SULFUR STUDIES USING THE HALOPHYTE *EUTREMA SALSUGINEUM*

SULFUR NUTRITION STUDIES USING THE HALOPHYTE *EUTREMA SALSUGINEUM*

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

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McMaster University MASTER OF SCIENCE (2016) Hamilton, Ontario

TITLE: Sulfur nutrition studies using the halophyte *Eutrema salsugineum*

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PAGES: xvi, 122

LAY ABSTRACT

Eutrema salsugineum is a model for studies of plant abiotic stress tolerance. I determined that the Yukon ecotype of *Eutrema* displays elevated expression of sulfur deficiency genes when grown in cabinets. Increasing soil sulfur level roughly 10-fold in the potting mix led to significantly increased biomass, leaf area and leaf number and reduced transcript abundance of sulfur deficiency genes. One sulfur-responsive gene encodes a novel long non-coding RNA. I adapted a method for transiently repressing its expression, an approach that can be used to explore the function of this and other novel genes in *Eutrema.*

ABSTRACT

Eutrema salsugineum is an extremophile plant that is naturally tolerant to abiotic stresses such as high salinity, drought and cold (Inan et al., 2004, Griffith et al., 2007; MacLeod et al., 2015). It is emerging as a stress tolerant model plant, due to its short life cycle and high genetic similarity to the model plant *Arabidopsis* (Inan et al., 2004). There are two well-studied ecotypes of *Eutrema,* the Shandong ecotype from the Shandong Province of China, and the Yukon ecotype that grows in the Yukon, Canada. Principal component analysis (PCA) comparing the transcriptomes of three Yukon cabinet plants, three Shandong cabinet plants, and three Yukon field plants revealed that Yukon cabinet plants load negatively along PC3, while the Shandong cabinet and Yukon field plants load positively. Of the top 50 genes that contribute to this negative loading, 12 are related to sulfur deficiency, leading to the hypothesis that the cabinet plants are deficient for sulfur. To test this hypothesis cabinet potting soil was supplemented with calcium sulfate dihydrate to raise the sulfur level to approximately that of Yukon field soil. Sulfur-treated plants were compared to those grown on unsupplemented soil and untreated plants had significantly reduced biomass and leaf area. Additionally, RT-qPCR showed that relative to sulfur-supplemented plants, untreated plants had 4 to 177-fold higher transcript levels of two sulfur deficiency marker genes, *Sulfur deficiency induced 1* and *γ-glutamylcyclotransferase 2;1.* While these findings are consistent with untreated plants experiencing a sulfur deficiency, there was no difference in chlorophyll content, and rosette sulfur levels were only 1.1-fold higher in the sulfur-

treated plants. The third most negatively loading gene on PC3 was identified as *XLOC_003912*, a long non-coding RNA of unknown function that is only expressed in Yukon cabinet plants. Because it shows a similar loading pattern to the 12 sulfur nutrition-related genes, it was hypothesized to be involved in sulfur homeostasis. RT-qPCR showed that *XLOC_003912* expression was lower in sulfur-treated compared to untreated plants. A method was developed to suppress *XLOC_003912* expression in Yukon *Eutrema* plants by Virus Induced Gene Silencing (VIGS). In addition to providing insight into the function of *XLOC_003912*, this technique can be used in future studies for determining the role of novel genes in *Eutrema salsugineum.*

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Elizabeth Weretilnyk for her support and guidance of my research. I would also like to thank the other members of my committee Dr. Robin Cameron and Dr. Brian Golding for their feedback and support. I am also very grateful for the help of Dr. Peter Summers.

I received help from many students of the Weretilnyk and Cameron labs including Vera Velasco, Caitlin Simopoulos, Mitch MacLeod, Phil Carella, and Irina Sementchoukova as well as Jennifer Faubert, who previously held the position of lab tech in Dr. Weretilnyk's lab.

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LIST OF ABBREVIATIONS AND SYMBOLS

µL	Microlitres(s)
µM	Micromoles per litre
µmol	Micromoles
ANOVA	Analysis of variance
APS	Adenosine 5'-phosphosulfate
C	Celsius
cm	Centimetres
Cq	Quantitation cycle
ddH_2O	Double-distilled H_2O
DHA	Dehydroascorbate
DW	Dry weight
EL	Expanding leaf
FE	Fully-expanded
FLC	Flowering locus C
FW	Fresh weight
g	Grams
GGCT2;1	γ-Glutamylcyclotransferase 2;1
GSH	Reduced glutathione
h	Hours
L	Litre
lncRNA	Long non-coding RNA

miRNA	MicroRNA
mL	Milliliters
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PC	Principal component
PDS	Phytoene desaturase
RISC	RNAi Silencing Complex
ROS	Reactive oxygen species
RPM	Rotations per minute
RT-qPCR	Real time quantitative polymerase chain reaction
SAM	S-adenosyl methionine
SDI1	Sulfur deficiency induced 1
SE	Standard error
siRNA	Short interfering RNA
SQDG	Sulfoquinovosyldiacylglycerol
TRV	Tobacco Rattle Virus
VIGS	Virus-induced gene silencing
wpi	Weeks post-inoculation

DECLARATION OF ACADEMIC ACHIEVEMENT

The research contained in this document has been completed by Amanda Garvin with the contribution of Dr. Elizabeth Weretilnyk, Dr. Peter Summers, Ms. Irina Sementchoukova, and Ms. Caitlin Simopoulos. Drs. Elizabeth Weretilnyk and Peter Summers assisted in the design of the research project and training of Amanda Garvin, and Caitlin Simopoulos aided with bioinformatic analysis of data. The cloning and preparation of VIGS vectors was done in collaboration with Ms. Irina Sementchoukova, and we acknowledge the advice and provision of some supplies for cloning and *Agrobacterium* transformation by Mr. Phil Carella. Amanda Garvin was responsible for carrying out the research, analyzing the data and writing the first draft of the thesis that was revised with the editorial assistance of Dr. Weretilnyk. Amanda Garvin formulated the sulfur-supplemented soil and tested its suitability for growing plants as part of a senior undergraduate thesis but the biological replicates and RT-qPCR analyses were completed in her Master's program

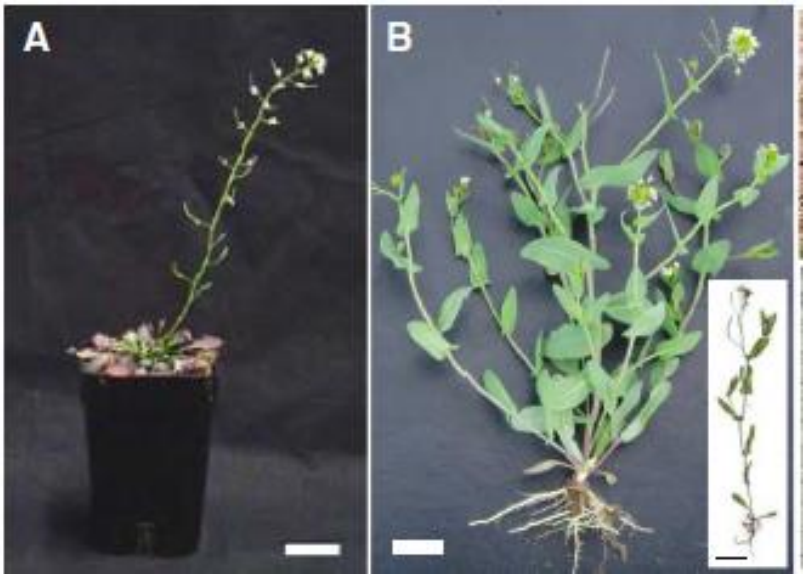
INTRODUCTION

I. *Eutrema salsugineum*

Eutrema salsugineum (*Thellungiella salsuginea*) is a close relative of *Arabidopsis thaliana*, and an excellent model system for stress tolerance research (Wong et al., 2005, Yang et al., 2013). *Eutrema* was identified as a potential model organism for its ability to tolerate extreme salinity as well as exhibiting many desirable traits such as short life cycle, small size, self-pollination, high seed number and an available annotated genome sequence (Bressan et al., 2001, Yang et al., 2013). In addition, *Eutrema* has 87.7% synteny with *Arabidopsis*, making protocols and mutants developed for *Arabidopsis* readily transferable to studies of *Eutrema* (Yang et al., 2013). The capacity for *Eutrema* to tolerate not only high salinity, but many other abiotic stresses such as drought or freezing temperatures (Inan et al., 2004, Griffith et al., 2007; MacLeod et al., 2015) and biotic challenges (Yeo et al., 2015) is now well established through the combined work of many research groups.

There are two *Eutrema* ecotypes that have been the subject of multiple studies. The Yukon ecotype is found on the highly saline soil of the Yukon, Canada, a semi-arid and subarctic region. The Shandong ecotype is found in the saline coastal areas of the Shandong Province, China. Under field conditions the Yukon ecotype displays prominent cauline leaves, clasping branching bolts that terminate in flowers, and either lack or display minimal rosette leaves. However, when grown in controlled-environment cabinets, Yukon *Eutrema* plants develop many large rosette

Figure 1: Phenotypic difference between *Eutrema salsugineum* plants of the Yukon ecotype when grown in the cabinet versus the field. *Eutrema salsugineum* plants of the Yukon ecotype A) grown in a controlled environment growth cabinet and B) found growing naturally in the Yukon, Canada. Figure from Guevara et al., 2012.



leaves and they also flower by bolting but often only a single, unbranched bolt emerges and the bolt has few, if any, cauline leaves (Fig. 1).

In order to look further into the difference between cabinet and field plants, Champigny et al. (2013) generated ten leaf transcriptomes, three each from Yukon and Shandong cabinet-grown plants and Yukon field plants collected at a field site known as "Dillabough's Grazing Lease", Yukon. They also prepared a transcriptome from the cauline leaves of a Yukon cabinet-grown plant. The authors identified a total of 27,000 genes by mapping RNA-Seq reads to the available reference genome of the Shandong ecotype (https://phytozome.jgi.doe.gov/). Only 3,000 of these genes were found to be differentially expressed between the three Shandong and Yukon plants under comparison, as determined using the DEseq Bioconductor package v1.6.1 (Champigny et al., 2013). Additionally, Principal Component Analysis (PCA) was used to compare transcript abundance between the datasets producing the results shown in Figure 2. In this analysis, PC1 explains 89.7% of the variance between the data, but all three groups had the same loading pattern and similar scores irrespective of where the plants grew (Champigny et al., 2013). However, PC2 and PC3 were reported as being more informative although these axes described far less of the variance between the ten datasets. In terms of information, PC3 explains only 2.5% of the variation yet this principal component separated the transcriptome data of Yukon cabinet plants from those of both the Yukon field and Shandong cabinet-grown plants. This makes PC3 a relevant component to look into regarding the difference in gene expression between Yukon field and cabinet plants. However

PC3 is not likely influenced by cabinet conditions per se due to the relative positioning of scores for the Yukon and Shandong transcriptomes describing plants that were both grown in a cabinet set under the same light and temperature conditions. Moreover, as discussed by Champigny et al. (2013), the different scores for the Yukon *Eutrema* plants found in the field versus cabinets is not a product of the different leaves found on field (cauline) and cabinet-grown (rosette) plants. The authors point out that the factor loadings for genes expressed in cauline leaves of cabinet plants show an even stronger negative loading along PC3, well apart from the more positive scores of transcriptomes from the cauline leaves of Yukon field plants. Another source of variation likely contributing to expression differences is environmental. Controlled environments are unable to replicate the complex and variable conditions found in the field and this was proposed as a significant influence over differentially expressed genes between the cabinet and field grown plants by Champigny et al. (2013).

Some insight into the environmental factors contributing to differences between Yukon plants in the field and Yukon plants grown in cabinets came to light following analysis of genes contributing to PC3 (Fig. 2). As discussed above, Figure 2 shows that the expression results for Yukon cabinet plants load negatively along PC3, whereas the data from Yukon field plants load positively. A list of the fifty top genes loading most negatively on PC3 was generated (Table 1; Champigny et al., 2013). Among the genes on this list are seven that are highlighted in yellow. These genes were identified by Watanabe et al. (2012) as being up-regulated in response

to sulfur deficiency. An additional six genes on the list are also denoted by green highlight as these are genes that are related to sulfur nutrition in some way, for example genes involved in the metabolism of sulfur-containing compounds or products that are cysteine-rich. This gene list offered the first evidence that Yukon plants grown in our controlled environment chambers with commonly used commercial soils were very likely sulfur deficient. Moreover, this condition is likely exacerbated as plants age given the higher expression levels of sulfur deficiency responsive genes in cauline leaves of older, flowering plants compared to rosette leaves of four-week-old plants (Champigny et al. 2013).

II. Yukon field conditions

There are several environmental differences between field and cabinet conditions. In June of 2014 I travelled to three Yukon field sites in order to collect plants samples. Some of the differences I observed were light-related. Light intensities in the field could reach 1200 μmol/m^2/s, whereas the cabinet light intensity is usually set to 300 μmol/m^2/s. Conditions such as temperature and light are also much more variable in the field, but constant in the cabinet. The site with the most *Eutrema* plants growing was Dillabough's Grazing Lease (DGL), a grazing site for horses. The animal grazing creates variability in the environment, as organic deposits from the animals affect the nutrient content of the soil. However, there are some variables in the soil that are fairly constant including the following conditions reported by Guevara et al. (2012) of highly alkaline soil with a pH exceeding 8.0

Figure 2: Biplot of PC2 versus PC3 generated from a PCA analysis using ten leaf transcriptomes from an RNA-Seq study of *Eutrema.* Transcriptomes analysed were from three Shandong cabinet-grown plants (SC), three Yukon cabinet-grown plants (YC), three Yukon plants collected from a field site (YF), and one from cauline leaves of cabinet-grown Yukon plants (cln). Library scores designated by red circles (●). Small grey points represent genes whose expression does not differ between the three sources (SC, YC, and YF). Circles representing genes expressed more highly in one source of tissue are shown for YF plants (●), YC plants (●) or SC plants (●). Figure generated from data reported by Champigny et al. (2013; Additional file 8).

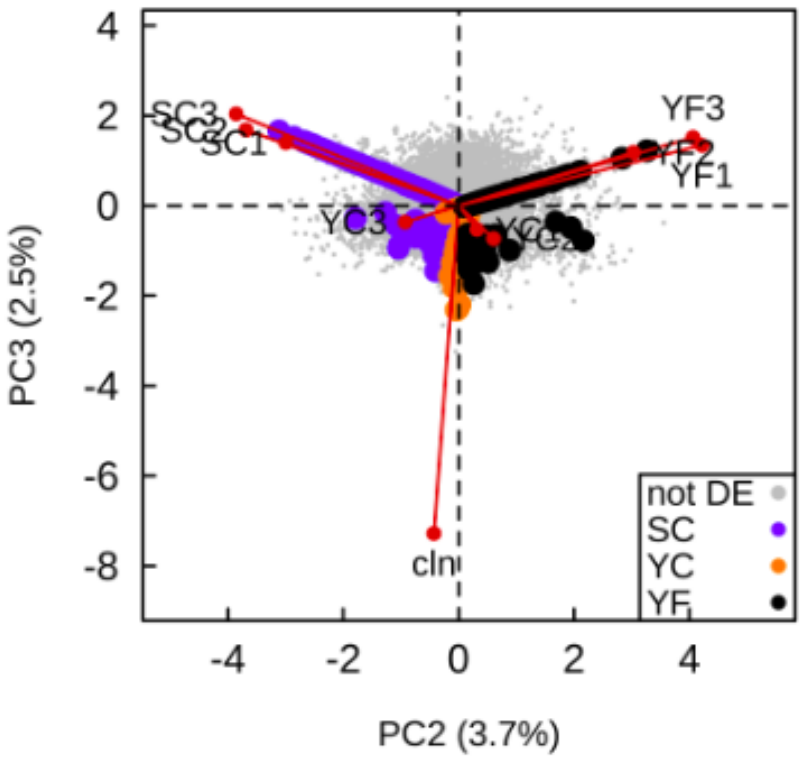


Table 1: List of the top 50 most negatively loading genes along PC3.

Top fifty genes contributing to the negative loading of Yukon cabinet plants on PC3 including the Yukon cabinet plant cauline leaf transcriptome (cln). The list corresponds to the PCA shown in Figure 2 based upon data reported by Champigny et al. (2013). Genes highlighted in yellow were previously reported by Watanabe et al. (2012) as being up-regulated during sulfur deficiency in plants. Genes highlighted in green have characteristics related to sulfur nutrition.

Gene	Ath loci	Ath desc
Thhalv10001756m.g	AT2G44460	beta glucosidase 28
Thhalv10009327m.g	AT1G12030	Protein of unknown function (DUF506)
XLOC_003912		
XLOC_017404	AT5G64140	ribosomal protein S28
Thhalv10020688m.g	AT3G05400	Major facilitator superfamily protein
Thhalv10004906m.g	AT5G66400	Dehydrin family protein
Thhalv10028733m.g	AT4G11320	Papain family cysteine protease
Thhalv10005154m.g	AT5G66780	
Thhalv10004135m.g	AT5G27360	Major facilitator superfamily protein
Thhalv10022994m.g	AT2G15220	Plant basic secretory protein (BSP) family protein
Thhalv10008003m.g	AT2G40920	F-box and associated interaction domains-containing protein
Thhalv10027060m.g		
Thhalv10003456m.g	AT5G33370	GDSL-like Lipase/Acylhydrolase superfamily protein
Thhalv10021737m.g	AT3G20450	B-cell receptor-associated protein 31-like
Thhalv10017840m.g	AT2G29460	glutathione S-transferase tau 4
Thhalv10019589m.g	AT1G67980	caffeoyl-CoA 3-O-methyltransferase
Thhalv10005858m.g	AT3G60140	Glycosyl hydrolase superfamily protein
Thhalv10008991m.g	AT1G24020	MLP-like protein 423
Thhalv10023787m.g	AT2G02100	low-molecular-weight cysteine-rich 69
Thhalv10024886m.g	AT4G20820	FAD-binding Berberine family protein
Thhalv10017193m.g	AT2G29470	glutathione S-transferase tau 3
Thhalv10004656m.g	AT5G48850	Tetratricopeptide repeat (TPR)-like superfamily protein
Thhalv10013645m.g	AT1G03410	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Thhalv10016927m.g	AT2G42840	protodermal factor 1
Thhalv10001180m.g	AT5G46610	Aluminium activated malate transporter

		family protein
Thhalv10026943m.g	AT5G53160	regulatory components of ABA receptor 3
XLOC_009118	AT4G38790	ER lumen protein retaining receptor family protein
Thhalv10002835m.g	AT4G09490	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
XLOC_005367		
Thhalv10026727m.g		
Thhalv10001647m.g	AT2G47770	TSPO(outer membrane tryptophan-rich sensory protein)-related
Thhalv10004888m.g	AT5G26220	ChaC-like family protein
Thhalv10029064m.g	AT1G35430	
Thhalv10011952m.g	AT1G54095	Protein of unknown function (DUF1677)
Thhalv10007399m.g	AT1G11600	cytochrome P450, family 77, subfamily B, polypeptide 1
Thhalv10010826m.g	AT3G51590	lipid transfer protein 12
Thhalv10018244m.g	AT1G78000	sulfate transporter 1;2
Thhalv10024976m.g	AT4G13310	cytochrome P450, family 71, subfamily A, polypeptide 20
Thhalv10025166m.g	AT4G28420	Tyrosine transaminase family protein
Thhalv10023929m.g	AT1G62420	Protein of unknown function (DUF506)
Thhalv10021448m.g	AT3G19590	Transducin/WD40 repeat-like superfamily protein
Thhalv10028292m.g	AT4G11700	Protein of unknown function (DUF626)
Thhalv10005180m.g	AT5G48210	Protein of unknown function (DUF1278)
Thhalv10014701m.g	AT5G53670	
Thhalv10015393m.g	AT5G09490	Ribosomal protein S19 family protein
Thhalv10000225m.g	AT2G23030	SNF1-related protein kinase 2.9
Thhalv10005451m.g	AT3G25080	Protein of unknown function (DUF626)
Thhalv10019752m.g		
Thhalv10022957m.g	AT2G15220	Plant basic secretory protein (BSP) family protein
Thhalv10004168m.g	AT5G61290	Flavin-binding monooxygenase family protein

compared to the slightly acidic cabinet potting mix (pH 6.5). Yukon soil also has a high sodium content (2224 ppm) compared to the cabinet potting mix (89 ppm), making soil in the field highly saline. Additionally, and perhaps most importantly for this study, the level of sulfur in the field soil can reach 9000 ppm, 45-fold higher than the level of sulfur in the cabinet potting mix of 200 ppm (Table A3, Appendix). While any of these factors could be responsible for differences between Yukon cabinet and field plants, the evidence points towards sulfur as an important contributor to this difference.

III. Overview of Sulfur Nutrition in Plants

A) Role of Sulfur in the Plant

Sulfur is an essential macronutrient for plants along with nitrogen, potassium, phosphate, calcium and magnesium. Sulfur usually comprises from 0.1 to 1% of the plant dry weight, however in halophytes it can reach as high as 3% (Thomas et al., 1950). Sulfur is a component of the amino acids cysteine and methionine, making it essential for protein synthesis (Takahashi et al., 2011) particularly through translation initiation of all proteins, which requires methionine (eukaryotic ribosomes) or *N*-formylmethionine (plastid and mitochondrial ribosomes) (Brosan and Brosan, 2006). Approximately 80% of the organic sulfur in plants that have received sufficient sulfur is present in proteins (Friedrich and Schrader, 1977). Methionine can also be modified to *S*-adenosyl methionine (SAM), which serves as a methyl donor for many necessary plant compounds such as the

cell wall component pectin, methylation of nucleic acids or proteins, and the biosynthesis of the photosynthetic pigment chlorophyll (reviewed in Moffatt and Weretilnyk, 2001). Adams and Yang (1977) showed that SAM is involved in the synthesis of ethylene, a volatile signaling molecule that participates in communication within and between plants allowing the plant to respond to both biotic and abiotic stresses. Sulfur is also important for membrane sulfolipids, a fraction that can account for up to 13% of total plant membrane lipids in Proteaceae species (Lambers et al., 2012). Glutathione is another important sulfur-containing molecule that works with ascorbate as an antioxidant to detoxify compounds such as hydrogen peroxide, a reactive product that can be harmful to the plant (Foyer and Halliwell, 1976). Sulfation is the addition of a sulfur molecule to substrates and chemical modifications through sulfation can inactivate compounds in the plant, as is the case for brassinosteroids and jasmonate (Rouleau et al., 1999; Gidda et al., 2003). In addition, there are several sulfur-containing specialized metabolites such as glucosinolates that accumulate in response to herbivory, particularly in cruciferous plants (Mewis et al., 2006). Human consumption of *Brassica oleracea* (including cabbage, broccoli, cauliflower) has been linked with a lower risk of some cancers, and beneficial glucosinolates have been identified as a contributor to this protection (Bellostas et al., 2007).

B) Sulfur Uptake and Assimilation by Plants

Sulfur is taken up by plant roots as sulfate by proton/sulfate transporters that use proton gradients as a driving force for sulfate uptake (Lass and Ulrich-Eberius, 1984). Both *Arabidopsis* and rice have 14 putative sulfate transporters divided into four groups with closely related sequences and a fifth more distant one (Buchner et al., 2004). The sulfate transporters can also be classified into high and low affinity with respect to their sulfate substrate. SULTR1;1 and 1;2 are high affinity transporters responsible for loading sulfate from the soil into the root (Yoshimoto et al., 2002; Takahashi et al., 2000). In keeping with their role, SULTR1;1 and 1;2 are expressed mainly in root hairs and cortical cells of *Arabidopsis* roots (Yoshimoto et al., 2002). Sulfate likely then moves to the central cylinder via plasmodesmata (Takahashi et al., 2000) and is loaded into the xylem by the low affinity transporter SULTR2;1 whose activity is enhanced by SULTR3;5, another transporter that co-localizes with SULTR2;1 (Takahashi et al., 2000; Kataoka et al., 2004).

Studies of sulfur assimilation began in yeast, where it was determined that sulfate must be converted to an active form known as 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Robbins and Lipmann, 1958). This is done in a two-step, enzymatic process wherein sulfate is first converted to adenosine 5'-phosphosulfate (APS) by an enzyme called ATP sulfurylase, and then phosphorylated to PAPS by APS kinase (Robbins and Lipmann, 1958). A similar pathway was later identified as operating in plants (Burnell and Anderson, 1973). However, while PAPS is used as

the substrate for sulfate reduction to sulfite in yeast, this was not found to be the case in plants (Schmidt, 1977). Gutierrez-Marcos et al. (1996) identified three enzymes in *Arabidopsis* that were able to complement a PAPS reductase *Escherichia coli* mutant, but each enzyme worked much more efficiently with APS than PAPS in generating sulfite, providing evidence that plants preferentially use APS as the sulfate donor for assimilation (Gutierrez-Marcos et al., 1996). Once sulfite is generated, it is reduced to sulfide by sulfite reductase (Bork et al., 1998). Sulfide is then incorporated into *O*-acetylserine to form cysteine by the combination of serine acetyltransferase and *O*-acetyl serine thiol lyase, that together form a cysteine synthase complex *in vivo* in higher plants (Bogdanoca and Hell, 1997). Serine acetyl transferase generates *O*-acetylserine from serine, and *O*-acetylserine thiol lyase combines this activated form of serine with sulfide to generate the amino acid cysteine (Bogdanoca and Hell, 1997). The other sulfur containing amino acid, methionine, is synthesized in plastids by two steps. Firstly, cysteine is converted to homocysteine by the enzymes cystathione γ-synthase and cystathionine β-lyase and then homocysteine is methylated by methionine synthase, using 5-methyltetrahydrofolate as a the methyl donor (Ravanel et al., 2004).

C) Regulation of Sulfur Homeostasis in Plants

Maruyama-Nakashita et al. (2006) created a line of *Arabidopsis* plants transformed with the gene encoding the Green Fluorescent Protein (GFP) reporter under the control of the sulfur-responsive *SULTR1;2* promoter. They used random

mutagenesis followed by screening to identify mutants that were unable to induce GFP expression under sulfur-limiting conditions in order to identify key transcriptional regulators of the sulfur-limitation response. They named a mutant identified by their screen *sulfur limitation 1* (*SLIM1*), and identified the gene they disrupted as encoding an ethylene-insensitive3-like3 family transcription factor. Mutation of this gene causes impaired plant growth and sulfur uptake under sulfur limiting conditions as well as excessive accumulation of glucosinolates (Maruyama-Nakashita et al., 2006). In order to identify genes under the control of SLIM1, Maruyama-Nakashita and co-workers also compared the expression profiles of *slim1* to wildtype plants under both sulfur-sufficient and limiting conditions. They identified several sulfur transporters *(SULTR1;1, SULTR1;2, SULTR3;4* and *SULTR4;2*) and a gene encoding thioglucosidase (At2g44460) involved in the degradation of glucosinolates as being up-regulated by SLIM1 under sulfur limitation. In addition, genes that code for the glucosinolate biosynthetic cytochrome P450 enzymes, *CYP79B2, CYP79B3* and *CYP83B1,* were shown to be down-regulated by SLIM1 in response to sulfur limitation. Maruyama-Nakashita et al. (2006) also found that SLIM1 regulates the expression of a previously identified MYB transcription factor, MYB34/APR1, that up-regulates indolic glucosinolate synthesis in *Arabidopsis* (Celenza et al., 2005).

Another well-known regulator of the sulfur limitation response in plants is miRNA395 (Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009). Jones-Rhoades and Bartel (2004) first identified miRNA395a-f computationally as a family

of six conserved miRNAs whose sequence identity is conserved between *Arabidopsis* and rice. The authors confirmed miRNA395 expression in *Arabidopsis* by using PCR to identify its presence in a library of small cDNAs, and identified that it was complementary to a 20 nucleotide sequence common to ATP sulfurylases (APS). Based on the complementarity they suggested that APS are probable target genes for miRNA395. They were able to confirm this hypothesis experimentally, identifying *APS4* mRNA as being cleaved by miRNA395. They also confirmed that miRNA395 expression was up-regulated in *Arabidopsis* during low sulfate conditions. Kawashima et al. (2009) subsequently found that miR395 was not induced in *slim1 Arabidopsis* plants suggesting that this miRNA is regulated by the SLIM1 transcription factor. They also found that the six different loci associated with miR395 vary in their level and location of expression within *Arabidopsis* plants.

IV. Plant Responses to Sulfur Deficiency

A) Growth of Plants and Sulfur Metabolism Under Low Sulfur Conditions

Arabidopsis plants subjected to a continuous sulfur deficiency show reduced total biomass after 13 days and reduced protein, chlorophyll, and total RNA content after 10 days (Nikiforova et al., 2003). When *Arabidopsis* is grown on sulfur-sufficient media then subjected to an induced sulfur deficiency, a decline in total biomass, chlorophyll, and protein content develops after just 6 days on insufficient media without a change in total RNA content (Nikiforova et al., 2003). Although sulfur deficiency decreases the total biomass and shoot to root ratio of *Arabidopsis*,

in contrast to many nutritional deficiency phenotypes, there is minimal impact of sulfur deficiency on root morphology (Gruber et al., 2013).

The impact of a long-term sulfur deficiency on plants has broad impacts on plant metabolism and morphology. A prolonged, constant nine-week exposure to sulfur deficient hydroponic conditions produced *Arabidopsis* plants with similar chlorophyll content as plants given sufficient sulfur. However, the activity of photosystems I and II in chloroplasts decreased by 30% in sulfur-starved plants compared to control plants (Ostazewska et al., 2014). The mitochondria of sulfur deficient plants were swollen with both a lower matrix density and fewer cristae than control plants (Ostazewska et al., 2014). The deficiency also led to a decrease in oxygen consumption by both leaves and roots of 40% and 35%, respectively, relative to controls (Ostazewska et al., 2014). In addition to adverse impacts on morphological and physiological traits, many important sulfur-containing compounds are significantly reduced in plants under sulfur deficiency, such as glutathione (7-16 fold), glucosinolates (up to 1000 fold), and SAM (3-30 fold) (Nikiforova et al., 2005). There is also an overall decline in lipid content in sulfur-deficient *Arabidopsis*, likely because of the involvement of the sulfur containing compounds acetyl-CoA and acyl carrier protein in fatty acid synthesis (Nikiforova et al., 2005).

B) Membrane Lipids

Sulfoquinovosyldiacylglycerol (SQDG) is a non-phosphorus glycerolipid synthesized by plants (Dormann and Benning, 2002). SQDG contains a sulfoquinovose in its polar head group, a glucose derivative with a sulfonate group replacing the 6-hydroxy (Yu et al., 2002). Among the structural lipids found in photosynthetic membranes, SQDG is one of the three most abundant, though it does not appear to have a direct role in photosynthesis in evolutionarily advanced plants (Yu et al., 2002; Kobayashi, 2016). When *Arabidopsis* is undergoing sulfur deficiency, the sulfolipid content of leaves decreases 10-fold (Nikiforova et al., 2005). Conversely, when *Arabidopsis* is undergoing a phosphorus deficiency, sulfolipid content increases. Evidence in support of this observation comes from use of the *Arabidopsis pho1* mutant, a plant that is unable to move phosphorus from roots to shoots and hence is used as a model for studies on phosphate deficiencies. For *pho1* plants the sulfolipid content is 300% higher than wild type plants given sufficient phosphorus (Essigmann et al., 1998). The relative content of sulfolipids increases with a corresponding decrease in the phospholipid phosphatidylglycerol when wild type plants are grown on media with a reduced amount of phosphate (Essigmann et al., 1998). In addition, the gene, *SQD1*, encoding a protein involved in sulfolipid biosynthesis has increased expression in the *pho1* mutant and in wild-type *Arabidopsis* plants exposed to reduced phosphate availability (Essigmann et al., 1998).

V. Molecular Genetic Responses to Sulfur Deficiency: Background and Research Approaches

A) Sulfur-Responsive Genes

In studies of nutrient deficiency in plants the phenotype that is displayed may be subtle, root-specific or visually imperceptible and not easily detected without destructive sampling. Our group recently described the lack of visible phenotypic traits associated with phosphorous deficiency in *Eutrema salsugineum* and relied upon the expression of phosphate-starvation-responsive genes to document phosphate stress in this species (Velasco et al., 2016). There are several sulfur-deficiency responsive genes, like *SLIM1* (described earlier), with well-documented responses to sulfur limitations that make them suitable reporters for this condition in plants. Indeed, several well-established sulfur deficiency marker genes exist and have been used to assess the sulfur status of *Arabidopsis* in sulfur homeostasis studies (Hubberten et al., 2012).

A gene associated with the locus At5g26220 was the first gene to be identified as sulfur responsive by Nikiforova et al. (2003) as it was 3.8 to 13.7-fold up-regulated in sulfur deficient compared to control seedlings. Watanabe et al. (2012) later identified the gene as encoding a ChaC-like family protein and reported it as showing 86-fold higher transcript levels in the roots of *Arabidopsis* plants grown on low (15 μM) sulfate for 10 days compared to control plants on sulfur-sufficient media. Expression of this gene is also increased in response to selenium treatment (40 μM for 10 days). Selenium interferes with sulfur metabolism because

it is taken up by the same system as sulfur and can be incorporated into amino acids in the place of sulfur, creating non-functional proteins (Watanabe et al., 2012). More recently, the product of At5g26220 was identified as γ-glutamyl cyclotransferase 2;1 (GGCT2;1), an enzyme involved in glutathione recycling (Paulose et al., 2013). Glutathione is a sulfur-containing tripeptide and antioxidant that is involved in many stress tolerance pathways, such as those involved in detoxifying heavy metals (Paulose et al., 2013). The first step in glutathione breakdown is catalyzed by γ-glutamyl transpeptidase (GGT), this reaction forms a cysteine-glycine dipeptide, and the remaining γ-glutamyl is transferred to a free amino acid to form a γ-glutamyl dipeptide. GGCTs then convert γ-glutamyl dipeptide to 5-oxoproline, which is converted to glutamate (Paulose et al., 2013). Given that the levels of glutathione are reduced during sulfur deficiency, it is not surprising to find that *GGCT2;1* is up-regulated under these conditions (Nikiforova et al., 2005) and indeed, it has been used as a sulfur deficiency marker gene in *Arabidopsis* (Hubberten et al., 2012).

The *SDI1* (*sulfur deficiency induced 1*) gene at the *Arabidopsis* locus At5g48850 is sulfur deficiency-responsive in both *Arabidopsis* and wheat (Watanabe et al., 2012; Howarth et al., 2009). In *Arabidopsis* roots from plants subjected to low sulfate (15 μM) for 10 days, *SDI1* expression was increased 89-fold and it also responded to selenium treatment (Watanabe et al., 2012). In wheat (*Triticum aestivum*), *SDI1* expression was induced in both leaf and root tissue in response to sulfate deficiency, but not deficiencies of nitrogen, phosphorus, potassium, or magnesium (Howarth et al., 2009). This provides evidence that *SDI1* is induced

specifically in response to sulfur deficiency. When plants have sufficient sulfur, they store sulfate in the vacuoles and deplete these stores in response to sulfur deficiency (Howarth et al., 2009). Arabidopsis *SDI1* knockout mutants grown on media lacking sulfur maintain a high leaf and root sulfur concentration compared to the wildtype, a finding consistent with the proposal that *SDI1* may play a role in the regulation of stored sulfate pools under deficient conditions (Howarth et al., 2009). Due to its consistent and selective expression in response to low sulfur *SDI1* has been used as a sulfur deficiency marker gene in studies of sulfur homeostasis (Hubberten et al., 2012).

The lack of annotation for several genes in Table 1 serves to emphasize that many genes, including sulfur-responsive genes, do not have a known function. Moreover, those designated as "XLOC" on the list represent products that were not annotated in *Eutrema salsulgineum* before the transciptome analysis done by Champigny et al. (2013). The third gene on this list, designated *XLOC_003912* is an example of a gene of unknown function and merits discussion because it is an example of a long non-coding RNA that shows co-expression with several known sulfur deficiency responsive genes (Table 1).

B) Long Non-coding RNAs (lncRNAs)

lncRNAs have recently been recognized as major regulatory components of many plant developmental pathways such as flowering and root development (Csorba et al., 2014;Lauressergue et al., 2015). One of the possible functions of an

lncRNA is a microRNA precursor. In plants, microRNAs are short, 21 to 24 nucleotide-long sequences of RNA that are cut by an enzyme called Dicer from a longer sequence of RNA called the pre-miRNA sequence (Llave et al., 2002; Park et al., 2002). The pre-miRNA sequence contains a stem-loop structure (Llave et al., 2002). One of the ways that these miRNAs can function is by being incorporated into an RNA-induced silencing complex (RISC) that is guided to target RNAs complementary to the miRNA, a complex that includes Slicer, an enzyme that cleaves the target RNA (Baumberger and Baulcombe, 2005). Aukerman and Hajirne (2003) identified an miRNA that regulates flowering time with a lncRNA precursor of 1.4 kb, though much shorter pre-miRNA sequences have been found as well (Llave et al., 2002). LncRNAs can also regulate genes via antisense binding. For example, Csorba et al. (2014) identified *COOLAIR* as a group of lncRNAs that is transcribed antisense to the *Flowering Locus C* (*FLC*) gene and regulates *FLC* expression in *Arabidopsis*. *COOLAIR* is alternatively spliced and polyadenylated so that it can either reduce or increase *FLC* transcription, as required, under different conditions. The *COOLAIR* lncRNA transcript has been shown to physically associate with *FLC* chromatin in regulatory regions. Antisense binding of a lncRNA to genomic DNA or other RNA transcripts is another mechanism of regulation by non-coding RNAs. Recently, some lncRNAs have been shown to code for short peptides less than 70 amino acids in length (Lauressergue et al., 2015). These peptides can play a regulatory role in the plant. For example, in *Medicago truncatula*, pre-miRNAs were shown to encode

regulatory peptides that enhance the accumulation of their own pre-miRNAs (Lauressergue et al., 2015).

C) Manipulation of Gene Expression Using VIGS

Elucidating functions of lncRNA is a challenge regardless of the organism under study, in part due to the lack of evolutionary conservation in DNA sequence (Ponting et al., 2009). This is equally true for the lncRNA *XLOC_003912* in *Eutrema*. One approach is to manipulate expression to reveal a phenotype. Virus Induced Gene Silencing or VIGS is one method of transiently manipulating gene expression.

VIGS is a method of inducing RNA interference in plants (Waterhouse and Helliwell, 2003). This approach takes advantage of a naturally occurring defense mechanism whereby plants are able to recognize viral RNA and degrade it into 21 nucleotide long, double-stranded RNA molecules called short interfering RNAs (siRNAs) (Baumberger and Baulcombe, 2005). The fragments are used as a guide for the RNAi silencing complex (RISC), which seeks out and degrades mRNAs complementary to the siRNA (Baumberger and Baulcombe, 2005). In VIGS, a viral vector containing a fragment of a gene of interest is transformed into *Agrobacterium tumefaciens*. The *Agrobacterium* is then used to inoculate plants with a needle-less syringe through the stomata (Burch-Smith et al., 2006). One of the most popular VIGS vectors is one derived from the tobacco rattle virus (TRV) and it has been tested in many systems such as *Arabidopsis*, tomato, pepper plants, and more recently, in Madagascar periwinkle (Burch-Smith et al., 2006; Liscombe and

O'Connor, 2011). TRV has the distinct advantage of infecting the meristem of host plants, allowing for the possibility of offspring in which the silencing persists (Burch-Smith et al., 2006). To date there are no reports on the use of VIGS vectors in *Eutrema* spp.

VI. The Role of Sulfur in Plant Responses to Stress

In 1976, Foyer and Halliwell isolated both the sulfur containing compound glutathione (GSH) and the enzyme glutathione reductase from spinach chloroplasts. They showed that GSH can reduce dehydroascorbate (DHA) to ascorbate at pH 7 and above, which is the pH found in the stroma of chloroplasts. They then proposed what is now known as the ascorbate-glutathione pathway, wherein ascorbate reduces the reactive oxygen species (ROS) superoxide to hydrogen peroxide, and then hydrogen peroxide to water. This generates DHA, the reduced form of ascorbate, which is then regenerated to ascorbate by the reduction of GSH (catalyzed by GSH reductase in a reaction involving NADPH). Generation of reactive oxygen species as a result of stress to the plant was first discovered in potato protoplasts infected with *Phytophthora infestans* (Doke, 1983). This response was later confirmed to occur in living potato leaves as well (Chai and Doke, 1986). Increases in the generation of reactive oxygen species have since been observed in plants experiencing abiotic stresses, such as salinity (Tanou et al., 2009). In addition to the accumulation of ROS during salinity, increases in reduced ascorbate and GSH were observed as well, suggesting that the ascorbate-glutathione pathway is increasing its activity in order

to combat the ROS (Tanou et al*.,* 2009). Additionally, the GSH content of plants is reduced under sulfur deficiency, suggesting that GSH synthesis is dependant on the sulfur status of the plant (Nikiforova et al., 2003). This means that the ability of plants to respond to stress depends to some extent on their sulfur status.

VII. Sulfur and Nitrogen: Relationship Between Two Macronutrients

In order for sulfur to be assimilated into cysteine, the nitrogen-containing compound *O*-acetylserine must be present, linking the two nutrient assimilation pathways very early on (Bogdanova and Hell, 1997). When plants are sulfur deficient, protein synthesis slows and excess nitrogen that would normally go into amino acids synthesis for protein, accumulates (Friedrich and Schrader, 1978). In *Arabidopsis* plants, this nitrogen is stored in the form of ureides (uric acid and allantoin) that accumulate in excess via purine catabolism under conditions of sulfur deficiency (Nikiforova et al., 2005). The combined accumulation of excess nitrogen and the reduction of sulfur content during sulfur deficiency leads to an increase in the nitrogen to sulfur ratio of plants (Nikiforova et al., 2005; Ostaszewska, 2014). Other complicating factors are physiological in nature. For example, sulfur deficiency may also increase photorespiration (Nikiforova et al., 2005), which occurs when Rubisco oxygenates instead of carboxylating its substrate ribulose 1,5-bisphosphate (Bowes and Ogren, 1972). The resulting two-carbon product, phosphoglycollate, must be metabolized in the chloroplast, peroxisome, and mitochondria involving the generation of carbon dioxide and re-generation of

phosphoglycerate (Bowes and Ogren, 1972). During this process, exacerbated under stress, excess ammonia is released by the mitochondria, further contributing to the sulfur/nitrogen imbalance during sulfur deficiency (Nikiforova et al., 2005). Nitrate reductase is responsible for the first step in nitrogen assimilation, the reduction of nitrate to nitrite (Migge et al., 2000). In maize plants, nitrate reductase activity decreases significantly within 9 days of sulfur starvation, and continues to decrease as the deficiency progresses (Friedrich and Schrader, 1978). When sulfur is added back in the soil, nitrate reductase activity recovers (Friedrich and Schrader, 1978). The sulfur-deficient maize plants also have a significantly increased nitrate content in leaf blades and stems, further establishing the link between nitrogen and sulfur (Friedrich and Schrader, 1978).

VIII. Biodiversity and Gene Discovery

Traditionally, sulfur deficiency has not been as great of a concern as other nutrient deficiencies (Coleman, 1966). Sulfur has a relatively low abundance in the plant, being an average of 30-fold lower than nitrogen, 8-fold lower than potassium and 2-fold lower than phosphorus (Lewandowska and Sirko, 2008). However, in the last few decades sulfur nutrition has become more of a concern. Beginning about fifty years ago, an increase in the prevalence of crop sulfur deficiency was observed (Coleman, 1966). Among the contributing factors, three are often cited as important considerations: 1) increased yields that, in turn, require more sulfur, 2) increased use of sulfur-free fertilizer, and 3) decreased use of sulfur-based insecticides

(Coleman, 1966). More recently, global sulfur emissions have decreased as a result of changes in various sulfur-emitting practices such as coal and fossil fuel combustion (Smith et al., 2011). All of these directly or indirectly impact the amount of sulfur deposited in soil. This trend of increasing sulfur deficiency in crops and the importance that this element has in stress tolerance discussed earlier has inspired this investigation of genes related to sulfur uptake and assimilation in plants. Although *Eutrema* is not a crop, the availability of two accessions, one requiring sulfur at elevated levels (Yukon ecotype) and a second without this apparent requirement (Shandong ecotype), sets an excellent stage from which the role of sulfur can be examined in an extremophile species. Thus the objective of this study was to lay the foundation for future research into the assimilation and metabolism of sulfur by *Eutrema* species. One promising outcome of this early work is the application of VIGS technology towards the identification of a novel, non-coding RNA whose expression pattern infers a role in sulfur nutrition specific to the Yukon ecotype of *Eutrema.*

HYPOTHESIS

Yukon cabinet plants have up-regulated expression of seven genes identified as being induced during sulfur deficiency compared to their field grown counterparts (Table 1). I hypothesized, based upon these expression results, that Yukon plants grown under cabinet conditions are experiencing a sulfur deficiency but Yukon plants in the field are not. The Shandong plants did not exhibit this pattern of gene expression, therefore I hypothesize that like the Yukon field plants, they are not experiencing a sulfur deficiency despite being grown under the identical cabinet conditions used for the Yukon ecotype. Finally, given the expression patterns of several sulfur-responsive genes listed on Table 1, I hypothesize that the lncRNA *XLOC_003912* is being expressed in the cabinet-grown Yukon plants and not the field plants because a) it is a sulfur-responsive gene product and b) plays a role in sulfur nutrition in *Eutrema salsugineum* plants of the Yukon ecotype.

MATERIALS AND METHODS

I. Materials

A) DEPC Treatment

All solutions, including those used for watering plants, were prepared with water purified on an ELGA Centra-S200/R200 system and, for the purpose of this thesis, this water is referred to as ddH_2O. For RNA work solutions were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, Missouri, USA, cat. no. D5758). ddH_2O and other solutions were filter-sterilized prior to DEPC treatment (EMD Millipore, cat. no. SLHV033RS). When DEPC was added, solutions were mixed thoroughly by swirling, incubated for a minimum of one hour at room temperature and then autoclaved to remove the DEPC.

B) Hot Borate Buffer

RNA extraction was performed using a hot borate buffer comprised of 200 mM sodium tetraborate (Caledon, cat#7360-1), 30 mM ethylenebis-(oxyethylenenitrilo)-tetraacetic acid (EGTA) (Fisher Scientific, cat#O2783-100), 1% (w/v) sodium dodecyl sulfate (SDS) (Life Technologies, cat# 15525-017), 1% (w/v) sodium deoxycholate (Sigma-Aldrich, cat. no. 0970), and 1% (w/v) polyvinyl-pyrrolidone (PVP40) (Sigma-Aldrich, cat. no. PVP40) were added to pre-heated ddH_2O in the order given. The solution was then adjusted to pH 9.0 using 1M NaOH, filter sterilized, and DEPC-treated.

C) General Use Buffers and Antibiotics

The Tris-EDTA (TE) buffer contained 10 mM Tris-HCl (Sigma-Aldrich, T6066), 1 mM Na_2-EDTA (Fischer Chemical, cat. no. S312), adjusted to pH 8 with 1 M HCl. Tris-acetate-EDTA (TAE) buffer used was 40 mM Tris-HCl, 20 mM acetic acid, 1 mM Na_2- EDTA, pH 8.5 (not adjusted). Stock rifampicin (Sigma-Aldrich, R7382) and acetosyringone (Sigma-Aldrich, D134406) were dissolved in dimethyl sulfoxide (BDH, 67-68-5) Rifampicin was protected from light exposure. Stock acetosyringone was stored at -20˚C and all other antibiotic stock solutions were stored at 4˚C.

II. Methods

A) Soil

Soil was prepared by mixing 6 L of Promix BX (Premier Horticulture, Rivière-du-Loup, PQ), 1 L of Turface (Profile Products LLC, Buffalo, NY) and 3.5 L of ddH_2O in an autoclave bin. The soil was then autoclaved for 35 minutes on a dry cycle and left to cool overnight. After cooling, 20 mL of Aquagro (Aqualtrois, Inc. Paulsboro, NJ) was added to the soil. Half of the soil was set aside for untreated plants. Calcium sulfate dihydrate (Sigma-Aldrich, cat. no. C3771) was added to the other half in a ratio of 6.6 g calcium sulfate per 700 g (approximately 1 L) of soil in order to achieve an estimated concentration of sulfate in the soil of around 5300 ppm, the average concentration of sulfur in the Yukon. The sulfur-treated soil and untreated soil was distributed into individual 5x5x7 cm pots and then placed in trays of 32 pots.

B) Seeds

Seeds of the Yukon (seed cat ref# T2021, T2025, T2090) and Shandong (seed cat ref# T755, T1514-T1533) ecotype that had been subjected to single seed decent for at least five generations were used. Seeds were surface-sterilized in 1.5 mL microfuge tubes by adding 1 mL of 70% (v/v) ethanol for 2 minutes, removing the ethanol and then adding 1 mL of 50% (v/v) bleach and 0.1% (v/v) Triton-X 100 (Sigma-Aldrich, cat. no. X100) for 8 minutes. The seeds were then washed with 1 mL of sterile ddH_2O at least ten times and suspended in sterile, 0.1% (w/v) Phytagel (Sigma-Aldrich, cat. no. P8169) overnight at 4˚C and used for seeding the next day.

C) Planting

The sterilized seeds were applied by pipette onto the soil using a P1000 tip with the end cut off, approximately five seeds were dispensed per pot. The pots were then covered with clear plastic lids and stored at 4˚C in the dark for 3 days before being moved to the growth chamber. Plants were grown at 300 μmol/m^2/s with a 21 hour day/3 hour night cycle and day/night temperatures of 22˚C/10˚C. Plants were watered daily to keep the soil moist. At 1.5 weeks post-germination the plastic lids were removed. Starting one week post-germination the plants were given 25 mL of 1 g/L 20-20-20 N:P:K fertilizer (Plant Products, 20-20-20) once-weekly.

D) Shoot Biomass Determination

At 4 weeks post-germination plants were removed from the growth chamber and severed at the junction between the root and shoot using a razor blade. The weight of the shoot was taken at this time and recorded as the shoot fresh weight. The plastic weigh boats containing the shoots were then placed on a tray and moved to an oven at 80˚C for one week. At this time, the dry weight of the shoot was obtained by weighing the boat with dried shoot together and subtracting the weight of the boat from this value.

E) Leaf Area and Number

At 4 weeks post-germination the plants used for fresh shoot weight measurements were used to determine leaf area. Leaves from the rosette were removed from the plant and placed adaxial-side down on a sheet of paper. Leaves were then taped to the paper with clear packing tape, the paper was scanned and the image imported into ImageJ version 1.41. In ImageJ the scale was set at 21.59 cm (8.5 inches) by drawing a line across the width of the paper and then selecting Analyze -> Set Scale to the value corresponding to the width of the paper. The image was then made binary by selecting Process -> Binary -> Make Binary and the leaf area was then determined in cm^2 by selecting Analyze -> Measure.

F) Chlorophyll Assay

Approximately 50 mg of leaf tissue (one or two fully-expanded leaves) was removed from the middle of the rosette of 4 week-old plants. These leaves were weighed and then placed in a chilled mortar with 1 mL of chilled 80% (v/v) acetone (Caledon, cat.no. 1201-2) and then the tissue was ground with a pestle until completely macerated. The suspension was transferred from the mortar to a 16 x 100 mm borosilicate glass test tube using a Pasteur pipette. Two 500 µL aliquots of 80% acetone were used to wash the mortar and combined with the suspension to yield a total volume of 2 mL. The extract was centrifuged in an International Clinical Centrifuge (model CL) for 60 seconds at Step 7 and the supernatant was transferred to a new 16 x 100 mm borosilicate glass tube. Using a glass cuvette the absorbance of the supernatant was measured with a spectrophotometer at 652, 663, 645 and 700 nm using 80% acetone as a blank. Chlorophyll content was calculated in mg/g fresh weight as follows:

Chlorophyll A = $(A_{663} - A_{700})$ x 8.02 x (mL acetone/mg fresh weight of tissue)

Chlorophyll B = $(A_{645} - A_{700})$ x 20.2 x (mL acetone/mg fresh weight of tissue)

Total Chlorophyll = chlorophyll A + chlorophyll B

The use of acetone as a solvent and the molar extinction coefficients used for calculations were as reported by Arnon, 1949.

G) RNA Extraction: Micro-scale Hot Borate

At 4 weeks post-germination, plants were removed from the growth chamber and 50 to 100 mg of leaf tissue, approximately two fully-expanded leaves, were removed from the middle of the rosette, weighed, and then wrapped in tin foil, immediately frozen in liquid nitrogen and then stored at –80˚C until RNA was extracted.

The method that follows is one suitable for small-scale extractions and is modified from one described by Champigny et al. (2013). For RNA extraction, leaf tissue was ground to a fine powder in a mortar using a pestle with approximately 10 mL of liquid nitrogen. To the ground tissue, 800 µL of Sigma Tri Reagent (cat. no. T9424) was added, the solution was allowed to thaw and then it was transferred to a 2.0 mL microfuge tube chilled on ice. The mortar and pestle were rinsed three times with an additional 200 µL of Sigma Tri reagent, with each rinse transferred to the microfuge tube containing the extracted tissue. The contents of the tube were mixed using a vortex for 30 seconds then centrifuged for 10 minutes at 4˚C at 16,000 RPM in a microcentrifuge. The supernatant was transferred to a new 2.0 mL centrifuge tube and allowed to sit at room temperature for 10 minutes. Next, 450 µL of chloroform (Caledon, cat. no. 3000-1) was added to the sample, the solution was mixed using the vortex for 30 seconds and the tube incubated at room temperature for 3 minutes. The tube was then centrifuged for 15 minutes at 16,000 RPM in a microcentrifuge at 4˚C. The upper aqueous phase was transferred to a 2.0 mL

centrifuge tube and 500 µL chloroform was added. The mixture was vortexed for 30 seconds and incubated at room temperature for 3 minutes then centrifuged at 16,000 RPM at 4˚C for 15 minutes. The aqueous phase was removed and transferred to a new 2.0 mL microcentrifuge tube and 1.2 mL isopropanol (Caledon, cat.no. 8600-1) was added to the aqueous solution. The sample was mixed by swirling and then allowed to sit at room temperature for 10 minutes. The tube was then centrifuged at 4˚C for 10 minutes at 16,000 RPM in a microcentrifuge, the supernatant was discarded and the tubes were inverted on a Kimwipe for about 20 minutes to let the pellet dry. When dry, the pellet was dissolved with 125 µL of DEPC-treated sterile ddH_2O. To the solubilized RNA a volume of 1.4 mL of hot borate buffer heated to 60˚C was added and the contents were mixed by vortexing for 30 seconds. The tube was placed on ice and 2 M potassium chloride (EMD, PX1330) was then added to a final concentration of 160 mM (133 µL), the solution was mixed well and then left on ice for 20 minutes. The sample was then centrifuged at 4˚C for 20 minutes at 16,000 RPM in a microcentrifuge. The supernatant was transferred to a new 2.0 mL microfuge tube, 430 µL of 7.5 M lithium chloride (Sigma, L9650) was added to yield a final concentration of 2 M lithium chloride, and the contents mixed in by inverting the tube. The sample was then left at -20˚C overnight. The sample was then centrifuged for 20 minutes at 4˚C at 16,000 RPM in a microcentrifuge. The supernatant was discarded and the pellet washed with 1.5 mL of chilled 2 M lithium chloride. The sample was then centrifuged at 4˚C at 16,000 RPM for 20 minutes in a microcentrifuge. The lithium chloride wash procedure was repeated until the pellet

went from opaque to clear, usually 2 to 4 times. The pellet was dissolved with 150 µL of 10 mM Tris-HCl (pH 7.5) and the solution transferred to a new 1.5 mL microfuge tube. The 2.0 mL tube was then rinsed once with 100 µL of 10 mM Tris-HCl (pH 7.5) and transferred to the 1.5 mL tube. To the pooled supernatant in the 1.5 mL microfuge tube, 65 µL of 1 M potassium acetate (pH 5.5) (EMD, PX1405) was added to a final concentration of 200 mM, and then 2.5 volumes of 100% ethanol was added. The sample was stored at -20˚C for 1 hour before centrifugation for 20 minutes 4˚C at 16,000 RPM in a microcentrifuge. The pellet was washed with 1 mL of 75% ethanol, the tube centrifuged again for 10 minutes at 4˚C and the supernatant was discarded. The pellet was allowed to air dry by leaving the tubes open at room temperature for about 5 to10 minutes. Then pellet was dissolved with 30 µL of DEPC-treated TE buffer and stored at -80˚C.

H) Two Approaches for the Determination of RNA Quality

The first method for assessing RNA quality and quantity used a Thermo Scientific NanoDrop 2000. The instrument determined RNA concentration (A_{260}) and the A_{260}/A_{280} and A_{260}/A_{230} ratios. The NanoDrop was cleaned by pipetting 1 µL of ddH_2O onto the lower sampling arm, closing then opening the lever arm and wiping excess water from the arm with a Kimwipe. The software program was then opened and the nucleic acids module was selected. The blank reading was taken by aliquoting 1 µL of TE buffer by pipetting onto the lower sampling arm and then closing the lever arm. The lower sampling arm and lever arm were them blotted dry

with a Kimwipe and 1 µL of the RNA sample was pipetted onto the lower sampling arm and nucleic acid content was measured. The lower sampling arm and lever arm were wiped with a Kimwipe between samples.

In the second approach, RNA quality and RIN (RNA Integrity Number) were assessed using an Agilent Technologies 2100 Bioanalyzer. This was done using the Agilent RNA 6000 Nano Kit and by following the manufacturer's Quick Start Guide. First, 550 µL of RNA 6000 Nano gel matrix was pipetted into the spin filter provided by the kit and then this matrix was centrifuged at 4000 RPM in a microcentrifuge for 10 minutes at room temperature. Next, 65 µL of the recovered gel matrix was pipetted into a 0.5 mL microfuge tube. While this was being done, the RNA 6000 Nano dye concentrate equilibrated at room temperature for 30 minutes. The Nano dye concentrate was then vortexed for 10 seconds, briefly spun down and 1µL of the Nano dye concentrate was then added to the filtered Nano gel matrix. The mixture was vortexed and then centrifuged at 14,000 RPM for 10 minutes at room temperature. The RNA 6000 Nano chip was placed in the priming station and 9 µL of the gel-dye solution was pipetted into the well that is marked with a G in a black circle. The plunger was positioned at 1 mL and then pressed until held by the clip. This was done for 30 seconds before the clip was released and then after an additional 5 seconds the plunger was pulled back to the 1 mL position and 9 µL of the gel-dye solution were then pipetted into the wells marked with a G, an additional 5 µL of RNA 600 Nano Marker was pipetted into all 12 sample wells while the ladder well was also loaded with 1µL of prepared ladder provided with the kit. Finally, 1 µL

of sample was then pipetted into the twelve sample wells. The chip was vortexed for one minute at 2400 RPM and then run on the Bioanalyzer.

I) DNase Treatment

DNase treatment was done using the Sigma Amplification Grade DNase I kit (cat.no. AMPD1). RNA samples were diluted to 1 µg of RNA in 8 µL of DEPC-treated ddH_2O in a PCR tube. Next, 1 µL of 10x reaction buffer and 1 µL of DNase I (Amplification grade, 1 unit/µL) was added to the tube and then samples were mixed gently by flicking and then spun down briefly. They were incubated at room temperature for 15 minutes and then 1 µL of Stop Solution was added. The samples were then heated in a Thermocycler (Bio-Rad, California, USA, C1000 Touch) to 70˚C for 10 minutes and then placed on ice. The samples were then immediately used for cDNA first strand synthesis (described below).

J) cDNA First Strand Synthesis

The first method used for cDNA synthesis used the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, 170-8840). Four µL of 5x iScript Reverse Transcription Supermix and 5 µL of nuclease-free ddH_2O (provided with the kit) were added to the 1 µg of DNase-treated RNA. For "no RT" controls, 4 µL of 5x No-RT Control Supermix and 5µL of nuclease-free ddH_2O were combined with 1 µg of RNA. The samples were then place in a Thermocycler (Bio-Rad, C1000 Touch) at

25˚C for 5 minutes, 42˚C for 30 minutes and 85˚C for 5 minutes. Tubes were then removed from the Thermocycler and stored at -20˚C.

A second method used for cDNA synthesis used the M-MLV Reverse Transcriptase Kit (Sigma- Aldrich, cat. no. M 302). RNA samples were diluted in DEPC-treated ddH_2O to 1 µg/10µL with the final volume being 10 µL. A reaction mix comprised of 2 µL 10 mM dNTP mix, 2 µL Oligo dT primer and 6 µL DEPC-treated ddH_2O was prepared and then 10 µL of the mix was added to each RNA sample. The solution was mixed gently, spun down and allowed to incubate at 70˚C for 10 minutes before the tube was placed on ice. A second mix of 4 µL of 10x M-MLV Reverse Transcriptase Buffer, 1 µL of Ribonuclease Inhibitor and 13 µL of DEPC-treated ddH_2O was made and 18 µL of this was added to each of the samples. Next, 2 µL of M-MLV Reverse Transcriptase was added to all of the samples. The samples were then left at room temperature for 10 minutes and then incubated at 37˚C for 50 minutes. The tubes were then heated to 80˚C for 10 minutes and then spun down and stored at -20˚C. A no-RT control was included. This control had RNA from all the samples pooled together such that the final volume was 10 µL. It was treated the same as the other samples except that no reverse transcriptase was added.

K) Primer Design

Primers were designed using the Primer-BLAST function on NCBI. Primers were designed to be 18 to 22 base pairs long, with a maximum melting temperature difference of 2˚C, and a GC content between 40 and 60%, and to provide a product

between 70 and 200 base pairs long. Primer-Blast returned fifteen primer pairs for each gene of interest and one or two pairs were selected from these fifteen. Secondary structure of each of the fifteen pairs was assessed using mfold RNA folding form, version 2.3 (Zuker, 2003). The primer sequence was entered in FASTA format into the text box and the temperature was adjusted to the melting temperature given by Primer-BLAST. Each primer pair was then analysed by BLAST on the Phytozome website against the masked genome labelled as *Thellungiella halophila* (correct current name is *Eutrema salsugineum*) Shandong accession. One or two primer pairs for each gene of interest that had minimal secondary structure and only one 100% match in the phytozome BLAST were selected. These genes were then analysed by BLAST against the *Eutrema salsugineum* Yukon accession genome by Wilson Sung to make sure that there were no secondary matches. All primers were obtained from the Mobix Lab at McMaster University.

L) Primer Optimization

Bio-Rad CFX Manager 3.0 was used to design templates for determining the optimal temperature of each primer. Four primers were tested per plate at eight different temperatures with two technical replicates per temperature. The MasterMix calculator on Bio-Rad CFX Manager 3.0 was used to determine the amount of LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich, cat. no. L6544) or SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad, cat. no. 1725018), as well as forward and reverse primers, and DEPC-treated ddH_2O to

include in the mastermix. Primers were diluted to a concentration of 10 µM before adding to the mastermix, for a final concentration of 200 nM. The well volume was 10 µL, and SYBR green concentration was 2x. The cDNA from all samples to be tested was pooled together and diluted five times and used as the template. A 96-well, clear Multiplate PCR Plate (Bio-Rad, cat. no. MLL9601) was loaded with 6 µL of mastermix and 4 µL of template pipetted into each well using a P10 pipettor that had been tested gravimetrically for accuracy. After the plate was loaded it was sealed with Microseal, 'B' Seals (Bio-Rad, cat. no. MSB1001). The plate was then run on a program with a two-step amplification, a temperature gradient, and a melt curve. The denaturation step was 95˚C for 3 minutes, followed by an annealing time of 10 seconds at 95˚C and an extension time of 30 seconds. The temperature gradient had a range of ±5˚C the melting temperature of the primer. The annealing and extension steps were then repeated 39 times before the melt curve. The melt curve had cycles of 5 seconds, with an increase of 0.5˚C per cycle that increased from 65˚C to 95˚C. Plate reads were assigned for the end of the extension step and after each cycle in the melt curve. The resulting data was analysed using Bio-Rad CFX Manager 3.0 and the optimal temperature range for each primer was determined as the range that generated the lowest Cq value.

M) Primer Efficiency

Primer efficiency was determined using a standard curve with an eight-point dilution of the cDNA pool. The dilution factor was calculated as $2^{(35-Cq)/8}$, where Cq was taken from the temperature optimization step above. Bio-Rad CFX manager 3.0 was used to design a template for each standard curve. Two or three primers were run on each plate in an eight-point dilution with three technical replicates per dilution and "no template" controls were included for each primer. The mastermix calculation and loading of the plate were done in the same way as for primer optimization. The plates were run with the same program used for temperature optimization except that instead of the extension step being a gradient, it was set to the optimum temperature determined above. The resulting data was analysed using Bio-Rad CFX Manager 3.0. Only primers with an efficiency between 90% and 110%, and an $R^2 > 0.98$ were used for RT-qPCR analysis.

N) RT-qPCR Analysis of Gene Expression

Bio-Rad CFX Manager 3.0 was used to design templates for determining relative expression of the genes of interest. The cDNA from each sample was diluted separately this time within the 8-point dilution series performed for the primer efficiency testing. Three sulfur-treated biological samples and three untreated biological samples were used for each gene, along with three technical replicates. Mastermix was made, as described above, for primers from each of the genes of interest, as well as reference genes, *YL8S*, *EF1α* and *UBQ10* that were selected

because their expression did not change between treatments. “No template” controls were included for each primer, and a “no RT” control was run from each round of cDNA synthesis with each of the primers. The plate was loaded and sealed as described for primer optimization, and the program run was the same as the one used for primer efficiency testing. The data was analysed in Bio-Rad CFX Manager. Target gene expression was normalized to the expression of the reference genes.

O) RT-qPCR Product Validation

After the RT-qPCR protocol was completed the products were purified using the MinElute PCR purification Kit (Qiagen, cat. no. 28004) and sequenced at least once. Five 10 µL RT-qPCR reactions were pooled together in a 1.5 mL microfuge tube. To the contents 250 µL of PB buffer (provided by the kit) was added to the reaction and the total 300 µL were transferred to a MinElute column in a 2 mL collection tube. The tube was centrifuged at 16,000 RPM in a microcentrifuge for 1 minute. The flow through was re-pipetted into the column and 700 µL of PB buffer was added. The column was centrifuged again for 1 minute at 16,000 RPM and the flow-through was discarded. The column was centrifuged again for 1 minute at 16,000 RPM to remove any residual fluid. The DNA was then eluted by adding 25 µL of ddH_2O water to the centre of the column. The column was inserted into a 1.5 mL microfuge tube and incubated at room temperature for 2 minutes. It was then spun at 16,000 RPM in a microcentrifuge for 1 minute. The elution step was repeated with the exception that it was spun for 2 minutes instead of 1 minute. 250 µL of the PB

buffer was then added to the eluate and the entire process was repeated again, not including the two elution steps. The final elution was done by adding 10 µL of ddH_2O water to the centre of the column, incubating it for two minutes at room temperature, and then centrifuging for two minutes at 16,000 RPM. The samples were then quantified using the Nanodrop 2000 and sent to the Mobix Lab for sequencing.

P) Determination of Nitrogen and Sulfur Content

Plants used for dry weight determination were ground in a mortar using a pestle until a fine powder was produced. The powder from seven plants of each treatment were pooled, weighed, and were analysed by Agri-Food Laboratories (Guelph, ON) for nutrient analysis. A minimum of 0.3 g of dried ground tissue was required, and the samples were analysed using combustion. Nitrogen determination was then done using the Dumas method, and sulfur determination was done using a LECO sulfur analyser.

Q) Statistical Analysis

All statistical analyses were carried out using the program R (R Core Team, 2014). One-way ANOVA comparisons were performed to test the significance of "between treatment" values using the type III ANOVA function of the car package. These significance tests were applied to measurements of shoot fresh weight and dry weight-associated calculations, leaf area, leaf number, chlorophyll content and

nitrogen and sulfur content. One-way ANOVAs were performed on data related to mean Cq analysis of *SDI1, GGCT2;1* and *XLOC_003912* transcript abundance using the aov Sufficient function of the HH package for tests of statistical significance between treatments.

R) Virus Induced Gene Silencing (VIGS)

a) Vectors and Strains

The vectors used for VIGS were pTRV1 (TAIR, stock#CD3-1039) and pTRV2 (TAIR, stock#CD301040) from tobacco rattle virus as described by Liu et al. (2002). The *Agrobacterium tumefaciens* strain used was GV3101.

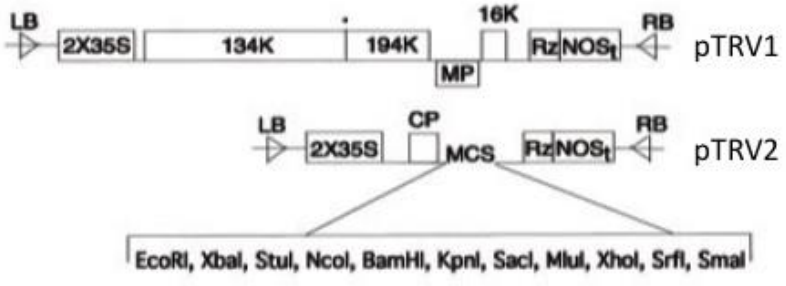


Figure adapted from Liu et al. (2002)

b) LB Plates and Media

LB liquid media was prepared using 1% (w/v) NaCl, 0.5% (w/v) Bacto Yeast Extract (Becton Dickenson, cat. no. 212750), and 1% (w/v) Bacto Tryptone (Becton Dickenson, 211705) dissolved in 1 L of ddH_2O. For plates, 1.5% (w/v) agar (Sigma, cat. no. A1296) was added to 1 L LB liquid media before autoclaving. After the

solution was cool enough to touch, antibiotics were added to final concentrations of 50 µg/mL rifampicin (Sigma-Aldrich, cat. no. R7382), 10 µg/mL gentamicin (Sigma-Aldrich, cat. no. G-3632), or 50 µg/mL kanamycin (Boehringer Mannheim GmbH, cat. no. 106 801). 100 x 15 mm polystyrene petri dishes (Fischerbrand, cat. no. S33580A) were used, and after the media solidified the plates were stored at 4˚C prior to use. Liquid LB media was stored at room temperature.

c) *XLOC_003912* Isolation

XLOC_003912 cDNA was amplified by PCR using the forward and reverse primers TCG*GGTACC*ATTATAACAAAGCCCAAAGGAC and GGAACA*GAGCTC*AAATTATATCGGCTTTTTGTTTCTG, respectively.
Underlined regions represent the restriction enzyme cut sites; *Kpn*I for the forward primer and *Sac*I for the reverse primer. Phusion High Fidelity DNA Polymerase (NEB, cat. no. M0530) was used for amplification of *XLOC_003912* from Yukon cDNA (cDNA isolation as described above). 30.5 µL DEPC-treated ddH_2O, 10 µL 5x Phusion HF Buffer, 2.5 µL of each the forward and reverse primer, 3 µL cDNA template, and 0.5 µL Phusion DNA Polymerase were added to a PCR tube. The tube was then placed in a Thermocycler and held at 94˚C for 3 minutes, followed by 34 repeats of 94˚C for 20 seconds, then 56˚C for 40 seconds, and 72˚C for 80 seconds. The tubes were then held at 72˚C for 10 minutes before being stored at -20˚C.

The PCR product was analysed on a 1% agarose (BioShop, cat. no. AGA001) gel with the GeneRuler 1kb DNA ladder (ThermoFisher Scientific, cat. no. SM0313)

size reference. The band was excised using a razor blade and collected in a microcentrifuge tube. The DNA was extracted from the gel using the GeneJET Gel Extraction Kit (ThermoFisher Scientific, K0691). 100 µL Binding Buffer was added to the tubes for every 100 mg of agarose gel. The mixture was incubated at 60˚C until the gel melted. 800 µL of the solubilized gel solution was transferred to a GeneJET purification column, the column was centrifuged for 1 minute at 16,000 RPM in a microcentrifuge and the flow-through was discarded. The column was placed over the collection tube, 700 µL of wash buffer was added and the column was centrifuged for 1 minute at 16,000 RPM. The flow-through was again discarded and the column was centrifuged over the same collection tube for an additional minute at 16,000 RPM in a microcentrifuge. The column was then placed over a new 1.5 mL tube and 50 µL of Elution Buffer was added to the column. The tube was centrifuged for 1 minute at 16,000 RPM, the column was discarded, and the DNA was stored at -20˚C.

d) Plasmid Preparation

E.coli with pTRV2 plasmids (ordered from TAIR) were incubated in 5 mL of LB media with 50 µg/mL kanamycin at 37˚C overnight with shaking at 250 RPM. The next day plasmid extraction was performed with the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, cat, no. K0502). Plasmid Minipreps were then run on a 1% agarose gel using gel electrophoresis to confirm the presence of a band.

e) Plasmid and *XLOC_003912* Digestion

Eight µL of plasmid from the Miniprep was pipetted into a PCR tube with 3 µL of Cut-Smart buffer (NEB, cat. no. B7204S), 15 µL of DEPC-treated water and 1 µL of KpnI-HF (NEB, cat. no. R3142S). The following steps were done in a Thermocycler at 37˚C. First, the PCR tube was incubated for 1.5 hours, then a second 1 µL aliquot of KpnI-HF was added and the tube was incubated for another 1.5 hours. Next, 1µL of SacI (NEB, cat. no. R0156S) was added, and the tube was incubated for 1.5 hours, then 1 µL more of SacI was added and the tube incubated for another 1.5 hours. For the *XLOC_003912* DNA, the protocol was the same except that 20 µL of the gel extraction product was used in place of plasmid, 3 µL of Cut-Smart Buffer and 3 µL of DEPC-treated ddH_2O were used for the same series of digestion reactions.

The digested *XLOC_003912* DNA and pTRV2 vector were then concentrated by adding 0.1 volumes of 3.0 M sodium acetate and 2 volumes of 100% ethanol, and after precipitation overnight at -20˚C the DNA was collected by centrifugation at 10,000 RPM at 4˚C for 10 minutes. The pellet was dissolved in 6 µL of TE buffer and an aliquot was resolved on a 0.8% agarose gel. Samples were analysed by nanodrop to confirm high concentration and quality of the DNA. Three µL of digested vector was diluted with 27µL of TE buffer and 2 µL of digested XLOC product was diluted with 18 µL of TE buffer.

f) Ligation of DNA Into pTRV2

The equation: $insert_{ng} = [(vector_{ng} \times insert_{kb})/vector_{kb}] \times$ insert/vector ratio, was used to approximate the amount of insert needed for the ligation reaction using ratios of insert/vector of 1/3, 1/2 and 1/1. The ng value was approximated using the nanodrop measurement. A negative control replacing the DNA to be inserted with DEPC-treated ddH_2O was included. Once the vector and insert had been added to the PCR tube, 1 µL of 10x Buffer for T4 DNA Ligase (NEB, cat. no. B0202S) and 1 µL of T4 DNA Ligase (NEB, cat. no. M0202S) were added to each of the tubes and incubated overnight at 16˚C in the Thermocycler.

g) Identifying Positive Clones

Subcloning Efficiency DH5alpha competent cells (Invitrogen cat. no. 18265-017) were used for transformation with 15 µL of thawed cells per reaction. In each 1.5 mL centrifuge tube, 2.5 µL of ligation reaction (including the negative control from the digestion above, and a positive control with 2.5 µL of uncut pTRV2 plasmid) were pipetted into separate tubes. The tubes were then incubated on ice for 30 minutes, then placed into a 42˚C water bath for 20 seconds followed by ice for 2 minutes. 950µL of LB media warmed to 37˚C was added to each tube and cells were then incubated for 1 hour at 37˚C in an incubator with shaking at 225 RPM. Cells were recovered by centrifugation at 2500 RPM for 4 minutes and pellets were resuspended with 200 µL LB. Cells were spread on LB plates containing 50 µg/mL of

kanamycin (LB kan) and incubated at 37˚C for two days. Colonies were selected, streaked onto LB kan plates and recovered colonies were numbered from 1-75.

A toothpick was used to transfer cells from the plates to 10 µL of ddH_2O in a PCR tube. Cells from 10 colonies were combined in one tube, the mixture was heated for 10 minutes at 95˚C then centrifuged at 16,000 RPM in a microcentrifuge at 4˚C for 1 minute to collect cell lysate. Two µL of the supernatant was used in a PCR reaction to amplify *XLOC_003912* (see PCR protocol in *XLOC_003912* isolation section), the PCR products were visualized using a 1% agarose gel, and tubes that were positive for *XLOC_003912* bands (800bp) were identified for a second screen to identify the source colony/ colonies carrying insert DNA of the appropriate size.

Once identified, the plasmids were sent to Mobix Lab for sequencing. The external forward primer used was GGACGAGTGGACTTAGATTC and the external reverse primer was CTTCAGACACGGATCTACT. The internal forward primer was CTGAGAAGATACCGCCGACA, and the internal reverse primer was TGGATTTGGTTATTCCGGCGA.

Colonies 1 and 34 had a 100% match to the *XLOC_003912* sequence and were then transformed into competent *Agrobacterium* cells.

h) Transformation of *Agrobacterium*

To make competent *Agrobacterium* for transformation, 3 mL LB containing 50 µg/mL rifampicin and 10 µg/mL gentamicin (LB rif, gent) were inoculated from a frozen glycerol stock of *Agrobacterium* and incubated overnight with shaking at 180

RPM at 28˚C. A 0.5 mL-volume of overnight culture was used to inoculate 50 mL LB rif, gent and incubated at 28 ˚C overnight with shaking at 180 RPM until the A_{600} was around 0.3. The culture was chilled on ice for 15 minutes, centrifuged at 4000 RPM for 5 minutes at 5˚C, and the cells were resuspended with 10 mL cold, sterile 100 mM $MgCl_2$. The cells were kept on ice for 1 hour then recovered by centrifugation at 4000 RPM for 5 minutes at 5˚C. The pellet was resuspended with 2 mL cold, sterile 20 mM $CaCl_2$ and then incubated on ice for 6 hours. Sterile glycerol was added to 20% of the total volume, 100 µL aliquots were made and immersed in liquid nitrogen for storage at -80˚C.

For Transformation, 1.5 µg of pTRV2 plasmid without the insert or containing the *XLOC_003912* DNA was added to a frozen 100 µL aliquot of competent *Agrobacterium* cells. The cells were then thawed on ice for 10 minutes and mixed gently. The tube was immersed in liquid nitrogen for 10 minutes and then thawed to 37 ˚C in a water bath for 5 minutes. One mL sterile LB rif, gent was added to the tube and the tube was incubated for 2 hours with shaking at 180 RPM. The aliquot was then spread on an LB plate containing 50 µg/mL rifampicin, 10 µg/mL gentamycin, and 50 µg/mL kanamycin and the plate was incubated at 28˚C for three days.

i) Inoculation of *Eutrema* Plants

A single colony from the transformation (see above) was streaked on a fresh LB plate containing 50 µg/mL rifampicin, 10 µg/mL gentamycin, and 50 µg/mL kanamycin. Plates were incubated for 2 days at 28˚C and on the evening of the

second day, 5 mL LB containing 50 µg/mL kanamycin, 10 µg/mL gentamycin, and 50 µg/mL rifampicin was inoculated with a single bacterial colony from the plate. There were five different strains used for the VIGS work: pTRV1 vector, pTRV2 vector lacking a DNA insert, pTRV2 vector containing the *Arabidopsis thaliana* phytoene desaturase (*PDS*) gene (TAIR, stock no. CD3-1047), and two independent isolates containing the pTRV2 vector each with an *XLOC_003912* cDNA insert cloned between the *Kpn*I and *Sac*I restriction sites. A single colony was used to inoculate 5 mL LB containing 50 µg/mL rifampicin, 10 µg/mL gentamycin, and 50 µg/mL kanamycin in 13 x 100 mm glass test tubes and bacteria were incubated overnight at 28˚C with shaking at 250 rpm. The next day, 2 mL of each bacterial culture was used to inoculate flasks containing 48 mL LB with 50 µg/mL kanamycin, 10 µg/mL gentamycin, and 50 µg/mL rifampicin, 10 mM MES buffer (pH 5.5), and 20 µM acetosyringone (Sigma-Aldrich, cat. no. D134406-5G). The cultures were incubated overnight at 28˚C and 250 RPM and the next day the A_{600} of each flask was measured using an aliquot of the culture. The remainder of the culture was transferred to a 250 mL polycarbonate centrifuge bottle and centrifuged at 3000 RPM for 5 minutes at room temperature (Beckman Avanti J-25 centrifuge with JLA-16.250 rotor). The supernatant was discarded, and the bacterial pellets were suspended using an inoculation buffer containing 200 µM acetosyringone, 10 mM $MgCl_2$ and 10 mM MES buffer adjusted to pH 5.5 with NaOH to produce an A_{600} of 1.5. The cultures were then left at room temperature for 3 to 4 hours. Next, 5 mL of each culture containing bacteria carrying the pTRV2 plasmid was combined and mixed well with an equal

volume of the culture carrying bacteria with the pTRV1 plasmid. The inoculum carrying bacteria with both plasmid vectors was then used to inoculate plants at the 2 to 3 leaf stage (1.5 weeks after germination).

To inoculate the leaves, a 1-mL syringe was filled with the bacterial mixture and the syringe barrel was pressed to the underside of a leaf. The plunger of the barrel was pushed down until the bottom of the leaf was filled with liquid (indicated by a darker green colour development in the inoculated versus un-inoculated leaves). Two leaves were inoculated per plant with leaves near the base of the plant selected in preference to less mature leaves above. The plants were then placed back into the growth chamber and covered with clear plastic lids for 3 days to increase the humidity to facilitate bacterial growth. The chamber settings were the same as stated above in the planting section.

j) Tissue Collection

Leaf tissue was collected from plants inoculated with the *XLOC_003912* cDNA VIGS vector, the uninoculated plants and plants inoculated with the empty vector once-weekly beginning 1.5 weeks after inoculation. Leaves were removed from the middle of the rosette using sharp tweezers and weighed before being placed in a foil packet for flash freezing in liquid nitrogen. The samples were then stored at -80˚C until use.

RESULTS

I. Comparisons Between Sulfur-Treated and Untreated Plants

The comparisons made for *Eutrema salsugineum* (Yukon ecotype) involved three sulfur treatment levels: (1) potting soil mix with no sulfur amendment (equivalent to 200 ppm sulfur), (2) potting soil mix amended with 1600 ppm calcium sulfate dihydrate (equivalent to 1800 ppm sulfur) and (3) potting soil mix amended with 3400 ppm calcium sulfate dihydrate (equivalent to 3600 ppm sulfur). Experiments using *Eutrema salsugineum* (Shandong ecotype) were tested using two sulfur levels: (1) potting soil mix with no sulfur amendment (equivalent to 200 ppm sulfur) and (2) potting soil mix amended with 1600 ppm calcium sulfate dihydrate (equivalent to 1800 ppm sulfur). In the experimental design used, plants on soil amended with sulfur were grown at the same time along with plants grown in untreated potting mix soil.

For ease of discussion, plants will be designated based upon the ecotype and sulfur amendment level and hence will be referred to as untreated "Yukon/Shandong 200 ppm" or sulfur-treated identified as "Yukon/Shandong 1800 ppm" or "Yukon 3600 ppm".

A) Biomass

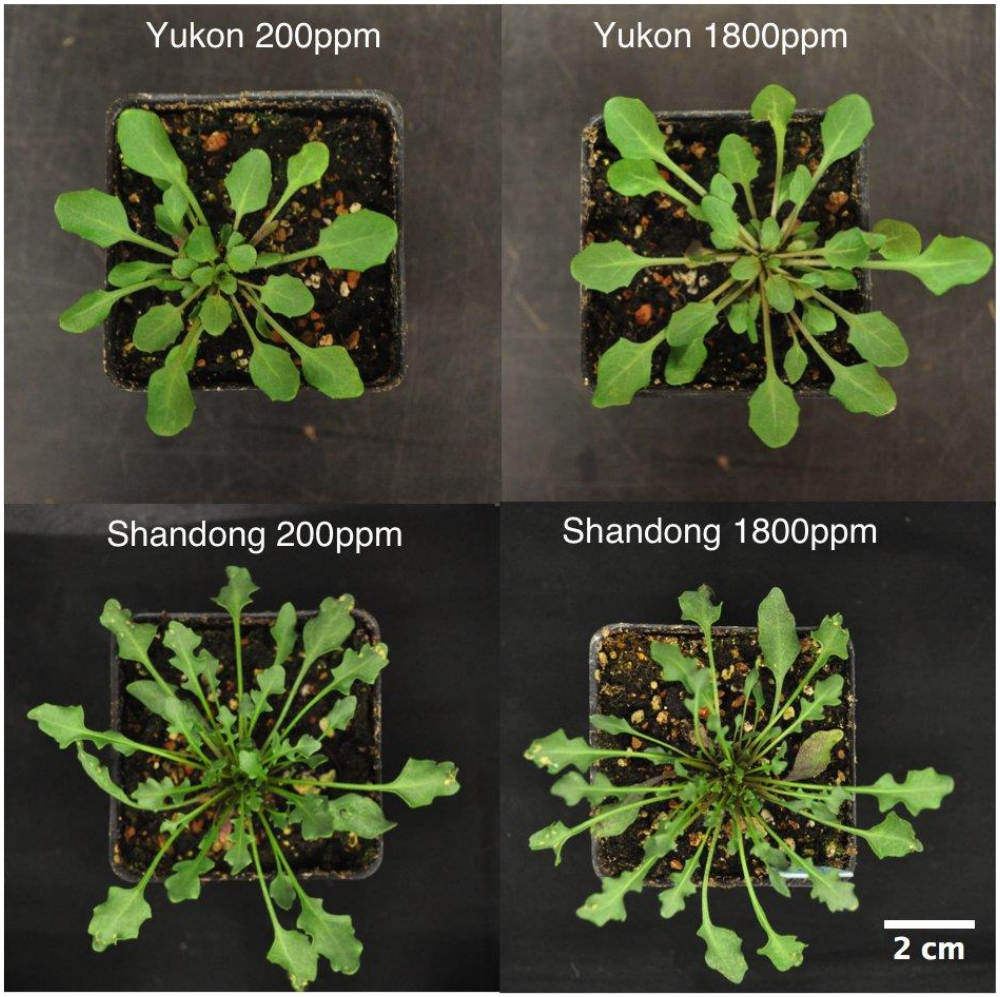
When the plants reached four weeks post-germination they were analysed for phenotypic differences between the sulfur-treated and untreated plants. Phenotypic analysis included taking photos of the plants to visually document their

development over time and to allow for measurements of leaf number and area. Plants were also destructively harvested to measure whole rosette fresh weight and dry weight.

Figure 3 shows that it was difficult to visually distinguish sulfur-treated from untreated plants and it was virtually impossible without the benefit of comparing plants grown at the same time and under identical conditions. However, Yukon 1800 ppm plants (Fig. 3A) were larger than the untreated plants grown at the same time and this visual impression was supported by whole rosette fresh weight and dry weight measurements shown in Figure 4 and again as Tables 2 and 3, respectively. Yukon 1800 ppm plants showed a 1.3-fold higher average for both fresh weight and dry weight, compared to untreated plants (Fig. 4A, B) and while this difference is modest, it is statistically significant. The Yukon 3600 ppm plants also showed higher fresh and dry weight measurements when compared to untreated plants grown at the same time with a 1.2 and 1.3-fold difference for fresh weight and dry weight between sulfur-treated and untreated plants, respectively (Tables 2,3 and Fig. 4E, F). In contrast to the modest increase in biomass when sulfur is added to the soil of Yukon *Eutrema*, there was no statistically significant difference between fresh weights for Shandong 1800 ppm and untreated plants (and Fig. 4C, D and Table 2). However, a statistically significant difference in dry weight was observed with sulfur addition leading to a slight, 1.1-fold increase in dry biomass (Table 3).

Figure 3: Representative four-week-old *Eutrema salsugineum* plants grown with or without added sulfur in the potting medium show similar phenotypes. A) Yukon and Shandong *Eutrema* plants grown in untreated or sulfur-treated soil with sulfur content approximately 200 and 1800 parts per million (ppm), respectively. B) Yukon plants grown in soil left untreated (200 ppm) or supplemented with sulfur to 3600 ppm. Both ecotypes were grown under the same settings in controlled environment growth chambers with soil sulfur amendments added as calcium sulfate dihydrate. Soil preparation and controlled environment settings are described in the Materials and Methods section.

A


Yukon 200ppm
Yukon 1800ppm
Shandong 200ppm
Shandong 1800ppm
2 cm

B


Yukon 200ppm
Yukon 3600ppm
2 cm

Figure 4: The impact of sulfur amendment on *Eutrema* rosette biomass.

Whole Yukon (A,B,C,D) and Shandong (E,F) rosette fresh (A,C,E) and dry (B,D,F) biomass of plants harvested four weeks post-germination. Mean +/- SE are plotted for four replicates from each experimental comparison between untreated and sulfur-treated plants. Fourteen plants per treatment per replicate were collected for fresh weight (n=56), and seven plants per treatment per replicate for dry weight (n=28). Asterisks denote level of significance: * = $P < 0.05$, ** =$P<0.01$, *** = $P<0.001$.

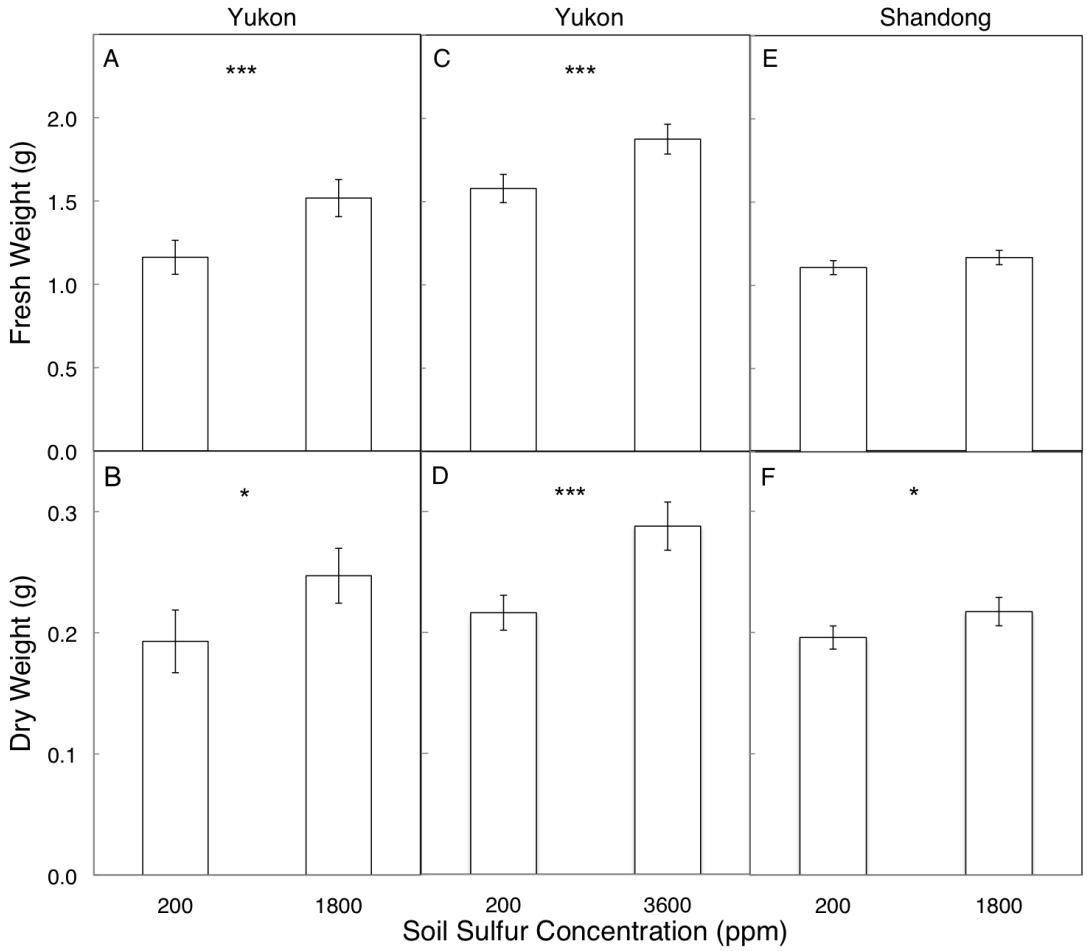

Yukon
Yukon
Shandong
A
***
C
***
E
B
*
D
***
F
*
Fresh Weight (g)
Dry Weight (g)
0.0
0.5
1.0
1.5
2.0
0.1
0.2
0.3
200
1800
200
3600
200
1800
Soil Sulfur Concentration (ppm)

Table 2: Whole rosette fresh biomass of four-week-old *Eutrema salsugineum* plants grown with or without added sulfur in the potting medium. Mean +/- SE for four replicates with 14 plants measured per treatment per replicate (n=56).

Ecotype	Trial	Soil Sulfur *ppm*	Fresh Weight *grams*	Fold-Change[a]	P-value
Yukon	1	200	1.162+/-0.102	1.3	<0.001
		1800	1.518+/- 0.112		
Shandong	2	200	1.107+/-0.042	1	0.198
		1800	1.167+/-0.043		
Yukon	3	200	1.579+/-0.090	1.2	<0.001
		3600	1.875+/-0.085		

[a]Sulfur-treated/untreated

Table 3: Whole rosette dry biomass of four-week-old *Eutrema salsugineum* plants grown with or without added sulfur in the potting medium. Mean +/- SE for four replicates with 7 plants measured per treatment per replicate (n=28).

Ecotype	Trial	Soil Sulfur *ppm*	Dry Weight *grams*	Fold-Change[a]	P-value
Yukon	1	200	0.193+/-0.023	1.3	0.013
		1800	0.247+/-0.026		
Shandong	2	200	0.196+/-0.010	1.1	0.04
		1800	0.217+/-0.012		
Yukon	3	200	0.216+/-0.020	1.3	<0.001
		3600	0.288+/-0.014		

[a]Sulfur-treated/untreated

Aside from the negligible benefit accruing from sulfur being added to the soil, the experiment evaluating the effect of 3600 ppm sulfur on Yukon plants had untreated plants that were, on average, larger than the sulfur-treated, Yukon 1800 ppm plants that were obtained in an earlier trial and shown in Figure 4A,B. We cannot easily attribute this to differences in growth conditions or batches of soil components. Rather, this difference likely reflects a seasonal effect that we observe in that plants of similar age grown in our controlled environment chambers during the summer are usually larger than plants grown during winter months. This observation shows the importance of growing untreated and sulfur-treated plants at the same time and in the same growth cabinet.

B) Response of Leaf Area and Leaf Number to Sulfur Supplementation

There was a 1.3-fold increase in the leaf area of Yukon 1800 ppm sulfur-treated compared to untreated plants (Fig. 5A, Table 4). Similarly, the leaf area of Yukon 3600 ppm plants also increased 1.2-fold with the sulfur treatment (Fig. 5C Table 4). These changes, albeit modest, were statistically significant between untreated and sulfur-treated plants. In contrast, there was no discernible change in leaf area between untreated and sulfur-treated Shandong plants (Fig. 5E, Table 4).

The Yukon sulfur-treated plants also showed around a 1.2-fold increase in leaf number from an average of about 35 leaves on untreated, 200 ppm plants to an average of over 42 leaves on Yukon 1800 or 3600 ppm plants. (Fig.

Figure 5: The impact of sulfur supplementation on leaf area and number of Yukon and Shandong *Eutrema* plants. Mean leaf area or number +/- SE for three replicates of each experimental group with 6 plants per replicate per treatment (n=18). Leaf area (A,C,E) and number (B,D,F) were measured using Yukon (A,B,C,D) and Shandong (E,F) plants at four weeks post-germination. Asterisks denote level of significance: * = P < 0.05, ** =P<0.01, *** = P<0.001.

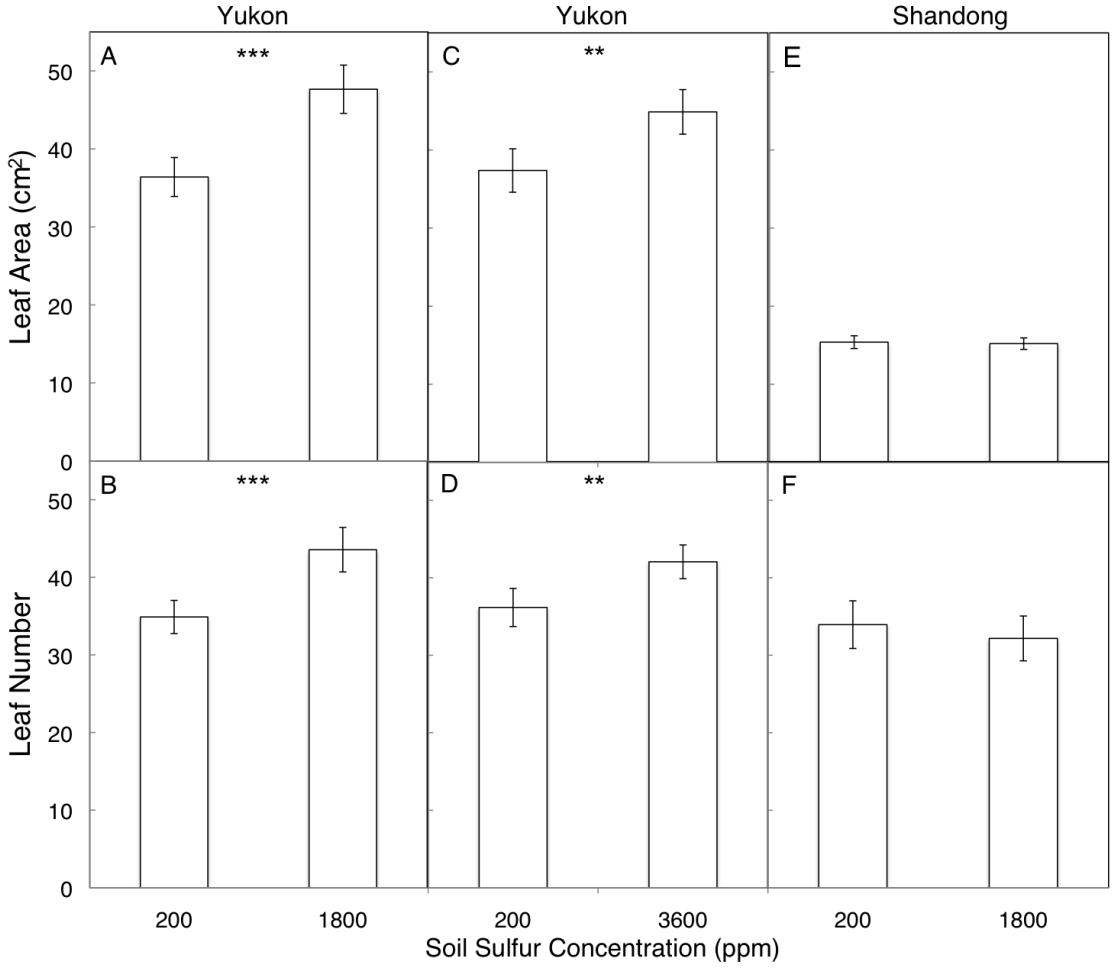


Table 4: Leaf area and number for *Eutrema salsugineum* plants grown with or without added sulfur in the potting medium. Data are mean +/- SE for three replicates with 6 plants measured per treatment per replicate (n=18).

Ecotype	Trial	Soil Sulfur	Leaf area		Leaf Number	
		ppm	*cm²*	*P*		*P*
Yukon	1	200	36.4+/-2.49	<0.001	34.9+/-2.14	0.002
		1800	47.7+/-3.09		43.6+/-2.86	
Shandong	2	200	15.2+/-0.74	0.86	33.9+/-3.07	0.45
		1800	15.2+/-0.74		32.2+/-2.89	
Yukon	3	200	37.4+/-2.78	<0.001	36.2+/-2.46	0.003
		3600	44.9+/-2.85		42.1+/-2.17	

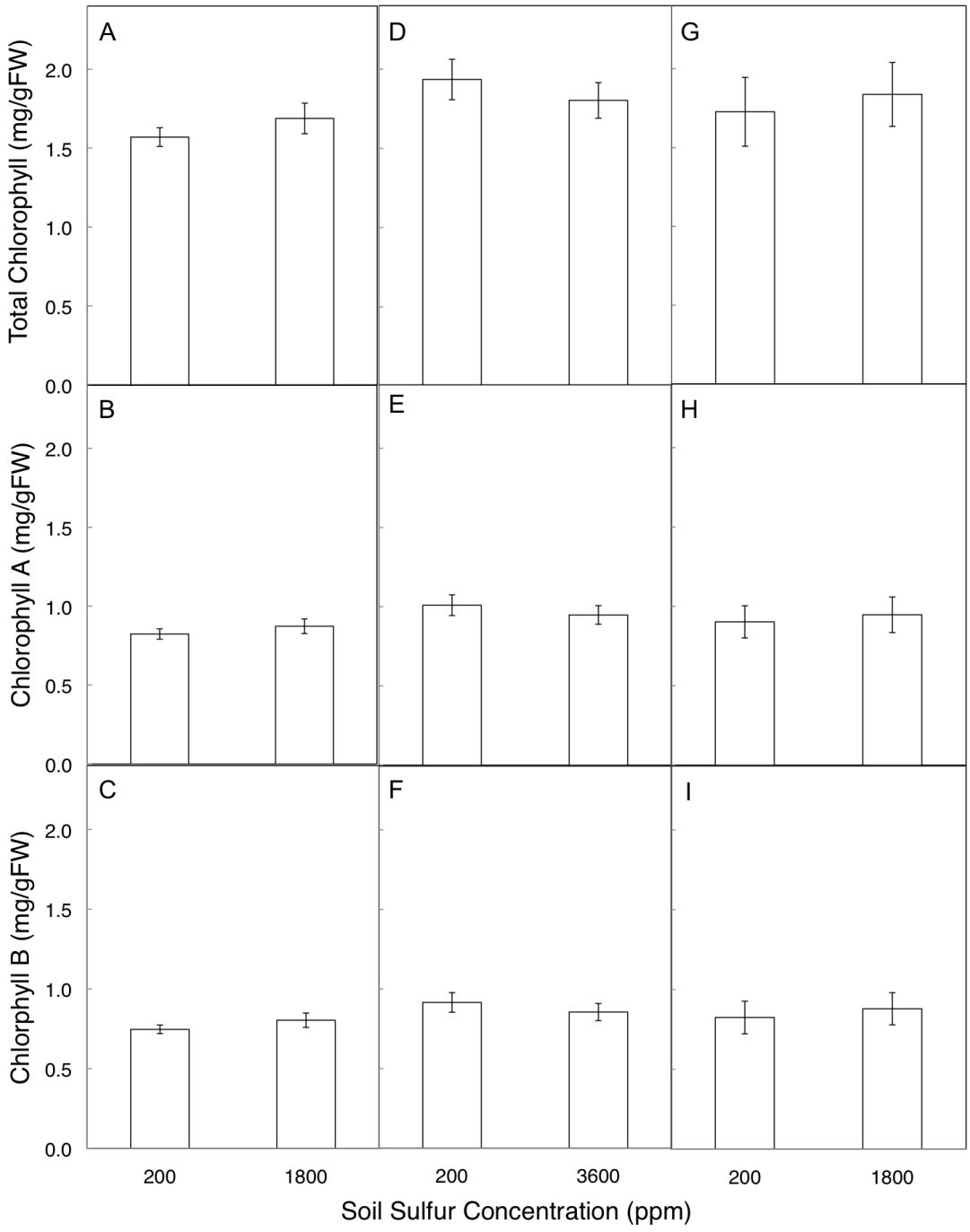
5B,D and Table 4). The Shandong plants, at around 32 to 34 leaves per plant on average, showed no difference in leaf number with sulfur treatment (Fig 5F and Table 4).

In summary, Yukon *Eutrema* plants showed an increase in both leaf area and leaf number when treated with either 1800 ppm or 3600 ppm whereas Shandong plants showed no difference with respect either property with sulfur treatment.

C) Chlorophyll Content of *Eutrema* leaves

Four weeks after germination plants were removed from the growth chamber and 50 mg of tissue (approximately one leaf) was removed from the middle of the rosette and analysed for chlorophyll A, B and total chlorophyll content. There were no significant differences between plants that received no additional sulfur and those that were grown on sulfur supplemented soil. Chlorophyll A, B and total chlorophyll levels were comparable between trials and ecotypes (Fig. 6). Under the controlled environment light conditions used the chlorophyll A/B ratio was approximately 1.1 for both ecotypes and all treatments.

Figure 6: Leaf chlorophyll content of sulfur-treated and untreated Yukon and Shandong *Eutrema* plants. Fully-expanded leaves positioned between the basal leaves of the rosette and the expanding leaves were harvested four weeks post-germination for chlorophyll A, chlorophyll B, and total chlorophyll measurements. Extracts were prepared as described in the Materials and Methods and values are reported as the mean mg chlorophyll per gram fresh weight (FW) +/- SE. Three plants from each treatment were analyzed and the entire experiment was repeated three times (n=9).


A
D
G
B
E
H
C
F
I
Total Chlorophyll (mg/gFW)
Chlorophyll A (mg/gFW)
Chlorophyll B (mg/gFW)
0.0
0.5
1.0
1.5
2.0
200
1800
200
3600
200
1800
Soil Sulfur Concentration (ppm)

D) Effect of Sulfur Amendment on *Eutrema* Leaf Nitrogen and Sulfur Content

As stated in section A of the Results section, fresh and dry biomass measurements of whole rosettes were made. In addition, the dried material was used for sulfur content determinations. The Yukon 1800 ppm and 3600 ppm plants both showed a modest, approximately 1.1-fold increase in sulfur content compared to plants that did not receive added sulfur (Fig. 7A,B). The change in sulfur content was statistically significant for the Yukon 1800 ppm plants but not the Yukon 3600 ppm plants, likely due to there being only two experimental replicates for the Yukon 3600 ppm group. The Shandong plants had a similar fold-increase of around 1.1 in sulfur-treated plants compared to untreated, but this was not statistically significant due to higher variability (Fig. 7C). Yukon plants generally had a higher sulfur content than the Shandong plants (Fig. 7A,B,C).

There was no significant difference in the nitrogen content between rosettes of sulfur-treated and untreated plants for either *Eutrema* ecotype (Fig.7D,E,F). As was found for the sulfur content, the nitrogen to sulfur ratio was only significantly different between Yukon 1800 ppm plants compared to their control, untreated plants. In this case the nitrogen to sulfur ratio was lower by 1.3-fold between the sulfur-treated plants compared to the untreated plants (Fig. 7G). This difference in the nitrogen to sulfur ratio was observed in the trials for the Yukon 3600 ppm and Shandong 1800 ppm plants but neither change was found to be statistically significant (Fig7G,H and Table 5).

Figure 7: Effect of sulfur amendment on *Eutrema* rosette nitrogen and sulfur content. Sulfur and nitrogen content were measured in four-week-old Yukon and Shandong plants. Seven entire rosettes from each treatment were pooled for analysis. Values are the mean +/- SE for three replicates (n=3) each for Yukon 1800 ppm and Shandong 1800 ppm samples and two replicates (n=2) for Yukon 3600 ppm samples. Asterisks denote level of significance: * = $P < 0.05$, ** =$P<0.01$, *** = $P<0.001$.

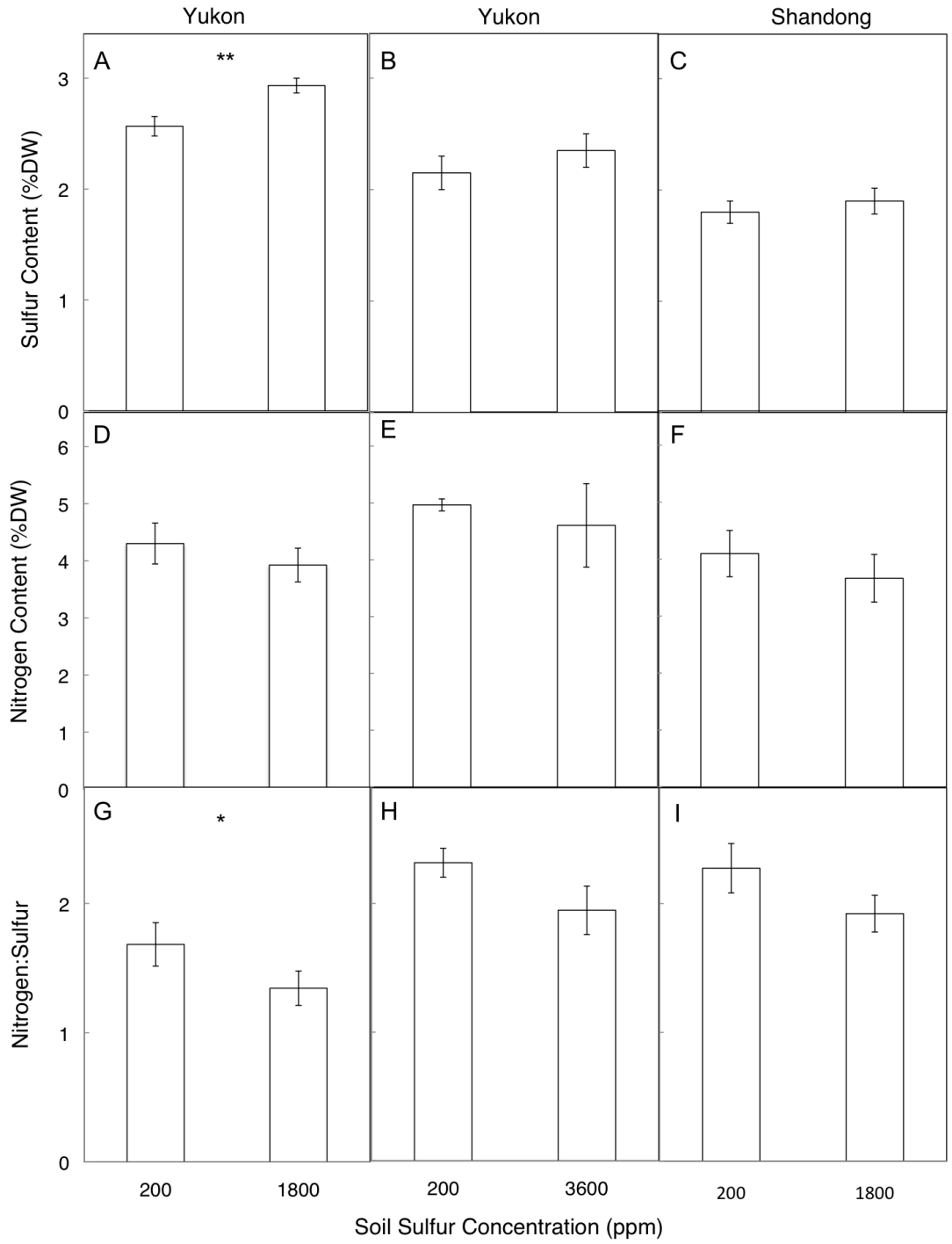

Yukon
Yukon
Shandong
A
**
B
C
D
E
F
G
*
H
I
Sulfur Content (%DW)
Nitrogen Content (%DW)
Nitrogen:Sulfur
0
1
2
3
4
5
6
200
1800
200
3600
200
1800
Soil Sulfur Concentration (ppm)

Table 5: Sulfur and nitrogen composition of *Eutrema* rosettes from four-week-old plants. Seven whole rosettes from each treatment were pooled for analysis. Sulfur (S) or nitrogen (N) reported as mean mg per rosette +/- SE with mean ratio of N to S (N/S) +/- SE for three replicates (n=3) Yukon and Shandong 1800 ppm plants and two replicates (n=2) for Yukon 3600 ppm plants. Asterisks denote level of significance: * = P < 0.05, ** =P<0.01, *** = P<0.001.

Ecotype	Trial	Soil Sulfur *ppm*	Rosette Nutrient Content		
			S *mg*	N *mg*	N/S Ratio
Yukon	1	200	4.01+/-0.44	6.88+/-1.40	1.68+/-0.17
		1800	5.48+/-0.58***	7.52+/-1.58	1.34+/-0.13*
Shandong	2	200	3.50+/-0.44	7.82+/-0.59	2.28+/-0.19
		1800	4.27+/-0.19	8.16+/-0.28	1.92+/-1.43
Yukon	3	200	3.71+/-0.96	8.45+/-2.53	1.95+/-0.19
		3600	5.32+/-1.27	10.5+/-2.87	2.32+/-0.11

Nutrients like nitrogen and sulfur are mobile in plants and, as a result, can be remobilized from expanded leaves established early in development (fully-expanded leaf, FE) to newly emerging leaves (expanding leaf, EL) (Abdallah et al., 2010). Conceivably, any pattern of sulfur in a whole rosette may mask a differential pattern of distribution among the leaves comprising the rosette. This was a consideration in our selection of leaves for down-stream analysis including RT-qPCR (discussed in the next section). In order to determine whether sulfur content differed between whole rosettes and FE leaves (used for RT-qPCR), sulfur content of both tissue sources was determined (Fig. 8, Table 6). Yukon 1800 ppm whole rosettes and FE leaves had comparable levels of sulfur while Yukon 200 ppm samples showed that the FE leaves have only about 70% of the sulfur content of the whole rosette (Table 6). This suggests that sulfur is remobilized in *Eutrema* plants, particularly under the low sulfur treatment that was used in this study. To address the consideration that leaves are likely variable with respect to sulfur content and that the older leaves in a rosette were most likely to be experiencing a sulfur deficiency, only FE leaves were selected for RT-qPCR. For the purpose of this study, large leaves positioned on the outside of the rosette were considered FE, while EL leaves were small and positioned closer to the centre of the rosette. At four-weeks-old, leaves 1 to 10 of Yukon rosettes are FE while leaves corresponding to 11 and above were EL (Fig. 8)

Figure 8: Yukon *Eutrema* plant labelled to show distinction between fully-expanded (FE) leaf and expanding leaf (EL).

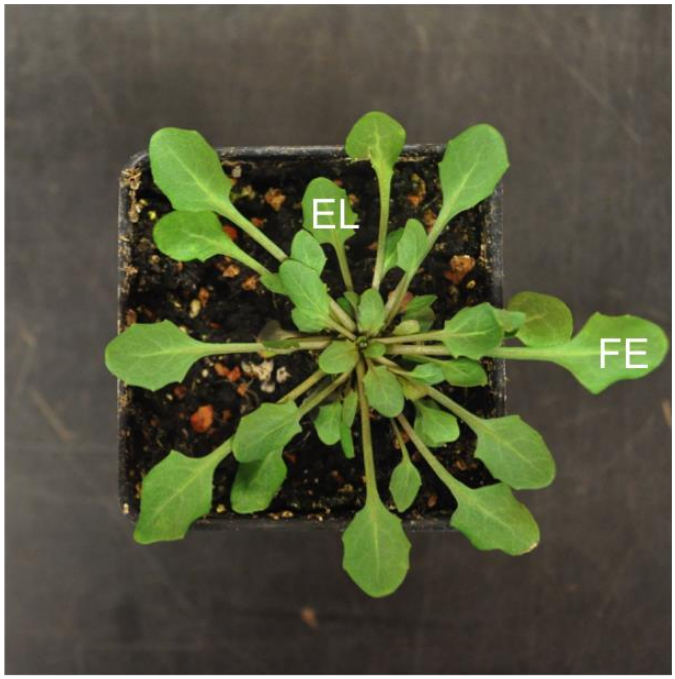


Table 6: Sulfur content of Yukon *Eutrema* rosettes and fully-expanded leaves.

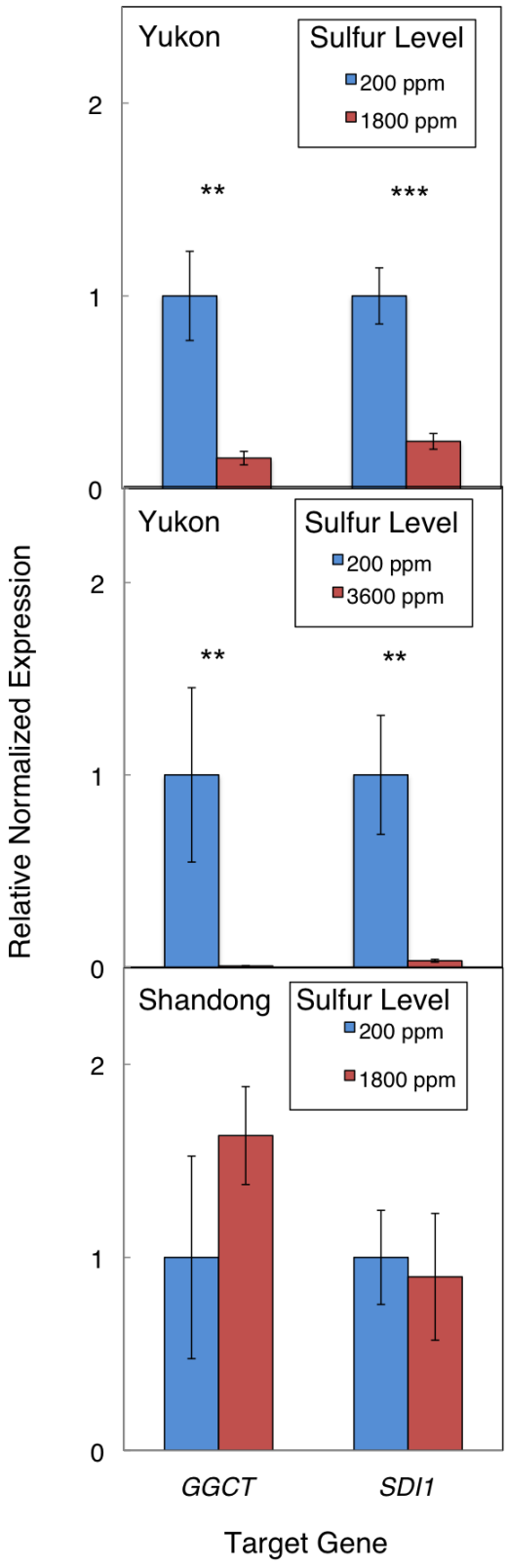
Tissue	Soil Sulfur *ppm*	Plant Sulfur Content *%DW*
Whole rosette	200	2.57+/-0.088[a]
	1800	2.93+/-0.067[a]
Fully-expanded leaves	200	1.80[b]
	1800	3.00[b]

[a] Mean +/- SE of three replicates (n=3) with 7 rosettes pooled from each treatment per replicate.
[b] Data for one replicate (n>10 leaves).

E) Expression of Sulfur Deficiency Marker Genes is Responsive to Sulfur Supplementation

The effect of sulfur supplementation on the visible phenotype of Yukon *Eutrema* was measureable but barely perceptible, an outcome that was not particularly surprising given the modest change in internal sulfur content in sulfur-supplemented plants (Fig. 3,7 and Table 5). This raised the question whether the sulfur content for the low sulfur soil was actually eliciting a sulfur deficiency in *Eutrema*. cDNA was prepared from leaf RNA and used for RT-qPCR measurements to determine the expression level of two sulfur deficiency marker genes, *γ-glutamyl-cyclotransferase 2;1 (GGCT2;1)* (Paulose et al., 2013) and *Sulfur Deficiency Induced 1 (SDI1)* (Howarth et al., 2009), and their relative normalized expression results are shown in Figure 9. For Yukon plants, transcripts associated with *GGCT2;1* and *SDI1* were 6.3 and 4-fold higher, respectively, for plants grown on 200 ppm relative to 1800 ppm sulfur. Similarly, there was a difference in transcript levels associated with *GGCT2;1* and *SDI1* between Yukon 200 and 3600ppm plants but the difference was greater in that the fold-changes between transcripts in leaves of plants grown on low versus high sulfur were 177 and 30.4-fold, respectively. In contrast, Shandong 200 and 1800 ppm plants showed no difference in the level of *GGCT2;1* or *SDI1* transcript abundance (Fig. 9).

Figure 9: Relative transcript abundance of sulfur deficiency marker genes in fully-expanded *Eutrema* leaves assessed using RT-qPCR. cDNA was prepared from RNA extracted from leaves of four-week-old Yukon 200, 1800, 3600 ppm soil sulfur plants and Shandong 200 and 1800 ppm plants. Three replicates were done for both the Yukon and Shandong 1800 ppm experiments with three plants harvested per replicate (n=9). One replicate comprised of three plants was done for the Yukon 3600 ppm experiment (n=3). *SDI1* and *GGCT2;1* mean relative expression values +/- SE were normalized for the expression of three reference genes *EF1a*, *UBQ10* and *YL8S*. Expression of both *SDI1* and *GGCT2;1* in the Yukon or Shandong 200 ppm plants was set to 1. RT-qPCR conditions were as described in the Materials and Methods using primers listed in Table A1 of the Appendix. Asterisks denote level of significance: * = $P < 0.05$, ** =$P<0.01$, *** = $P<0.001$.


Yukon
Sulfur Level
200 ppm
1800 ppm
**
***
Yukon
Sulfur Level
200 ppm
3600 ppm
**
**
Shandong
Sulfur Level
200 ppm
1800 ppm
Relative Normalized Expression
0
1
2
GGCT
SDI1
Target Gene

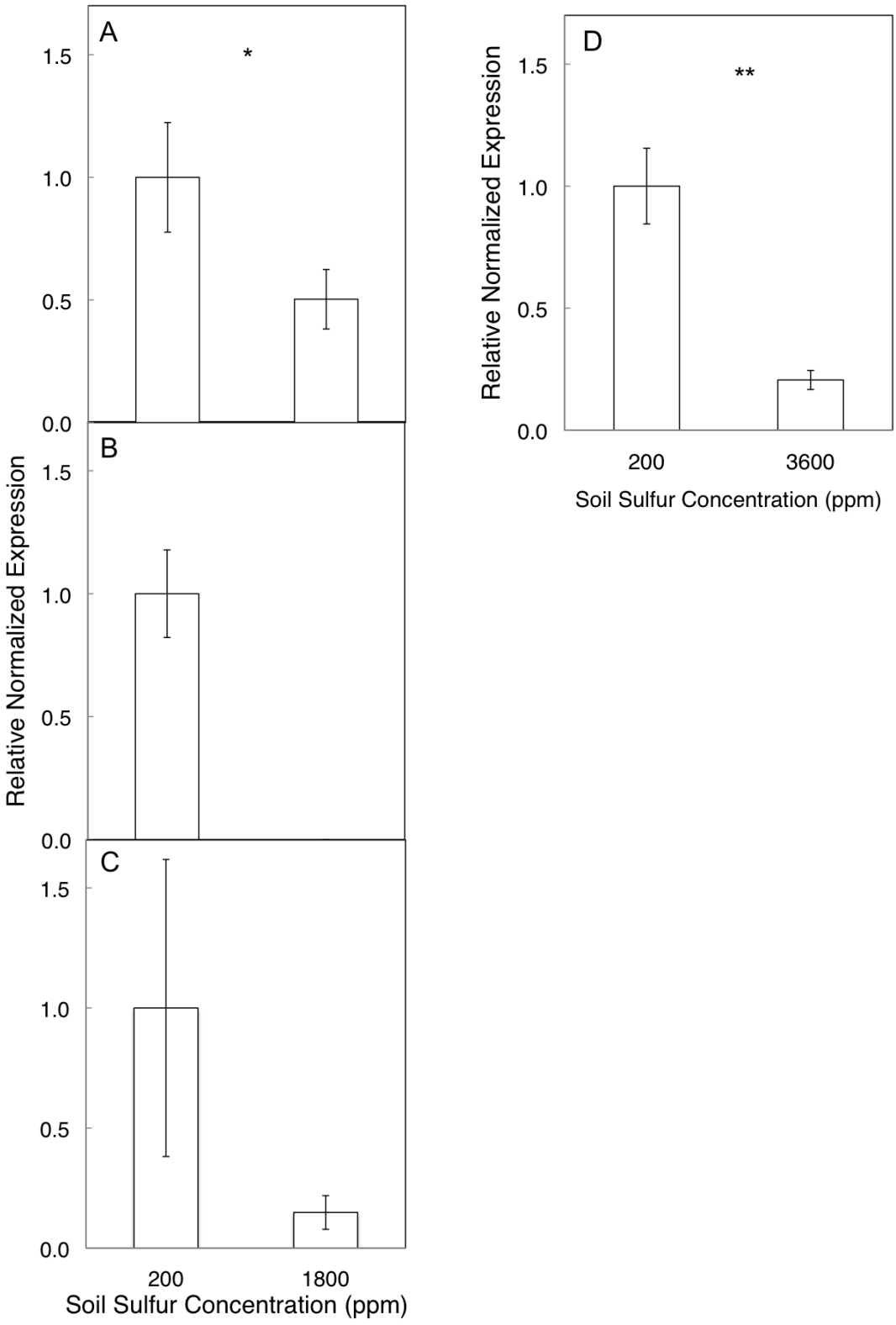
II. Exploring the Function of a Long-noncoding RNA

As noted in the introduction, the long non-coding RNA designated *XLOC_003912* was identified by Champigny et al. (2013) as being highly expressed in Yukon cabinet plants and having no expression in either Yukon field plants or Shandong cabinet plants. We hypothesized that the gene was sulfur-responsive based upon its co-expression with sulfur deficiency marker genes identified by PCA analysis (Table 1). The following work was done to explore this hypothesis and the function of *XLOC_003912* in Yukon plants.

A) Effect of Sulfur Supplementation on *XLOC_003912* Transcript levels

RT-qPCR was used to assess the relative expression levels of *XLOC_003912* in four-week-old Yukon 200 and 1800 ppm plants. In three experimental repeats involving three plants per treatment (n=3) *XLOC_003912* had elevated transcript levels in Yukon 200 ppm relative to 1800 ppm plants (Fig. 10). Thus *XLOC_003912* displayed a sulfur-responsive pattern of expression as found for *SDI1* and *GGCT2;1* (Fig. 9) but the response between experimental repeats was variable. In the first replicate, *XLOC_003912* transcript abundance was 2-fold higher in the untreated plants compared to sulfur-treated plants (Fig. 10A) but in the second replicate *XLOC_003912* transcripts in sulfur-treated plants were below the detection limits for RT-qPCR (Fig. 10B). In the third replicate, *XLOC_003912* transcripts were 6.75-fold higher in untreated compared to sulfur-treated plants but this difference was not statistically significant due to the high variability in *XLOC_003912* expression levels

Figure 10: Relative *XLOC_003912* expression levels estimated by RT-qPCR using cDNA from four-week-old Yukon 200, 1800, and 3600 ppm plants. For each treatment, fully-expanded leaves were removed from three individual plants once (n = 3). Three repetitions were done for Yukon 1800 ppm plants (A,B,C), and one repetition was done for Yukon 3600 ppm (D). Leaf tissue was used for RNA extraction and cDNA synthesis. cDNA was used to analyze gene expression by RT-qPCR following the PCR conditions reported in Materials and Methods and the primers listed in Table A1 of the Appendix. *XLOC_003912* mean relative expression values +/- SE were normalized for the expression of three reference genes *EF1a*, *UBQ10* and *YL8S*. *XLOC_003912* expression in the Yukon 200 ppm plants was set to 1. *P* values were: A) 0.0265, B) N/A, *XLOC_003912* not detected, C) 0.137 and D) 0.007.


A
*
B
C
D
**
1.5
1.0
0.5
0.0
Relative Normalized Expression
200
1800
3600
Soil Sulfur Concentration (ppm)

between plants (Fig. 10C). *XLOC_003912* expression was also compared between untreated Yukon 200ppm and 3600ppm plants. In this single trial, *XLOC_003912* relative transcript levels were 4.9-fold higher in plants grown with 200 ppm compared to 3600 ppm sulfur in the soil (Fig. 10D).

B) Virus-Induced Gene Silencing

The results described in the forgoing section are consistent with the proposal that *XLOC_003912* expression is sulfur-responsive in Yukon plants but how and the extent to which it is involved in sulfur nutrition is not known. Manipulating the expression of a gene can help elucidate the function of a gene or its product. In this study, I tested the effect of transiently repressing *XLOC_003912* expression using Virus Induced Gene Silencing (VIGS) in Yukon *Eutrema salsugineum* plants. However, before testing VIGS-mediated suppression of *XLOC_003912*, it was important to demonstrate that VIGS would operate in Yukon *Eutrema*. As such we tested VIGS-mediated suppression of *phytoene desaturase* (*PDS*), the product being a very visible bleaching of leaves. Indeed, given the lack of phenotype found between cabinet-grown plants with high versus low sulfur, I did not anticipate a strong phenotype if VIGS successfully repressed *XLOC_003912* expression. One option was to incorporate *PDS* either as part of the same construct as *XLOC_003912* or as separate constructs used in separate plants to indicate when and where the silencing was occurring. The decision was to use separate constructs in different plants because sulfur is inevitably tied to many stress events including the

interaction involving resistance to pathogens (Bloem et al., 2015). Thus we wished to minimize the stress to the plant caused by the combination of the unknown action of *XLOC_003912* and *PDS*-deficiency. Inoculation with the VIGS vectors was performed when the plants were 1.5 weeks old, approximately at the four-leaf stage (Fig. 11). *PDS* silencing first became evident about 1.5 weeks later when the plants were 4-weeks-old as the appearance of a few chlorotic spots (Fig. 11). At 2.5 weeks post-inoculation the silencing was more obvious with chlorotic zones progressing from a light yellow colour to a bleached-white appearance, often including many, but usually not all rosette leaves, and was frequently only visible on one-half of the plant. At 3.5 weeks post-inoculation the plants were 5-weeks-old and zones of white could often be seen on both halves of the rosette although they were not seen on newly emerging leaves, suggesting that the VIGS-mediated repression of *PDS* expression was either losing efficacy over time and/or newly formed leaf tissue was not an accessible target for viral movement. At 4.5 weeks after inoculation, when the plants were 6-weeks-old, lower leaves of the rosette were beginning to senesce and the visible phenotype associated with *PDS* silencing showed little progression until the plant started to flower at approximately 8 weeks post-inoculation and plants were 10-weeks-old (Fig. 12). At this point, some newly emerging leaves at the center of the rosette displayed photobleaching as did the occasional bolt and silique produced by the plant. Figures 11 and 12 show a typical pattern for the progression of the VIGS-mediated *PDS* silencing but there was considerable plant-to-plant variability with respect to where and when *PDS* silencing was evident (Fig. 13).

Moreover, raising the sulfur content of the soil from 200 to 1800 ppm had no effect on the VIGS-mediated photobleaching and did not change the inherent plant-to-plant variability of the phenotype generated, indicating that VIGS-mediated silencing can be effective in both sulfur-treated and untreated plants (Fig. 13).

With a procedure for VIGS tested and, while variable for *PDS*, the approach was shown to work, the next step involved testing for VIGS-mediated repression of *XLOC_003912.* Given that the *PDS* insert was approximately 1,000 base pairs, *Agrobacterium* strains containing the entire 786 base pair coding sequence of *XLOC_003912* inserted in the cloning site of the VIGS plasmid vector were generated. After sequencing to confirm the sequence of the insert, two independent pTRV2 clones corresponding to *XLOC_003912* were generated and designated XLOC Vector 1 and XLOC Vector 34, respectively. DNA sequence analysis showed that the two vectors were identical. To test for VIGS-mediated repression of *XLOC_003912*, a set of four plants were used for each test: an uninoculated control plant, an empty vector inoculated control, and two plants, one each inoculated with XLOC Vector 1 or Vector 34. Additionally, plants were tested on soil with and without sulfur supplementation to 1800 ppm such that each repeat was comprised of eight plants.

Figure 11: Yukon *Eutrema salsugineum* showing the VIGS-mediated progression of *PDS* silencing over time. *Agrobacterium tumefaciens* GV3101 carrying pTRV1, which encodes the replication and movement functions of the tobacco rattle virus (TRV), was combined 1:1 with the same *A. tumefaciens* strain carrying pTRV2 encoding the TRV coat protein either without (Empty Vector Control) or with a cloned insert encoding *PDS* (*PDS* Insert). Methods for vector preparation and inoculation of leaves as described in Materials and Methods. Wpi = weeks post-inoculation.

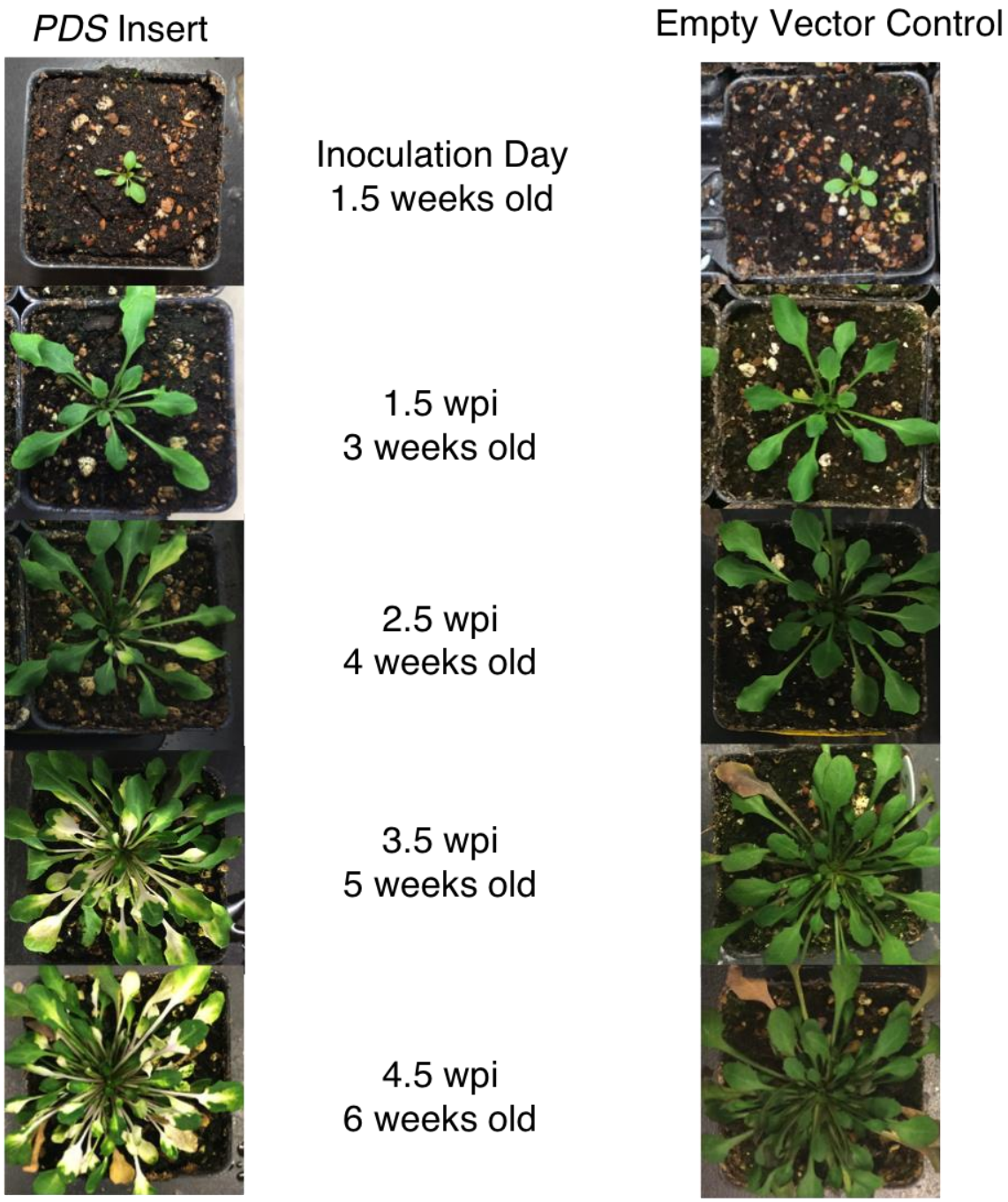
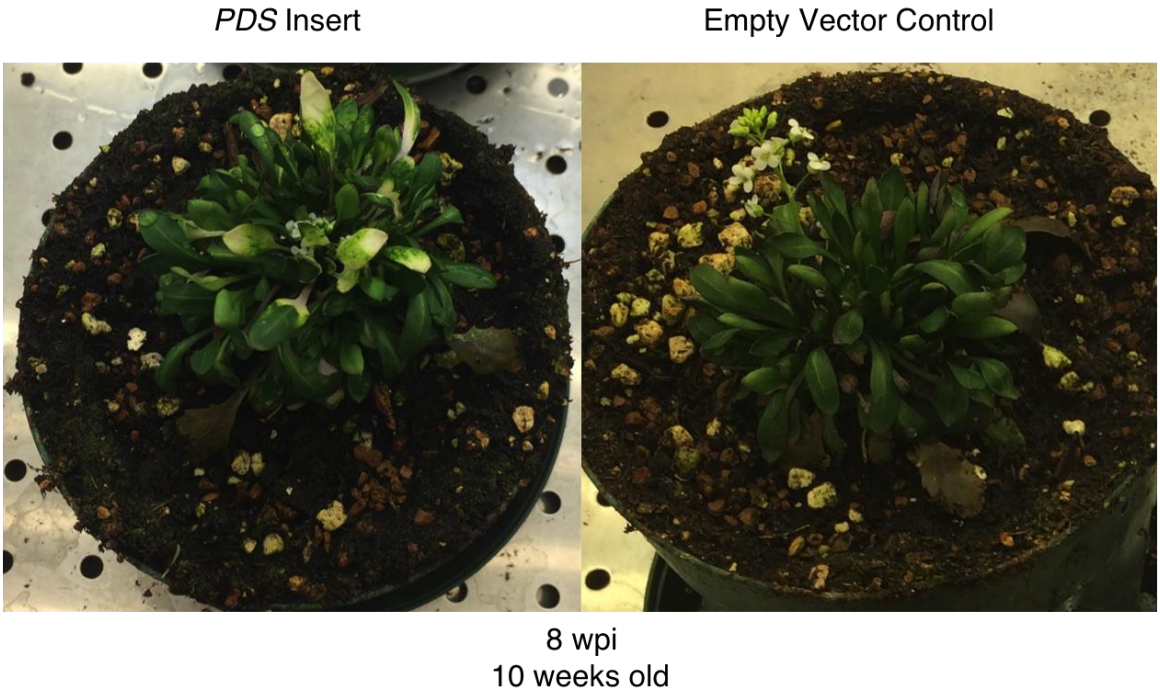

PDS Insert
Empty Vector Control
Inoculation Day
1.5 weeks old
1.5 wpi
3 weeks old
2.5 wpi
4 weeks old
3.5 wpi
5 weeks old
4.5 wpi
6 weeks old

Figure 12: Yukon *Eutrema salsugineum* showing VIGS-mediated *PDS* silencing at the flowering stage of development. *Agrobacterium tumefaciens* GV3101 carrying pTRV1, which encodes the replication and movement functions of the tobacco rattle virus (TRV), was combined 1:1 with the same *A. tumefaciens* strain carrying pTRV2 encoding the TRV coat protein either without (Empty Vector Control) or with a cloned insert encoding *PDS* (*PDS* Insert). Methods for vector preparation and inoculation of leaves as described in Materials and Methods. Wpi = weeks post-inoculation.



Relative *SDI1* and *XLOC_003912* transcript abundance was assessed by RT-qPCR using cDNA prepared from RNA of a fully-expanded leaf collected between 2.5 and 3.5 wpi. In two experimental trials, *SDI1* expression was lower in the sulfur-treated uninoculated plant compared to its counterpart on soil without added sulfur (Fig 14A, B), the expected response for *SDI1* based upon previous results (Fig. 9). Each trial yielded a single plant that had significantly repressed *XLOC_003912* expression compared to the uninoculated or empty vector controls. In the first trial the plant displaying repressed *XLOC_003912* expression was inoculated with XLOC Vector 1 (Fig. 14A), whereas in the second trial the plant with repressed *XLOC_003912* expression was inoculated with XLOC Vector 34 (Fig. 14B), evidence that both vectors had an equal potential to repress *XLOC_003912*. Neither of the repressed plants exhibited an observable phenotype. The *XLOC_003912* repressed plants were confirmed through two separate rounds of RNA extraction and RT-qPCR. In both cases, the material used for RNA extraction was collected at 2.5 wpi when the plants were at 4 weeks post-germination.

Interestingly, Figure 14 shows that the reduced expression of *SDI1* and *XLOC_003912* with 1800 ppm sulfur compared to 200 ppm sulfur is not the response seen in the plants inoculated with the empty vector or the *XLOC_003912*-VIGS vector (Fig 14A, B). In *Agrobacterium*-inoculated plants the expression of *XLOC_003912* and *SDI1* both remain as high or even higher relative to the expression levels found in plants on low sulfur. Given that there were no changes to in the expression of the reference genes *EF1α* or *UBQ10* between inoculated and uninoculated plants, this

Figure 13: Four-week-old Yukon *Eutrema salsugineum* plants showing variability with respect to the extent of the photobleaching phenotype produced by VIGS-mediated *PDS* silencing. Shown are six different Yukon plants at 2.5 weeks post-inoculation with four grown on 200 ppm soil (A), and two plants grown on 1800 ppm soil (B). For inoculation, *Agrobacterium tumefaciens* GV3101 carrying pTRV1 was combined 1:1 with the same *A. tumefaciens* strain carrying pTRV2 encoding the TRV coat protein and a cloned insert encoding *PDS*. Methods for soil preparation, vector construction, and inoculation of leaves as described in Materials and Methods.

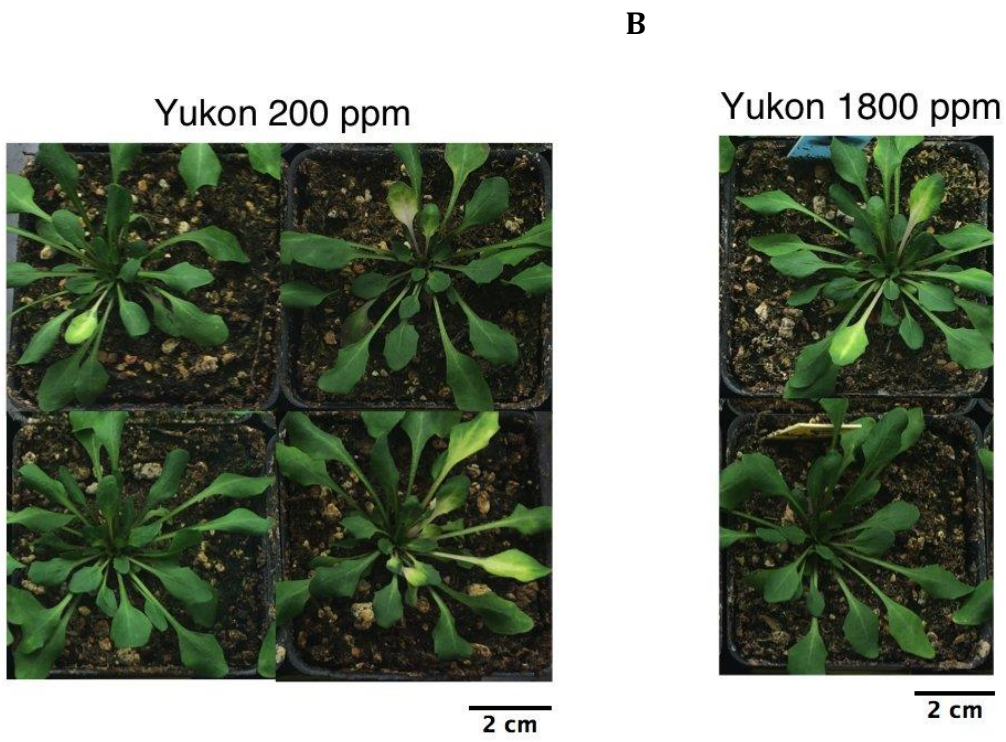
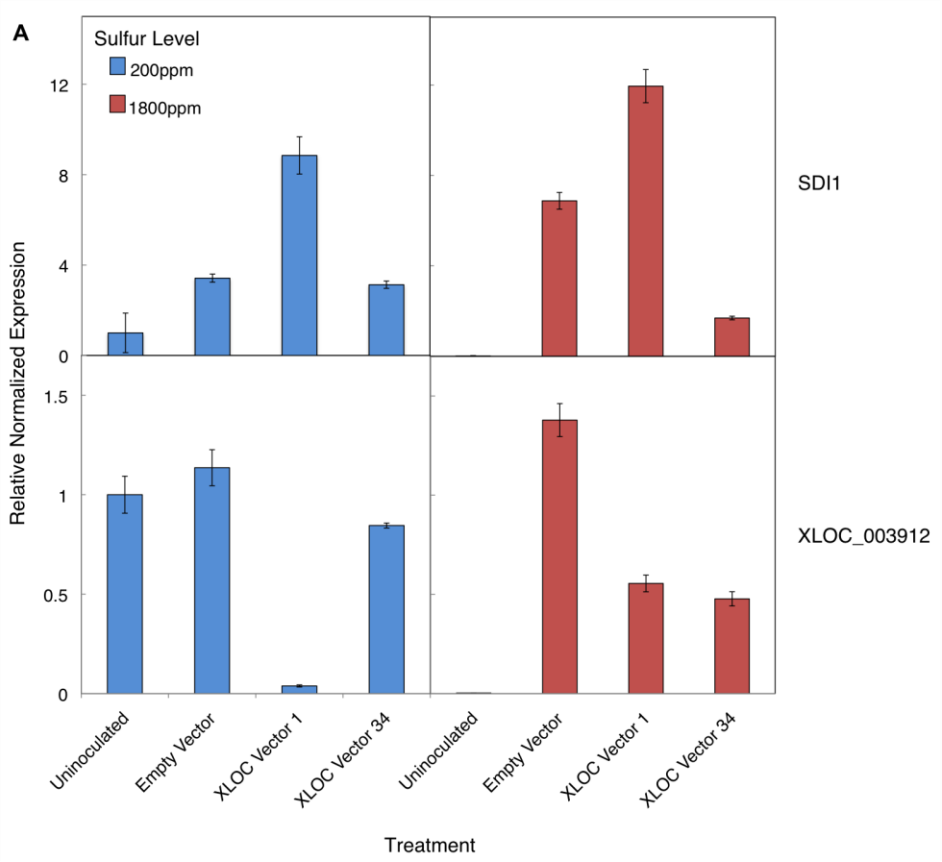
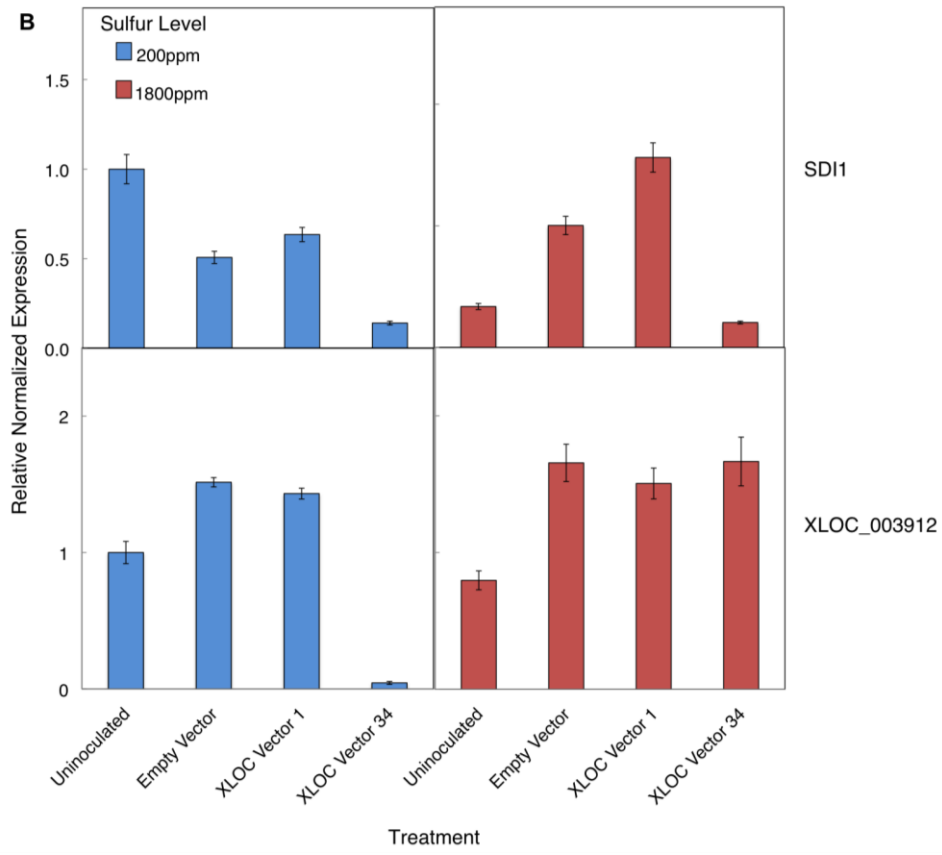


Figure 14: *XLOC_003912* expression shows VIGS-mediated repression.

XLOC_003912 and *SDI1* expression in Yukon 200 ppm and 1800 ppm *Eutrema* plants inoculated with *Agrobacterium tumefaciens* GV3101 containing the VIGS vector pTRV2 along with the same strain carrying the pTRV1 vector in a 1:1 ratio. There were two experimental trials (A, B) comprised of four treatments involving four plants: control plant (Uninoculated), plant inoculated with an empty pTRV2 vector (Empty Vector), and two plants inoculated with pTRV2 carrying sequence associated with a full-length *XLOC_003912* (XLOC Vector 1 and XLOC Vector 34). XLOC Vectors 1 and 34 refer to two independent constructs of pTRV2 carrying full-length *XLOC_003912* (see Results). The expression of *XLOC_003912* and *SDI1* is normalized to the expression of two reference genes, *EF1a* and *UBQ10*. Both *XLOC_003912* and *SDI1* expression in the uninoculated, untreated control are set to 1. RT-qPCR conditions as described in Materials and Methods with information on primers found in Table A1 of the Appendix.


A
Sulfur Level
200ppm
1800ppm
Relative Normalized Expression
SDI1
XLOC_003912
Uninoculated
Empty Vector
XLOC Vector 1
XLOC Vector 34
Treatment

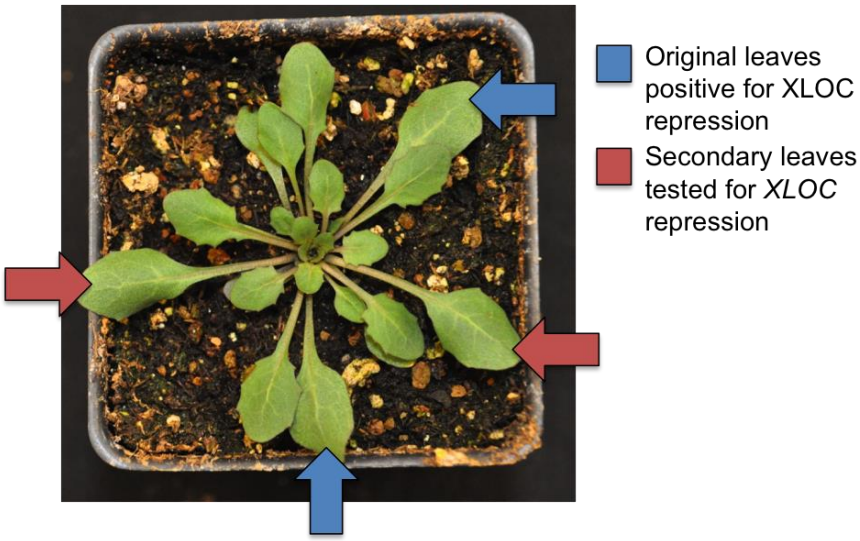

B
Sulfur Level
200ppm
1800ppm
Relative Normalized Expression
SDI1
XLOC_003912
Uninoculated
Empty Vector
XLOC Vector 1
XLOC Vector 34
Treatment

difference suggests that bacterial or viral infection may impact the expression of *XLOC_003912* and *SDI1.*

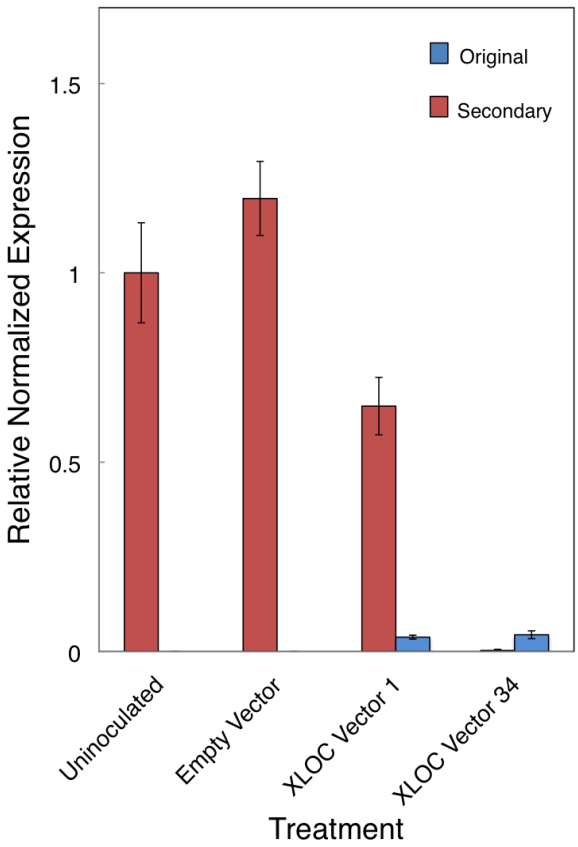
Given the sporadic appearance of bleaching when VIGS was used to repress *PDS* (Fig. 13), it was of interest to determine whether VIGS-mediated suppression of *XLOC_003912* was more widely distributed in the plant than the leaves selected for RNA extraction. With the absence of any *XLOC_003912*-associated phenotype, the approach used to address this question involved extracting RNA from two different leaves than those already removed from the two *XLOC_003912*-repressed plants originally identified (Fig 14A,B). For the *XLOC_003912*-repressed plant shown in Figure 14A, the secondary leaves that were removed provided a different assessment in that the 26-fold reduction in *XLOC_003912* expression shown in Figure 14A was only about 1.5-fold in the secondary leaves (Fig. 15; XLOC Vector 1). However, the opposite was true for the plant shown in Figure 14B where the 22-fold reduction seen in the original leaf was now found to be about 350-fold reduced (Fig 15B; XLOC Vector 34). This variability in *XLOC_003912* expression between different leaves of plants inoculated with the *XLOC_003912*-VIGS vector suggests that the level of *XLOC_003912* repression is variable across different leaves of the rosette. This finding is consistent with the variability in photobleaching across rosettes where leaves showed a differential and seemingly unpredictable pattern of VIGS-mediated repression of *PDS* (Fig. 13).

Figure 15: Variability in VIGS-mediated repression of *XLOC_003912* across rosettes of Yukon *Eutrema.* Leaf samples of plants previously shown to display VIGS-mediated repression of *XLOC_003912* were tested by RT-qPCR for *XLOC_003912* transcript abundance. A) Outline of strategy used for leaf selection in re-testing *XLOC_003912* expression showing position of original leaf tested relative to secondary leaves used for RNA extraction. B) Relative *XLOC_003912* expression for treatments involving four plants: control plant (Uninoculated), plant inoculated with an empty pTRV2 vector (Empty Vector), and two plants inoculated with pTRV2 carrying a DNA sequence encoding a full-length *XLOC_003912* (XLOC Vector 1 and XLOC Vector 34, corresponding to plants identified in Figure 14). The expression of *XLOC_003912* is normalized to the expression of one reference gene, *EF1a*. *XLOC_003912* expression in the uninoculated control is set to 1. PCR conditions as described in Materials and Methods with information on primers found in Table A1 in the Appendix.

A


Original leaves positive for XLOC repression
Secondary leaves tested for *XLOC* repression

B


Original
Secondary
Relative Normalized Expression
1.5
1
0.5
0
Uninoculated
Empty Vector
XLOC Vector 1
XLOC Vector 34
Treatment

DISCUSSION

Eutrema salsugineum (Yukon ecotype) plants express several genes associated with sulfur deficiency when grown in controlled environment growth cabinets compared to Shandong *Eutrema* plants grown under identical conditions or Yukon plants found in the field (Table 1). A possible difference in sulfur status between field and cabinet-grown Yukon *Eutrema* plants is perhaps not surprising given that the field soil in the Yukon has a 10 to 50-fold higher sulfur content than the cabinet potting mix (Appendix, Table A3). The difference between Yukon and Shandong plants is perhaps more difficult to explain as phenotypically both ecotypes seem equally healthy (Fig. 3). Typically sulfur-deficient plants display phenotypic features relative to plants with sufficient sulfur including reduced biomass and chlorophyll deficiency, consequences that culminate in reduced yield in crop plants (Nikiforova et al., 2005). Thus one objective of my research was to determine whether Yukon *Eutrema salsugineum* plants are experiencing a sulfur deficiency when grown under cabinet conditions as inferred by their transcriptome profiles. Secondly, among the differentially-expressed genes between *Eutrema* field and cabinet-grown plants were products that have not been previously annotated in *Eutrema* and hence were designated by the prefix "*XLOC*" by Champigny et al. (2013). One such product, "*XLOC_003912*", is a gene encoding a lncRNA that is co-expressed with a number of sulfur-responsive genes (Table 1) and the possible role of this gene in sulfur homeostasis in Yukon *Eutrema* plants was also explored in this thesis.

I. Sulfur supplementation and the Yukon *Eutrema* phenotype

With respect to biomass, Yukon 200 ppm plants displayed a 16 to 23 % reduction in fresh weight and a 22 to 25% reduction in dry weight compared to the 1800 ppm or 3600 ppm sulfur-treated plants (Fig. 4; Tables 2,3). While this is a barely discernible difference in fresh and dry biomass between the sulfur-supplemented and untreated plants (Fig. 3), it was reproducible over several replicates and it was statistically significant. Studies using *Brassica* plants show that they may not respond to sulfur deficiency with a severe reduction in shoot biomass. Indeed, Abdallah et al. (2010) found no reduction in fresh weight observed during a 35-day sulfur starvation treatment of *Brassica napus L.* compared to plants given adequate levels of this nutrient. In *Arabidopsis,* Nikiforova et al. (2005) reported a 20% decline in total biomass (root and shoot dry weight) in seedlings exposed to a sulfur-depleted medium for 13 days or for seedlings grown on sulfur-sufficient medium and then transplanted to deficient media for six days. Similarly, a more recent study of *Arabidopsis* plants deprived of sulfur for 9 weeks led to a 30% reduction in fresh weight and a 28% reduction in dry weight when compared to control plants (Ostaszewska et al., 2014). Taken together, the difference in fresh and dry biomass between untreated and sulfur-treated Yukon plants is in the same order of magnitude as the changes found for *Arabidopsis* and *B. napus* experiencing a sulfur deficiency, and is therefore consistent with the Yukon plants having a higher sulfur requirement to overcome a deficiency. By analogy, the lack of sulfur-

associated changes in biomass for Shandong *Eutrema* rosettes suggests that these plants are not experiencing sulfur deficiency (Fig. 4; Tables 2,3).

Reduced biomass is not the only indicator of a sulfur deficiency in Brassicaceae plants. Figure 5 and Table 4 shows that Yukon plants grown without sulfur supplementation had 24% less leaf area and 20% reduced leaf number compared to plants supplemented with 1800 ppm sulfur. Yukon 3600 ppm plants also had a greater leaf area and leaf number relative to the untreated controls of this trial although the magnitude of the difference was slightly less at a reduction of 17% and 14% for leaf area and number, respectively. The difference between the Yukon 1800 ppm and 3600 ppm plants is likely a result of seasonal changes in the growth cabinets as plants grown during winter months tend to grow more slowly and are smaller (unpublished observation). Again, a relatively modest albeit significant difference in leaf area and number distinguishes sulfur-treated from untreated plants. Similar reductions in leaf area and number have been reported for other plants. Notably, *Arabidopsis* depleted of sulfur for 9 weeks were reduced in leaf area and leaf number by 31% and 12.5%, respectively, relative to plants receiving adequate sulfur (Ostaszewska et al., 2014). The lack of changes with respect to leaf area or number between Shandong 200 ppm and 1800 ppm plants is further evidence that the Shandong 200 ppm plants are not deficient for sulfur and this finding supports the proposal that Yukon plants have a higher requirement for sulfur to achieve optimal growth.

Chlorosis is a classic symptom of sulfur deficiency used to diagnose this nutritional deficit in crops (Taiz & Zeiger, 2002). In this study no difference was found between the Yukon 200, 1800 or 3600 plants with respect to total chlorophyll, its components, chlorophyll A and B and the ratio between chlorophylls A and B was unchanged (Fig. 6). Nikiforova et al. (2005) reported a 2 to 3-fold reduction in total chlorophyll content in *Arabidopsis* seedlings grown on sulfur-depleted media relative to controls that were provided with sulfur. However, when Ostaszewska et al. (2014) starved *Arabidopsis thaliana* plants for sulfur for 9 weeks they found no difference in chlorophyll A or B content between control and sulfur-starved plants. These authors also pointed out that studies reporting reduced chlorophyll content with sulfur deprivation also used relatively shorter periods of sulfur depletion (Nikiforova et al., 2005, Zuchi et al., 2009). Given that plants grown for this study meant extending low sulfur exposure for a period of four weeks, it is possible that the conditions used would also not produce a chlorophyll deficiency as was proposed by Ostaszewska et al. (2014) to explain their similar results.

In summary, the phenotypic evidence that Yukon *Eutrema* requires more sulfur for growth includes significantly higher rosette biomass, leaf area, and leaf number in sulfur-treated compared to untreated plants. In contrast, the similar levels of chlorophyll between plants from different sulphur treatments shows that chlorosis is not a suitable marker for Yukon plants experiencing a long-term sulfur deficiency.

II. Physiological and Molecular Traits Distinguishing Sulfur-Treated From Untreated Plants

A) Nutrient Content

Yukon plants grown in untreated soil showed only an 8 to 12% decline in total sulfur content (%DW) compared to the sulfur-treated plants, with the comparison for the Yukon 3600 ppm plants not being statistically significant (Fig. 7A,B). One consideration on the matter of statistical significance is that only two replicates were done for the Yukon 3600 ppm plants whereas three were done for the Yukon 1800 ppm plants so it is possible that further replication is required to obtain a statistically significant difference for the former. Nonetheless, the results of the sulfur content measurements of the Yukon plants is not consistent with information of sulfur deficiency studies reported in the literature. For example, Nikiforova et al. (2005) found a 43% reduction in sulfur content in *Arabidopsis* plants grown for 10 days on sulfur-deficient media and Ostaszewska et al. (2014) reported a 52% reduction in sulfur for *Arabidopsis* plants deprived of sulfur for 9 weeks. The similar magnitude in the reduction of sulfur in plants grown for either a short period of 10 days to a long period of 9 weeks is consistent with the interpretation that *Arabidopsis* plants establish a sulfur content early in the deficiency and then sustain that level around 50% that of sulfur-sufficient plants for the duration of the deficiency, presumably by using acclimation strategies that maintain a critical sulfur homeostasis when sulfur is limiting.

The comparison between the Yukon and Shandong plants with respect to rosette sulfur content is also of interest. Firstly, in contrast to the Yukon *Eutrema,* Shandong 200 ppm plants showed no difference in sulfur content compared to the Shandong 1800 ppm plants, again suggesting that the Shandon *Eutrema* plants are not responding to a sulfur deficiency (Fig. 7C). Secondly, expressed as %DW, Yukon *Eutrema* has a higher rosette total sulfur content than the Shandong ecotype for both sulfur-treated and untreated plants (Fig. 7A,B,C). This suggests that when grown on soil with the same sulfur concentration, Yukon *Eutrema* accumulates more sulfur in its leaves than Shandong *Eutrema* does.

Placed in the context of other species, Yukon and Shandong plants have a relatively high sulfur content compared to many plants (Table 7). Many common plants maintain a sulfur content (expressed as a % DW) of less than 1% DW whereas Shandong and Yukon 200 ppm plants have a sulfur content of 1.8% and 2.6 % DW, respectively (Table 7). Notably, Table 7 shows that both *Eutrema* ecotypes have a sulfur content that falls within the range of sulfur reported for other halophytes found in their native habitats. Even with the exception of sugar beet (*Beta vulgaris L.*) considered, Table 7 suggests that halophytes may tend to have or require a naturally higher sulfur content than glycophytes.

Sulfur is often coupled with N in plants in the form of free amino acids, incorporated as peptides (eg. glutathione), oligomers (eg. phytochelatin) or small and large molecular mass proteins (eg, thioredoxin or Fe-S electron carriers). Thus in plants, sulfur and nitrogen levels in the plant are inevitably linked with a 2 to10-

fold decrease in the nitrogen:sulfur ratio a reported outcome of a sulfur deficiency for both *Brassica napus* and *Arabidopsis* (Abdallah et al., 2010; Oskazewska et al., 2014).

With respect to the nitrogen content of the *Eutrema* plants, there was no change in the nitrogen content between the ecotypes in response to the sulfur treatments (Fig. 7D,E,F). This is comparable to results in the literature for *Brassica napus* and *Arabidopsis thaliana* showing no change in nitrogen content with sulfur deficiency (Abdallah et al., 2010; Oskazewska et al., 2014). For the Yukon 1800 ppm plants there was a statistically significant decrease in the nitrogen:sulfur ratio between untreated plants and sulfur-supplement plants and while this difference was also apparent in the comparisons involving Yukon 3600 ppm and Shandong 1800 ppm plants as well, in neither of these cases were the changes statistically significant (Fig. 7G,H,I; Table 8). Overall, the comparison of sulfur and nitrogen content suggests that whole rosette sulfur content is fairly consistent, that changes in absolute and relative values are barely perceptible and hence sulfur, nitrogen, and nitrogen:sulfur ratios are collectively not very sensitive indicators to diagnose a sulfur deficiency in *Eutrema* plants. Figure 7A, however, does provide evidence that Yukon 200 ppm plants can take up more sulfur if provided with a source of this nutrient in the potting soil.

Table 7: Sulfur content is variable among plants and often high in halophytes.

Plant	S Content *%DW*	Comment	Source
Suaeda suffrutescens (ink weed)[a]	2.94	halophyte	Thomas et al., 1950
E. salsugineum Yukon ecotype[b]	2.57	halophyte	This study
Tamarix [a]	2.36	halophyte	Thomas et al., 1950
Salicornia [a]	2.04	halophyte	Thomas et al., 1950
Allenrolfea (bushy samphire)[a]	1.48	halophyte	Thomas et al., 1950
E. salsugineum Shandong ecotype[b]	1.8	halophyte	This study
Atriplex [a]	0.88	halophyte	Thomas et al., 1950
Sarcobatus (greasewood)[a]	0.58	halophyte	Thomas et al., 1950
A. thaliana	0.77		Ostaszewska et al., 2014
cabbage	0.62		Thomas et al., 1950
rutabaga	0.51		Thomas et al., 1950
sugar beet	0.38	halophyte	Thomas et al., 1950
alfalfa	0.18-0.56		Thomas et al., 1950
deciduous trees	0.18-0.54		Thomas et al., 1950
radish	0.17-0.4		Thomas et al., 1950
shrubs and bushes	0.16-0.31		Thomas et al., 1950
rapeseed (dried)	0.16-0.49		Thomas et al., 1950
clover	0.11-0.41		Thomas et al., 1950
conifers	0.10-0.11		Thomas et al., 1950

[a] Measurements from saline field sites.
[b] Measurements from four-week-old *Eutrema* plants grown on 200 ppm sulfur.

B) Gene Expression

GGCT2;1 and *SDI1* have been used as markers of sulfur deficiency in *Arabidopsis* and wheat. *GGCT2;1* was found by Nikiforova et al., 2005 to be 10.6-fold up-regulated in seedlings grown for 13 days on sulfur-deficient media while *SDI1* was identified as a marker gene that is up-regulated in response to sulfur deficiency in the leaves and roots of wheat and *Arabidopsis* (Howarth et al., 2009) and has since been used as a sulfur deficiency biomarker in *Arabidopsis* (Hubberten et al., 2012; Leustek and Zheng, 2015). *SDI1* was found to be 57 to 200-fold up-regulated in the roots of sulfur deficient *Arabidopsis* (Hubberten et al., 2012; Leustek and Zheng, 2015). Yukon 200 ppm plants had a 6.35 and 4-fold increase in *GGCT2;1* and *SDI1* expression, respectively, when compared to Yukon 1800 ppm plants and far greater 177 and 30.4-fold increases, respectively, relative to expression levels in Yukon 3600 ppm plants (Fig. 9). By way of comparison, both *GGCT2;1* and *SDI1* were identified as being highly responsive to sulfur deficiency in *Arabidopsis* by Watanabe et al. (2012), with *GGCT2;1* being 86-fold up-regulated, and *SDI1* being 89-fold up-regulated in *Arabidopsis* roots. Given this range of fold-changes, the greater differential between Yukon 200 and 3600 ppm as opposed to Yukon 200 and 1800 ppm plants with respect to the expression of both marker genes (Fig. 9) suggests that the Yukon 1800 ppm plants still lack sufficient sulfur to suppress the deficiency-responsive expression of these markers. In contrast, the Shandong 200 and 1800 ppm plants did not display difference in the expression of *GGCT2;1* or *SDI1*

reaffirming that the Shandong 200 ppm *Eutrema* are not experiencing a sulfur deficiency (Fig. 9).

An unexpected finding in this research relates to the expression of *GGCT2;1* and *SDI1* in that their fold-levels of expression in Yukon plants changed to a much greater extent in response to the sulfur treatment than the comparatively minor change in sulfur content between untreated and sulfur-supplemented plants would have predicted (Fig. 7). Tissue selection was one possible source of this finding in that sulfur determinations required relatively large quantities of tissues so whole rosettes were sent away for sulfur content analysis whereas fully-expanded leaves in the middle of the rosette were selectively used for RNA extraction and RT-qPCR determinations. This possibility was tested by collecting leaves similar to those used for RNA from four-week-old Yukon 200 ppm and 1800 ppm plants and sending them away for sulfur content analysis. The leaves from the Yukon 200 ppm plants were found to have a sulfur content of 1.8% DW, a lower level than seen for the whole rosette (Fig. 7B). However, the fully-expanded leaves from the Yukon 1800 ppm plants grown at the same time had a similar sulfur content of 3.0% DW, representing a 1.7-fold different sulfur content between leaves of 200 and 1800 ppm plants compared to the 1.1-fold difference found for whole rosettes (Fig. 7A,B). In a recent study where *Arabidopsis* was deprived of sulfur for 9 weeks, there was a 2.1-fold difference in sulfur content between sulfur-starved and sulfur-sufficient plants (Ostaszewska, 2014), a number in close agreement to the 1.7-fold difference in the level of sulfur found between expanded leaves of Yukon 200 and 1800 ppm plants in

this work. The difference between the sulfur content estimated from expanded leaves versus whole rosettes suggests that the plants are mobilizing sulfur in response to the deficiency. That is, the sulfur content remains basically unchanged across the rosette but is differentially distributed away from fully-expanded leaves into other sites on the rosette, likely the younger, expanding leaves.

Although the relatively low sulfur content and high differential in gene expression may seem contradictory, these findings are supported by previous results published by Guevara et al. (2012) and Champigny et al. (2013). Figure 16 is a re-work of Figure 7 replacing the Shandong data with the sulfur and nitrogen content data of Guevara et al. for Yukon *Eutrema* plants grown in the cabinet as well as Yukon plants found growing naturally in the field. In their study they showed that at a Yukon field site where the level of sulfate in the soil was recorded at 9379 ppm the plants present had a leaf sulfur content of 1.92% DW while plants grown in a cabinet using potting soil with a soil sulfate level of 200 ppm had a leaf sulfur content of 1.71% DW (Fig. 16C). In this study the change in sulfur levels was comparable: 1800 ppm plants had an average sulfur content of about 2.93% DW and plants grown on 200 ppm soil sulfur had an average sulfur content of 2.57% DW (Fig. 16A). However, the same was not the case for nitrogen. The nitrogen content of field plants reported in the study by Guevara et al. (2012) is lower relative to the content of their cabinet plants and all of the plants used in this study (Fig. 16D,E,F). Although both field and cabinet soil have low nitrogen levels, cabinet-grown plants are fertilized regularly and hence receive nitrogen-containing fertilizer weekly,

which may explain the differences seen in nitrogen content as a function of growth conditions (cabinet versus field). The nitrogen to sulfur ratio estimated using the data of Guevara and co-workers is lower for field plants compared to the cabinet plants, matching the pattern that I observed in Yukon 1800 ppm and 3600 ppm plants compared to 200 ppm plants (Fig. 16G,H,I). Overall, the data published by Guevara et al. (2012) and my results both suggest that the leaf sulfur content of Yukon *Eutrema* plants does not respond strongly to changing levels of soil sulfate. That is, a high content of sulfur in the soil does not translate to a high internal leaf sulfur content in the plant. Conversely, the gene expression level of two sulfur deficiency marker genes, *GGCT2;1* and *SDI1* were shown to be strongly responsive to the level of sulfate provided in the soil (Fig. 9). These results were also observed by Champigny et al. (2013) when they compared transcriptomes between leaves of Yukon field and cabinet plants. They observed 30.1 and 7.6-fold differences in *GGCT2;1* and *SDI1* transcript levels between plants grown in cabinets with soil containing 200 ppm sulfur and fertilized weekly versus field plants growing with >2000 ppm sulfur in the soil (Table 8). These fold-differences are in agreement with those that I detected by RT-qPCR between sulfur-treated and untreated Yukon plants (Fig. 9) of about 177 and 30.4-fold for *GGCT2;1* and *SDI1* between leaves of Yukon 200 and the 3600 ppm plants. Additionally, in my results as well as those reported by Champigny et al. (2013), *GGCT2;1* showed a greater difference between the cabinet as compared to the field plants and my Yukon 200 ppm compared to sulfur-supplemented plants. Thus although the sulfur content of the plants does not

Figure 16: Sulfur and nitrogen content of Yukon *Eutrema* plants from this study compared to previously published values for cabinet-grown and field-sourced plants. Panels A, B, D, E, G and H reproduced from Figure 7. See legend of Figure 7 for details on plant analysis and replication. Data shown in Panels C, F, and I are directly from (sulfur and nitrogen content) or calculated (nitrogen:sulfur ratio) using the data reported by Guevara et al. (2012). Asterisks denote level of significance: * = $P < 0.05$, ** =$P<0.01$, *** = $P<0.001$.

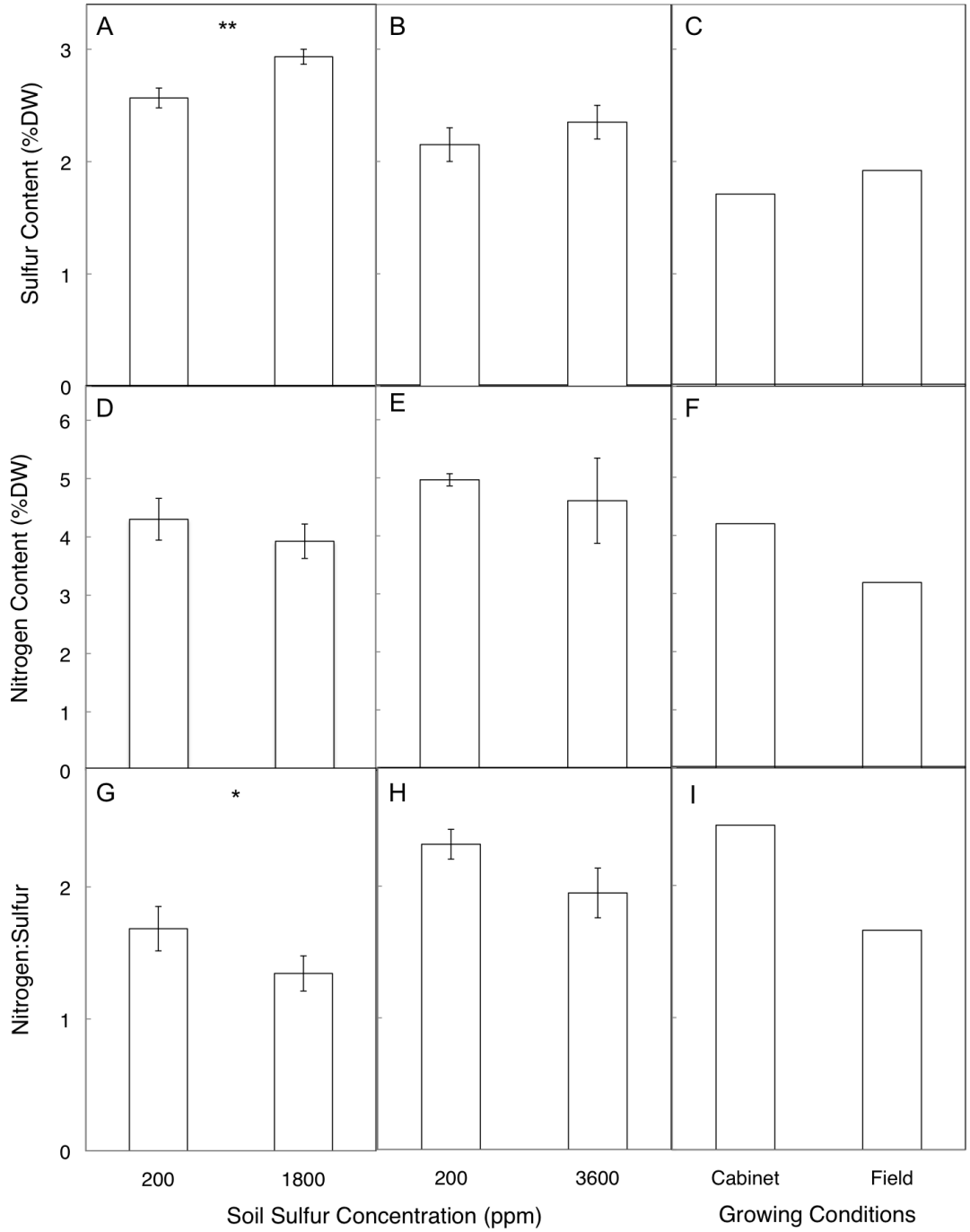

A
**
B
C
D
E
F
G
*
H
I
Sulfur Content (%DW)
Nitrogen Content (%DW)
Nitrogen:Sulfur
0
1
2
3
4
5
6
200
1800
200
3600
Cabinet
Field
Soil Sulfur Concentration (ppm)
Growing Conditions

Table 8: Soil sulfur content in field and cabinet growth locations related to the differential expression of Yukon *Eutrema* biomarkers *GGCT2;1* and *SDI1.*

Comparison	S Content		Gene Expression		Source
	Soil *ppm*	Plant *%DW*	*GGCT2;1* *Fold Δ*	*SDI1* *Fold Δ*	
Field vs Cabinet	>2000[a] 200	1.92 1.71	30.1	7.6	Champigny et al. 2013[b]
Cabinet vs Cabinet	200 1800	2.57 2.93	6.4[c]	4.0[c]	This study
Cabinet vs Cabinet	200 3600	2.15 2.35	177.0[c]	30.4[c]	This study

[a] A. Garvin, unpublished data
[b] Calculated from published Reads Per Kilobase of transcript per Million mapped reads for cabinet and field data for *GGCT2;1* (177.3 vs 5.9) and *SDI1* (38 vs 5)
[c] Data from Figure 9 based upon RT-qPCR

undergo a many-fold increase when plants are exposed to high soil sulfur, the same is not true for the markedly different expression levels of the two sulfur deficiency marker genes. As a result, *GGCT2;1* and *SDI1* offer a sensitive means of detecting low plant sulfur conditions for *Eutrema* plants, and arguably a far more sensitive approach than measuring changes in rosette biomass, leaf area, leaf number, or internal sulfur content of either elected leaves or whole rosettes.

III. Analysis of *XLOC_003912* Expression in Response to Soil Sulfur Content

In order to test the hypothesis that the lncRNA *XLOC_003912* is sulfur-responsive and plays a role in sulfur nutrition in Yukon *Eutrema* plants I looked at *XLOC_003912* expression in sulfur-supplemented and untreated plants. In all cases *XLOC_003912* expression was repressed in sulfur-treated plants compared to untreated plants although the degree to which it was repressed varied greatly between replicates (Fig. 10). Additionally, *XLOC_003912* was not more highly suppressed in the 3600 ppm than the 1800 ppm plants compared to the 200 ppm plants. This data suggests that *XLOC_003912* may be responding to sulfur treatment of the soil, but not likely sulfur alone. That is, *XLOC_003912* expression must be influenced by other factors (or combination of factors) with many possible candidates that could include humidity, water availability, or even the availability of other nutrients. The variation in gene expression between trials serves as a reminder of the importance of monitoring environmental conditions for reproducibility between experiments and ensuring that the experimental design

includes a sufficient number of untreated plants to provide estimates for high-confidence baseline gene expression determinations.

IV. VIGS as a Method of Silencing *Phytoene Desaturase (PDS)* and *XLOC_003912* in Yukon plants

In order to determine whether *XLOC_003912* expression is involved in sulfur nutrition, I wanted to develop a means of manipulating the expression of this gene. I elected to use Virus Induced Gene Silencing (VIGS), a method that does not require the development of stably transformed RNAi or overexpression lines. I was able to successfully silence the phytoene desaturase gene (*PDS*), creating a photo-bleached phenotype (Fig. 11,12). By following the progression of photo-bleached zones in leaves of Yukon *Eutrema* I was able to determine that silencing was best when plants were selected for inoculation at 1.5 weeks post-germination, that the most pronounced phenotypic indicators of bleaching emerged as early as 1.5 weeks post-inoculation, and that the effects of silencing dissipated by 3.5 weeks post-inoculation. Through trial and error I determined that the best time for tissue collection was 2.5 weeks post-inoculation, when the plants are 4-weeks-old. This information was used to direct the VIGS-mediated suppression of *XLOC_003912.*

To prepare the VIGS vector for experimentation the entire 786 bp sequence of *XLOC_003912* was cloned into the VIGS pTRV2 vector, the sequence was confirmed by sequencing and then the plasmid carrying the transgene (or empty vector construct) was transformed into *Agrobacterium* for inoculation of plants at

1.5 weeks after germination. The inoculated plants showed variable levels of *XLOC_003912* suppression from considerable to no apparent silencing (Fig. 14A,B). When I extracted RNA from leaves for RT-qPCR analysis I used two to three fully-expanded leaves in the middle of the rosette (Fig. 15A) but subsequent extraction of RNA from other leaves showed that silencing is more wide-spread in the plant (Fig. 15B). In many respects, this sporadic pattern of repression resembled the variability seen for VIGS-mediated silencing of *PDS* (Fig. 13).

Of interest, only Yukon 200 ppm plants were found to have significantly reduced *XLOC_003912* expression compared to the uninoculated control. The association between sulfur and VIGS suppression of *XLOC_003912* may be physiological, but this would require more extensive testing with a greater number of plants as "n" may be a factor. An unanticipated finding was the high expression of *SDI1* and *XLOC_003912* in Yukon 1800 ppm plants when they were inoculated with *Agrobacteria* containing empty vector or the *XLOC_003912* construct (Fig. 14A). This suggests that a biotic interaction was elicited by the inoculum and that this had the unexpected effect of altering the expression of the both the target and biomarker gene. While this may be a complication in studying *XLOC_003912* using VIGS, the discovery of a couple plants showing suppression of *XLOC_003912* expression is evidence that VIGS can be used as a method of repressing *XLOC_003912* and that it has potential to work as a way of studying the function of other unknown genes in *Eutrema.*

ONGOING PROJECTS AND FUTURE DIRECTIONS

Over the period of my MSc program I have collaborated on a number of projects related to sulfur nutrition in *Eutrema salsugineum*. The first of these projects involved looking into the way that sulfur nutrition impacts *Eutrema*'s response to herbivory, specifically by green peach aphids (*Myzus persicae*). These projects include "Herbivore Defence Mechanisms in *Eutrema*—A pilot study" by Jacqueline Rotondi (2014), work that began to explore differences in aphid herbivory on Yukon 200 and 1800 ppm plants. A second study "Metabolic analysis following nutrient manipulation of *Eutrema salsugineum* and its corresponding effects on *Myzus persicae"* by Adelle Strobel (2015) delved further into these differences, and began to explore possible mechanisms behind decreased herbivory on sulfur-treated plants. A third study, "The Impact of Soil-Sulfur on Glucosinolate Content of *Eutrema salsugineum"* by Julianna Stangroom (2016) measured the glucosinolate content of sulfur-treated and untreated plants, though found there was no difference between the two. We concluded that *Eutrema* may be using other sulfur-derived phytochemicals to deter herbivory or possibly the plant-animal interaction is needed to induce glucosinolate synthesis. These hypotheses should be investigated further in the future.

I have been a part of an investigation on the effect of sulfur and phosphorus availability on the lipid composition of Yukon *Eutrema* plants. Under low phosphate conditions plants can remodel lipids to increase the proportion of glycolipids (including sulfolipids) and decrease phospholipids. I worked with Ms. Vera Velasco

to develop a method of varying the soil composition of sulfur and phosphate to alter nutrient levels for plants: low sulfur and low phosphorus (-S-P), low sulfur and high phosphorus (-S+P), high sulfur and low phosphorus (+S-P), and high sulfur and high phosphorus (+S+P). We used RT-qPCR to determine relative expression levels of *SDI1* and *IPS2* (*Induced by Phosphate Starvation2*), we measured the total phosphorus and sulfur content of the plants to confirm that the plants were responding to the treatment, measured rates of photosynthesis, and Dr. Peter Summers oversaw the lipid analyses. This work is now being prepared for publication.

Lastly, transcriptomes from control and *XLOC_003912*-repressed plants created using VIGS and identified by RT-qPCR have been prepared and will be used to explore the function of *XLOC_003912* as well delve further into differences in gene expression caused by sulfur treatment, and possibly to explore the impact of bacterial and viral infection on the gene expression of *Eutrema* plants by comparing inoculated plants to uninoculated plants. Additionally, plants corresponding to the lipid-remodeling project were also sent for RNA-seq analysis and these transcriptomes will be available as valuable tools for exploring interactions between the phosphorus and sulfur nutrition pathways in *Eutrema*.

SUMMARY

In conclusion, Yukon but not Shandong *Eutrema salsugineum* plants experience symptoms of sulfur deficiency when grown under conventional lab conditions even when fertilized regularly. Yukon *Eutrema* sulfur content of leaves is not strongly responsive to soil sulfur levels but the expression of biomarker genes associated with sulfur deficiencies (*GGCT2;1* and *SDI1*) can be used as reliable indicators of insufficient sulfur. In addition, the expression of a novel lncRNA (*XLOC_003912*) was shown to be responsive to the sulfur content of the soil in Yukon *Eutrema* plants although the variability in its expression suggests that it is responsive to other factors as well. Lastly, a method for suppressing genes by VIGS was tested using *XLOC_003912* as a target. This method will be useful for exploring the function of other novel genes in *Eutrema.*

APPENDIX

Table A1: Sequence information for primers used in RT-qPCR analysis.

Primer Name	Gene	Gene ID	Sequence	Product Length	GC (%)	Temp (˚C)
SDI1-1F SDI1-1R	SDI1	Thhalv10004656	**Forward:** ACAAGAACCCAG AGATGGCG **Reverse:** GATGGCTTCTTC GGAACGGT	125	55	55.7-61.4
GGCT-3F GGCT-3R	GGCT2;1	Thhalv10004888	**Forward:** GCTTCTGGACCT TGTGGGAA **Reverse:** TCCTCGTGCTCA ATGTCGTG	77	55	55.7-65
XLOC-2F XLOC-2R	XLOC	XLOC_003912	**Forward:** AGCCCAACATCA ACCCATTCA **Reverse:** ACGAAGCTCTGT TCGGCTAC	138	50	61.4-65

Table A2: RT-qPCR information and validation sequences for primers used for RT-qPCR.

Primer Name	Cq	Efficiency (%)	R^2	Description	Validation sequence	% identity
SDI1-1F SDI1-1R	24	93.5 at 61˚C	0.98	Sulfur Deficiency induced 1 S responsive	ACAAGAACCCAGAGATGG CGATAGTATGGTTTTGGA AAGCCATTAACACAGGAG ACAGAGTAGACAGTGCTC TCAAGGACATGGCTGTTG TAATGAAACAACTTGACC GTTCCGAAGAAGCCATC	100
GGCT-3F GGCT-3R	27	109.9 at 61˚C	0.994	γ-glutamyl cyclotransferase 2;1 S responsive	GCTTCTGGACCTTGTGGG AACAAACCGGGAGTACTT CAAGCTTGANAANCAATG CACGACATTGAGCACGAG GA	91
XLOC-2F XLOC-2R	25	95.3 at 61˚C	0.986	Unknown function	AGCCCAACATCAACCCAT TCAAATTAGAGGATAGCG ATTAACAAAGATCAAACA CATGTAACACACATGGTA GGCTAAGGTGAAGATTAG GATGATACTGAGAAGATA CCGCCGACAAGTAGCCGA ACAGAGCTTCGT	100

Figure A1: pTRV2 insert sequences used for VIGS.

PDS:

TTCTGCGGCGAATTTGCCTTATCAAAACGGGTTTTTGGAGGCACTTTCATCTGGAGGTTGTGAACTAATGGG
ACATAGCTTTAGGGTTCCCACTTCTCAAGCGCTTAAGACAAGAACAAGGAGGAGGAGTACTGCTGGTCCTTT
GCAGGTAGTTTGTGTGGATATTCCAAGGCCAGAGCTAGAGAACACTGTCAATTTCTTGGAAGCTGCTAGTTT
ATCTGCATCCTTCCGTAGTGCTCCTCGTCCTGCTAAGCCTTTGAAAGTTGTAATTGCTGGTGCTGGATTGGC
TGGATTGTCAACTGCAAAGTACCTGGCTGATGCAGGCCACAAACCTCTGTTGCTTGAAGCAAGAGATGTTCT
TGGTGGAAAGATAGCTGCATGGAAGGATGAAGATGGGGACTGGTATGAGACTGGTTTACATATTTTCTTCGG
TGCTTATCCGAATGTGCAGAATTTATTTGGAGAACTTGGGATCAATGATCGGTTGCAGTGGAAGGAACACTC
CATGATTTTTGCTATGCCAAGTAAACCTGGAGAATTTAGTAGATTTGACTTCCCAGATGTCCTACCAGCACC
CTTAAATGGTATTTGGGCTATTTTGCGGAACAACGAGATGCTGACATGGCCAGAGAAAATAAAGTTTGCTAT
TGGACTTTTGCCAGCCATGGTCGGCGGTCAGGCTTATGTTGAGGCCCAAGATGGTTTATCAGTCAAAGAATG
GATGGAAAAGCAGGCAATACCTGAGCGCGTGACCGACGAGGTGTTTATTGCCATGTCAAAGGCGCTAAACTT
TATAAACCCTGATGAACTGTCAATGCAATGCATTTTGATAGCTTTGAACCGGTTTCTTCAGGAAAAACATGG
TTCCAAGATGGCATTCTTGGATGGTAATCCTCCGGAAAGGCTTTGTATGCCAGTAGTGGATCATATTCGATC
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TTTCT

XLOC_003912:

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GTGAAGATTAGGATGATACTGAGAAGATACCGCCGACAAGTAGCCGAACAGAGCTTCGTCGAAAAGCAGAAA
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TAAACTCCTTTCACTGCAAGATCACGATTAACCAAAAGTCTTCTTGCATCCGTAAGATTAGCCACCGCTCAT
CTCAAACTCAGAGACCAAATACCCTTCACCTTCAAAAGGCTCTTTAGAAAGGACTTTAGGGAGAAACAACTC
TCAGCCAAGAGAGGATGATGCCAATTGTTGGAGAATATGGTGCTATCACATCATCGGATATGAACTGTAAAA
AAAGGAGACAAGTTTCAAAAACAGATACCTTTAACATTATCGCCGGAATAACCAAATCCATATTTCAATCTG
TGAAGAATCTTCGCTCCACTAAGGATTAGCAAAACTTAATACTCTTGATATAAGAAGAACCGATTTTAATAT
GGCATAAGAAGAGCATCAAAACATAAACCCACAAATACTCAGAAAAAGGTAATTCGCAAAACGGAAAGCAAA
AACAATCTAATCAAGCCGGAACTTTATTATCCGATCAAAATCAGAAACAAAAAGCCGATATAATTT

Table A3: Sulfate concentration of soils collected from Yukon field sites compared to soil used in the cabinet. Modified from Amanda Garvin undergraduate thesis.

Yukon Field Soil Location	**Collection Year**	**Sulfate (ppm)**
Cracker Creek	2011	4409
Takhini Salt Flats	2011	1393
Takhini Salt Flats	2011	1990
Dillabough's Grazing Lease - outside fence	2011	7199
Dillabough's Grazing Lease - outside Fence	2011	6852
Dillabough's Grazing Lease - inside fence	2011	5006
Dillabough's Grazing Lease - stored at Mac	Approx. 2008	5862
Sample analysed by Ping	Approx. 2006	9379
Average		**5261**
Cabinet Soil	**Collection Year**	**Sulfate (ppm)**
A - mix	2012	502
B - mix	2012	197

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