed in Table 4.1. Method of making the constructs and plant transformation are described in the text of Chapter IV. For each genotype, picture of fluorescence microscopy (Flu), bright-field microscopy (Bri) and the overlapped (Ove) are shown sequentially from left to right. The last graph is the Lamda scan (LamSca) between wavelength of 500-600nm, which is applied to guarantee the fluorescence signal is from GFP (with the emission peak at 509nm as indicated in the picture), other than from autofluorescence (with the emission peak around 550nm).

Chapter 5

Other observations and future perspectives

5.1 *sel1* mutations override the C and N requirement in inducing S deficiency response of At2g44460 expression.

As shown in Fig. 2.8A, the At2g44460 promoter activity was highly up-regulated by –S stress, but this up-regulation was strongly dependent on the C availability or strongly enhanced by +C. With the addition of N to its sufficient level the promoter activity reached the peak level. The above signal interaction was also observed on the mRNA level of the endogenous reporter gene, At2g44460 (Fig. 2.8B). The responsive characteristic of reporter line GHF1 to the three nutrients C, N and S provides a sensitive tool in identifying any signal alteration by the mutations.

The altered C-N-S signaling of the two mutant alleles (*sel1-15* which is a synonym of 11p101h and 11p201h, sel1-16 synonym of 11p34h) is displayed in Fig. 3.3A. Results of WT (the reporter line, GHF1) displayed identical responsive pattern as in the previous chapter, i.e. the –S responsive At2g44460 promoter activity is highly regulated by C presence, and also synergistically regulated by N presence (Fig. 2.8A; and Fig. 3.3A). However the mutants severely altered the above C-N-S crosstalk. Of interest is that all mutants seem to have a constitutive strengthened GUS response under all tested nutrient conditions. Therefore from this experiment we conclude that no matter what nutritional conditions are, all mutants 'feel like' they still lack S, or are in a sulfur-starved condition.

By carefully checking the results shown in Fig. 3.3A, some sophisticated yet also important differences can be identified. First, the mutant has a higher GUS strength at +C+N-S than at +C+N+S, this is especially clear in 11p201h. When compared to the wild type (WT), the results show that the *sel1-15* and *sel1-16* mutation does not completely wipe off its ability to detect change in sulfate concentration even though its sensing ability has been severely crippled. Another noticeable comparison is between -C+N-S and +C+N-S, which shows the C difference. The addition of C source increased the GUS strength in response to –S stress. This result also indicates the retaining of C sensing capability of the *sel1* mutants. Similarly, the GUS strength increased from +C-N-S to +C+N-S in all the mutants, although the increase is much weaker than in WT. By this, we can also identify the N-sensing capability of sel1 mutants. Therefore the *sel1* mutations severely cripple the sensing ability of mutants to the change of C, N, and S, but still retain the capability to some extent. In chapter 4, we applied all these mutants together with the WT to the qRT-PCR tests under a series of sulfate concentration treatments, and we determined that *sel1* mutants kept the original –S response, but had a general defect in sensing the differences in sulfate concentration (Fig. 4.6; Table 4.4).

Instead of the above observation on the nutrient sensing, we can still find some information of nutrient interaction among the C-N-S tested here (Fig. 3.3A). The increased GUS strength from -C+N-S to +C+N-S indicate that the –S response is still C-dependent. And the GUS increase from +C-N-S to +C+N-S shows the up-regulation of –S response by N source. Combine with the observation that +C+N-S treatment still shows the highest GUS staining among all the 8

combinatory treatments, it suggest that the C-N-S crosstalk is still working inside the mutants tested. Another possibility is that the *sel1* mutation did not alter the C-N-S interaction and instead mutations of *sel1-15* and *sel1-16* altered the C, N, and S sensing ability.

Since the GUS tests did not provide the direct genetic evidence for us, we subjected the WT and the two mutants, *sel1-15, sel1-16* to RT-PCR in order to test the mRNA change of the reporter gene At2g44460. As shown in Fig. 5.1, there was an increase in gene expression in all mutants tested in various nutrient conditions, however the –S response (comparing –C-S to –C+S; and +C-S to +C+S), and + C response (comparing –C-S to +C-S; and –C+S to +C+S) are still observable. Also the –S, and +C responses in mutants are much weaker than those in WT (GHF1). Considering the C-S interaction, we found that the gene expression level at +C-S is still the highest in all treatment concluding that the C-S interaction still exists in mutants the same as in the WT. All the above gene expression tests are consistent with the GUS tests, and support that sel1 mutation altered the sensitivity of nutrient sensing, but not the nutrient interaction. We tested only C and S here because we identified in the previous experiments that in the –S responses, C plays the predominant role, while N plays a synergistic role (Fig. 2.8A and the corresponding chapter).

5.2 *sel1* alleles altered the responses to hormones

In Chapter II, we gave evidence that support a negative regulatory role for auxin in –S responses. Our results also indicate a similar regulatory role for two other phytohormones, ABA and BA. Therefore, we decided to investigate the hormonal response in the *sel1* mutants.

We began our experiment by testing on a natural nutrient medium, i.e. without –S stress. We sowed both mutants and WT seeds on the MS plates, which had a pre-added gradient concentration of IAA as shown in Fig. 5.2A. The plates were vertically incubated at a programmed growth chamber for 11d, and then seedlings were subjected to GUS staining directly. Under this condition (+S), both *sel1* mutants displayed a strengthened GUS staining in comparison to WT (see column of Fig. 5.2A, '[0] μM IAA'). This is not surprising since it is the characterization of *sel1-15*, and *sel1-16* that's capable of strengthen the GUS staining even at +S condition. But we notice that by the addition of different concentration of IAA, no obvious difference can be observed. The mutants' response to IAA at +S is the same as WT, at least at the At2g44460 promoter::*GUS* changing level (i.e. not change at all. See also 2.5A second panel, 5.2A).

We next subjected both mutants and WT seedlings from MS plates to different hormonal treatments under both –S and +S conditions (Fig. 5.2B). The WT, GHF1 displayed the same as shown in chapter II (Fig. 2.5A), especially under –S conditions, all the three tested hormones, IAA, ABA, and BA severely weakened the GUS staining of the WT. It is also important to note that no difference can be observed under +S conditions between the untreated (CK) and three hormonal treated conditions (Fig. 5.2A, B). The difference was observed when we used all three

mutants to test the three hormonal treatments, 11p101h, 11p201h, and 11p34h, strongly restored GUS weakening by the three hormones. The restoration was especially strong on the BA treatment. This phenotype suggest that the negative role of auxin, including ABA and BA tested here, on the –S responses is *SULTR1;2*-dependent. If the transporter gene *SULTR1;2* was mutated as in *sel1-15*, or *sel1-16*, the negative regulation of IAA on –S response is weakened. Notice that under +S condtions, all three hormones tested here had no obvious effect on GUS strength. We show another independent experiment by using different batch of mutant seeds in Fig.5.3, just to display the mild differences between different batches of seeds on the root length and on the GUS staining. Both experiments revealed that auxin' down-regulation of –S response is *SULTR1;2*-dependent. In the later experiment (Fig. 5.3), we applied not only IAA, ABA and BA in the tests as shown above, but also other commonly used plant hormones such as ACC (1-Amino-Cyclopropane-1-C arboxylic acid, ethylene precursor), BR (brassinosteroids), JA(methyl jasmonic acid), and SA (salicylic acid). However, we did not observe an obvious modification of hormonal responses by the *sel1* mutants. It is the same as WT shown here and in Chapter II (Fig. 2.5A).

5.3 More evidence in support of the dual role of SULTR1;2

The severely crippled GUS responsiveness in the *sel1* mutants provides the strongest evidence for the sensing function of SULTR1;2. The mutated At2g44460 promoter::*GUS* reporter lines expressed a strong GUS staining even in the condition of S sufficient (Fig. 3.2B, C; 3.3; 3.4A, B), the GUS strength was almost the same as WT at S deficient. One possible reason is the loss of 'sensing' function of the mutated gene product SULTR1;2. So that even under a +S condition,

the mutated sensor could not identify it, the sensor still 'feels like' –S, so that the reporter will display as a –S phenotype, that is stronger GUS staining. This enhanced effect in the sel1 mutants was not only identified on the promoter::GUS level, but also on the mRNA level as shown in Fig. 3.4C, where, all tested mutants displayed stronger gene expression of the reporter, At2g44460 than that of the WT.

It is still possible that the GUS strength increased by *sel1* mutants is solely due to the disruption of the sulfate transporting function, and has nothing to do with the 'sensing' function of SULTR1;2. Because a crippled SULTR1;2 can also let cell suffer from S deficiency, even under a +S condition. Under this condition, the reporter gene At2g44460 and the promoter::*GUS* can also express strongly so as to show a –S phenotype. It is true, our experiments proved the S-transporting functional disruption in the two mutants, *sel1-15*, and *sel1-16* in the yeast complementation test (Fig. 4.3). But in other experiments, especially in the gene expression in response to sulfate dose changes, we observed the in-accordance between cellular sulfate state and the gene expression level. An example is that under high sulfate treatment, for example at 10mM sulfate feeding experiment, intra-cellular S level was much similar between WT and mutants, but we could still identify 2-7 fold higher of At2g44460 expression in mutants than that in WT (Fig. 4.4B; 4.6A), which means even under a sufficient cellular S state, the mutated cell couldn't identify that, but 'felt' like S-starving. This phenomenon can only be explained as *sel1-15*, and *sel1-16* mutation disrupt the S sensing function of the SULTR1;2, which also suggests a sensor function of SULTR1;2. *sel1* mutants decreased the sensitivity of cells to extra-cellular S states was observed not only on the mRNA level of the reporter gene, At2g44460, but also on

that of a few other –S-responsive genes tested in this project, such as *SULTR4;2, LSU1*, and *SDI1* (Fig. 4.6; and Table 4.4).

The feeding plants with alternative S sources, such as sulfide, Cys, and GSH, which are all S nutrients that do not need the sulfate transporters to get inside the plant cells, although we don't know exactly which transporter is responsible for the transportation of each of them. Under this condition, there will be no difference between WT and sel1 mutants in absorbing these S sources, i.e. both genotypes (WT and mutant) will in a +S state. But GUS staining from these treatments displayed obvious GUS strength difference between WT and mutants (Fig. 4.8). All mutants displayed some extent of S starving. The explanation for this phenotype will have nothing to do with the crippled S transporting function, because all these tested nutrient don't need the mutated SULTR1;2. So the sole possibility is that the mutants crippled their sensing function of SULTR1;2. If this is the case, the reporter system will still indicate as a –S state, so as to display as an increased GUS staining as in Fig. 4.8.

Taken together, all above experimental evidence suggest the possible dual function of SULTR1;2 both as a sulfate transporter, and as a S sensor.

Not only this, in the 8 combinatory tests with C-N-S three nutritional sources and 6 nutritional states [(+ or -) X 3=6], we observed the S-sensing function of SULTR1;2, and at the same time, we also observed the sensing of C and N state by the same sensor, SULTR1;2 (Fig. 3.3 and 5.1).

In the hormonal tests for the S-status responses, we observed that the negative regulatory role of auxin was SULTR1;2-dependent (Fig. 5.2B, and 5.3). And the *sultr1;2* mutants, *sel1-15*, *sel1-16* strongly restored the GUS weakening by 1μM IAA at –S condition. This result indirectly supports the hypothesis that SULTR1;2 serves as a sulfur sensor; because it helps to complete the possible signaling pathway from the S status sensing by SULTR1;2, to signal transduction by some types of transcription factors, finally reach the regulated region of the target gene. It is already known that many members of the auxin-inducible gene family, *Aux/IAA* encode proteins involved in –S stress responses, and among which is the transcription factor IAA28 (Rogg et al., 2001; Falkenberg et al., 2009). IAA28 has been identified as an regulatory 'hub' in an computerized transcriptome and metabolome analysis (Nikiforova et al., 2005a) and it was directly detected to be negatively responsive to sulfate transporters (Falkenberg et al., 2008). The binding model of IAA28 with another transcription factor ARF (auxin response factor) in the gene regulation is well characterized (Weijers and Jurgens, 2004; Falkenberg et al., 2009). Thus, by the integration of auxin and the IAA transcription factors, the whole picture for a S status sensing, signal transduction, gene regulation, and finally functional rebalancing becomes possible. Although it is only a possibility, and is in need of experimental evidences.

The STAS (sulfate transporter and antiSigma antagonist) domain in the C-terminal of SULTR1;2 and the many other SULTRs in Arabidopsis was functionally characterized by previous works (Shibagaki and Grossman, 2004; 2006; and 2010). In SULTR1;2, this 131amino-acid-containing protein sequence is not only important for sulfate transportation, but also in the membrane

localization, and most recently, in regulation of the some enzymatic reactions in the intracellular S assimilation. Structural and functional analysis of the STAS domain revealed the two functionally important sites, the putative phosphorylation site at T587, which is essential for the function of SULTR1;2. And another important site, which contains two Cys (C645/C646) and putatively involved in protein-protein interaction by red-ox of the disulfide bond (Rouached et al., 2005). By using a yeast two-hybrid system, Shibagaki and Grossman, 2010, showed the direct experimental evidence of the protein-protein interaction between SULTR1;2 STAS domain and the Cys synthase, OASTL. The binding of which resulted in a deduction of the transporter activity of SULTR1;2. The direct binding between SULTR1;2 and the downstream enzyme so as to give a regulatory result, is in agreement with the hypothesis that SULTR1;2 is a S sensor, which interact with the down-stream key enzymes when it senses the alteration of S availability in the environment, so as to adapt to the changed S level intracellularly.

5.4 Future perspectives

Plant nutritional sensing and signaling in plant adaptation is the frontier of plant science research. Addressing this topic is important not only in understanding molecular mechanisms, but also in agricultural practice, increasing the crop productivity, increasing food quality, and also in environmental protection. The identification of the first plant nutrient sensor, CHL1 in 2009 (Ho et al., 2009) was a milestone in plant science in the aspect of understanding nutrient sensing and signaling. In Ho's work, the so-called dual-affinity nitrate transporter which transports nitrate by an adapting mechanism depending on the surrounding nitrate availability. If the environmental nitrate concentration is low (typically at 0.2mM), the nitrate binds to the high-affinity binding

site of the N transporter, CHL1, and triggers the phosphorylation of CHL1, which active the low-level primary nitrate response by some unknown mechanism. If the environmental nitrate concentration is high (typically 10mM), nitrate molecules bind on both the low- and high-affinity binding sites of the N transporter, CHL1, and inhibit CHL1 phosphorylation, which activates the high-level primary nitrate response. IN the above model, the N transporter CHL1 serves both as a transporter and a nutrient sensor, and more interestingly, these two functions can be uncoupled.

Structural and functional analysis of SULTR1;2 indicates that a phosphorylation site, T587 on the STAS domain is essential for the function of SULTR1;2. If SULTR1;2 acts both as a sulfate transporter and a sensor like CHL1, addressing the following two questions can support our present model: 1) Is there a sulfate binding site in SULTR1;2 for both transport and sensing? Alternatively, SULTR1;2 may have two distinct sites specifically for transport and sensing. 2) Is there a phosphorylation mechanism involved in the sulfate sensing? It was reported by a site-directed mutagenesis test, that the putative phosporylation site (T587) in the STAS domain of SULTR1;2 is critical for the transformed yeast mutant to grow in a –S (100μM) medium (Rouached et al., 2005), However, more experiments are needed to identify the responsible kinase that phosphoylates SULTR1;2, and also evidence is needed to verify the binding of the enzyme to its target, SULTR1;2.

In addition, if SULTr1;2 acts as a transceptor, what is the downstream signaling event? To provide some clues, epistatsis tests with known –S-responsive genes, especially those genes that encode transcription factors will be helpful. These include SLIM1(Sulfur LIMitation1), member

of the MYB(MYeloBlastosis) family, and the Auxin Responsive Factors (ARFs) (Maruyama-Nakashita et al., 2006; Van der Ent et al., 2008; Nikiforova et al., 2003; Kasajima et al., 2007; Falkenberg et al., 2008). Among these, of great importance is the –S-responsive transcription factor SLIM1, which is believed to play a central role in transcriptional regulation, and controls both the sulfate uptake and degradation of glucosinolates so as to re-balance the S availability (Maruyama-Nakashita et al., 2006). SLIM1 is demonstrated to be necessary in activation of of some important –S-responsive genes, such as *SULTR1;2* and At2g44460. The GFP-SLIM1 protein was localized in the nucleus of root vasculatures. Due to this, the authors hypothesized that sulfur status was first sensed by an unknown sensor in the central vascular region, and then the signal was transmitted to SLIM1, then to epidermal/cortical localized SULTR1;2 through some intermediate signals(Maruyama-Nakashita et al., 2006). It is interesting that this proposed model is just the opposite of our experimental deduction. An epistatic study by using a *sel1 x slim1* double mutant can elucidate this question. If *SULTR1;2* works at the up-stream of *SLIM1*, we can add the latter in the signaling cascade of S sensing and regulation.

The newly characterized protein-protein interaction between the STAS domain of SULTR1;2 and the Cys synthase, OASTL(O-acetylserine thiol-lyase), provides an intriguing possibility how OSULTR1;2 regulates OASTL in S acquisition and S assimilation (Shibagaki and Grossman, 2010). The binding between these two proteins was demonstrated to increase the enzyme activity of OASTL, i.e. to enhance the Cys synthesis. It however decreased the transporter activity in an assay of yeast mutant system that co-expression of OASTL with SULTR1;2. This protein-protein interaction is believed to be important in coordinating the internalization of sulfate in plant root cells (Shibagaki and Grossman, 2010). Based on this regulatory model it will be interesting to test the impact of *sel1* mutation on the enzymatic activity of OASTL and the impact on the

accumulation of internal S-containing compounds. Any results of these tests can help us to understand more about the altered transporter function, the sensing capability or both. In one of our previous tests with the S-containing metabolites, sulfide, Cys, and GHS, we found all treatments with these compounds displayed a similar GUS strength as the treatment of 1.6mM sulfate, both in WT and the two mutants, *sel1-15* and *sel1-16* (Fig. 4.8). We explained this result in Chapter IV as the interruption of sensing capability by the *sel1* mutations. It will be interesting to determine how SULTR1;2 also senses these internal sulfate metabolites.

Clearly, a critical question remains, what is (are) sensed by SULTR1;2? Is it simply sensing external sulfate or internal sulfate and its metabolites such as sulfite, sulfide, Cys, Met, and GSH? There is no direct experiment that has addressed these questions, but it has been proposed that there must be more intracellular S-compounds that are sensed other than only the outside sulfate status (Hawkesford and De kok, 2006; Hawkesford, 2007). Our experimental results tend to support the latter, especially in the treatment shown in Fig.4.6 of Chapter 4. The experiment shown in Fig. 3.3 and 5.1 displayed a capability of sensing the C and N source change by the SULTR1;2. More experiments such as that of direct binding between the sensor and the sensed are needed before we can answer correctly what is actually sensed by SULTR1;2.

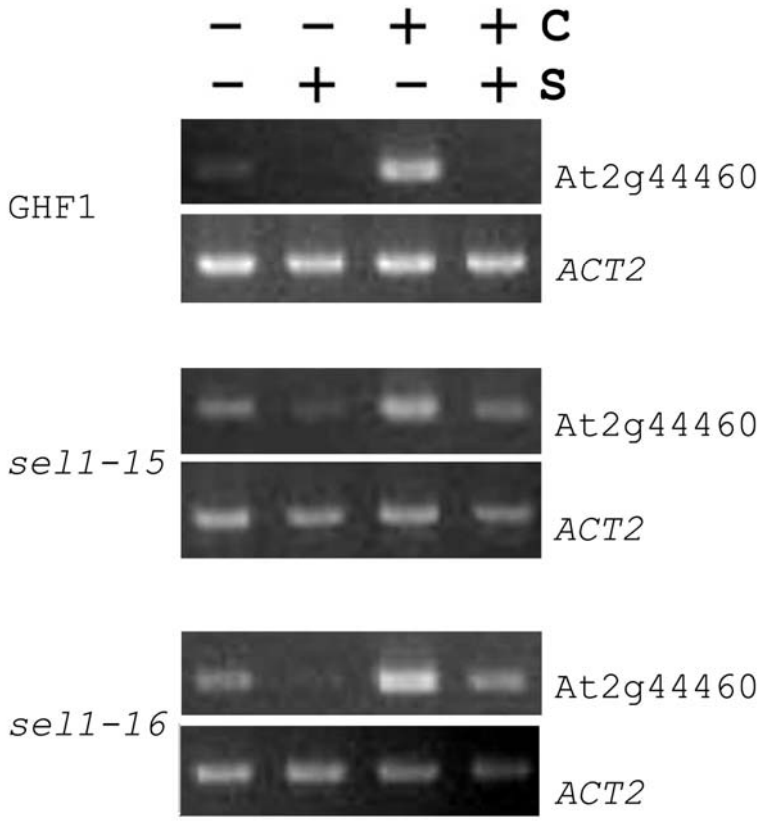

Figure 5.1 Alteration of C-S crosstalk in *sel1* alleles: RT-PCR on the endogenous reporter gene, At2g44460.

Seeds of the three genotypes were grown and treated as above. After treatment, seedlings were harvested and ready for RNA extraction, reverse transcription and RT-PCR. Primers for RT-PCR are the same as in Table 4.1 (YZP 63, 64 for At2g44460; and ACT2S, ACT2A for *ACT2*). Notice that *sel1-15* is the mutant originally designated as 11p201h.

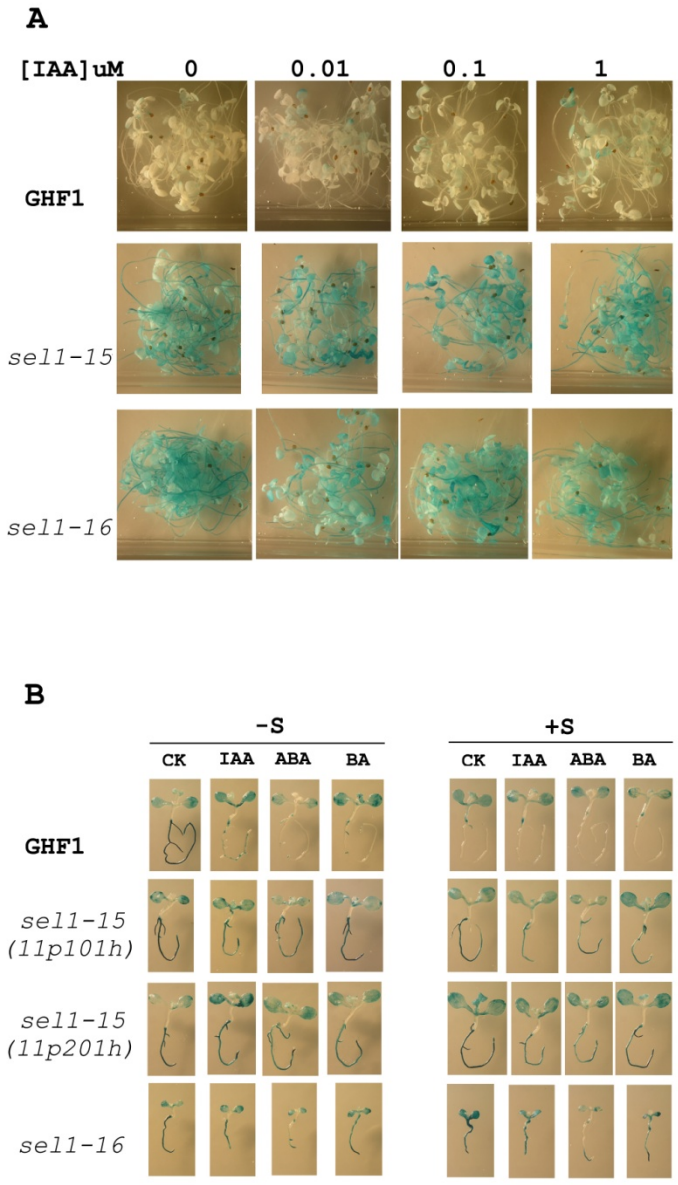

Figure 5.2 *sel1* alleles altered the hormonal response under –S condtions

A. Seeds of WT and mutants were sowed on MS plates with the four concentrations of IAA pre-added inside the solidified media directly. Germinated seedlings were vertically grown in programmed growth chamber for 11d, and then subjected to GUS staining.

B. Seeds of WT and mutants were sowed on MS plates for 5d growth, and then treated with –S and +S media for 2d. The indicated hormones were applied in a 1μM final concentration by directly added inside the liquid media for treatment. CK means without hormones. IAA, Indole-3-acetic acid (auxin); ABA, Abscisic acid; BA, Benzyladenine (cytokine).

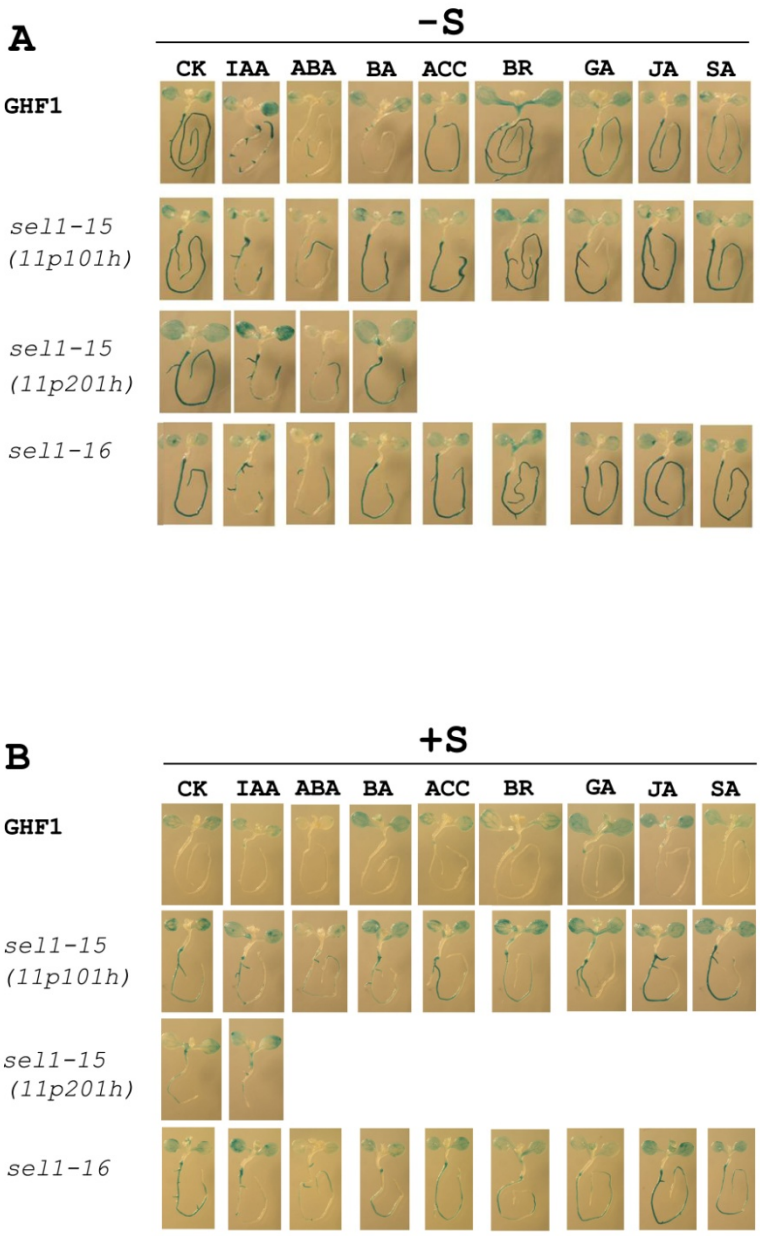

Figure 5.3 More tests on hormonal responses of *sel1* alleles

This experiment is used to show the mild differences between difference experiments on the same topic. Pay attention to the IAA, ABA reaction under –S condtion, which is stronger at Fig. 5.2B but weaker at Fig. 5.3A.

A. Tests on –S condition; B. Tests on +S condtions. This is another experiment using different batch of seeds from the experiment of Fig. 5.2. All experimental methods are the same as in Fig. 5.2. IAA, Indole-3-acetic acid (auxin); ABA, Abscisic acid; BA, Benzyladenine (cytokine); ACC, 1-Amino-Cyclopropane-1-Carboxylic acid (ethylene precursor); BR, brassinosteroids; JA, methyl jasmonic acid; SA, salicylic acid.

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