

Table of Contents	
1 Introduction	1
1.1 Role of sulfur in plants	1
1.2 Sulfur access and assimilation in plants	2
1.3 History of plant S deficiency	3
1.4 Plant responses to S deficiency	3
1.5 S deficiency and nitrogen fixation of legumes	4
1.5.1 Leguminous and nitrogen fixation	4
1.5.2 Nodule formation	5
1.5.3 Genes related to nitrogen fixation in legumes	6
1.5.3.1 Nif gene	7
1.5.3.2 Leghemoglobin	8
1.5.3.3 Ferredoxin	10
1.6 S deficiency responses of legumes	11
1.7 Sulfate transporter gene family in plants	12
2 Material and methods	15
2.1 Equipments and chemicals	15
2.1.1 Equipments	15
2.1.2 Chemicals	16
2.2 Enzymes	17
2.3 Molecular biology Kit	18
2.4 Plant Cultivation of <i>Pisum sativum</i> and growth conditions	18
2.4.1 Seeds infection and germination	18
2.4.2 Fertilization and treatment	18
2.4.3 CNS measurement	20
2.5 mRNA quantification using quantitative real-time PCR	20
2.5.1 RNA Isolation and purification	20
2.5.2 RNA quality controls	20
2.5.2.1 Total RNA quantity measurement	20
2.5.2.2 Total RNA purity	20
2.5.2.3 Total RNA integrity	20
2.5.2.4 Determination of RNA integrity factor (RIN)	21
2.5.3 First strand cDNA synthesis	21

2.5.4 mRNA expression	22
2.5.5 Data analysis of real-time PCR	22
2.5.6 Reference gene selection for normalization of genes of interest	22
2.5.7 Gene of interest	24
2.5.8 Statistical analyses	24
2.6 Identification of symbiotic sulfate transporter gene in <i>Pisum sativum</i>	24
2.6.1 Preparing electrocompetent cells of <i>Escherichia Coli</i>	24
2.6.2 Transformation of <i>Escherichia coli</i>	25
2.6.3 Plasmid preparation from <i>Escherichia Coli</i>	25
2.6.4 Degenerated primer designing	26
2.6.5 Isolation of partial cDNA corresponding to symbiotic sulfate transporter	27
2.6.6 Cloning of partial cDNA of SST for sequencing	27
2.6.6.1 Extraction of partial cDNA from an agarose gel	27
2.6.6.2 Ligation	27
2.6.6.3 Purification of ligation mixture	28
2.6.6.4 Blue/white screening	28
2.6.6.5 Restriction Analysis	28
2.6.7 Designing specific sense and anti sense primer from partial cDNA	29
2.6.8 Rapid Amplification cDNA 3' End (3' -RACE)	29
2.6.9 Rapid Amplification cDNA 5' End (5' -RACE)	31
2.7 Functional complementation of SST in yeast double mutant	33
2.7.1 Amplification of ORF fragments	33
2.7.2 Cloning Strategy	33
2.7.3 Ligation of Blunt End SST ORF with the pJET 1.2 cloning vector	33
2.7.4 Ligation of SST ORF with the pESC_His yeast expression vector	34
2.7.5 Generation of electrocompetent of <i>Saccharomyces cerevisiae</i>	34
2.7.6 Transformation of <i>Saccharomyces cerevisiae</i>	35
2.8 Biochemical methods	37
2.8.1 Protein isolation	37
2.8.2 Protein quantification	37
2.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)	38

2.8.4 Western blotting and immunodetection	39
2.8.5 Chlorophyll determination	40
2.8.6 Leghemoglobin measurement	41
2.9 Next generating sequencing	41
2.9.1 Biological samples for transcriptional analysis	41
2.9.2 RNA isolation and purification	42
2.9.3 Transcriptome de novo assembly	42
2.9.4 Data analysis and sequence homology for transcriptome identification of sulfate transporter gene family in <i>Pisum sativum</i>	43
3 Results	44
3.1 Sulfur supply and growth conditions	44
3.2 Sulfur content of plants	45
3.3 Sulfur supply and nodule development	46
3.4 Sulfur supply and chlorophyll content	47
3.5 Sulfur supply and leghemoglobin content in nodules	48
3.6 Expression of symbiotic nitrogen fixation (SNF) related genes	49
3.6.1 Expression of leghemoglobin genes in the nodule tissue	49
3.6.2 Expression of the symbiotic sulfate transporter (SST) gene in the nodule tissue	50
3.6.3 Expression of the nitrogenase (Nif) gene in the nodule tissue	51
3.6.4 Expression of the ferredoxin (Fd) gene in the root, nodule and leaf tissues	52
3.7 Western blotting and immunodetection of ferredoxin	54
3.8 Identification of a symbiotic sulfate transporter cDNA in <i>Pisum sativum</i>	55
3.8.1 Isolation of a partial cDNA corresponding to the symbiotic sulfate transporter	55
3.8.2 Isolation of the 3' end of the SST from pea by 3'-RACE	56
3.8.3 Isolation of the 5' end of the SST from pea by 5'-RACE	57
3.9 Functional complementation of symbiotic sulfate transporter in a yeast double mutant	60
3.10 Identification of the sulfate and molybdate transporter gene family in <i>Pisum sativum</i> by transcriptome identification	63

4 Discussion	66
4.1 Plant performance and changes in the contents of chlorophyll, leghemoglobin and sulfur to S deficiency	66
4.2 Expression of SNF related genes under S deficiency condition	69
4.2.1 Leghemoglobin	69
4.2.2 Ferredoxin	70
4.2.3 Nitrogenase	72
4.3 Identification of sulfate transporter genes in <i>Pisum sativum</i>	72
4.3.1 Symbiotic sulfate transporter in <i>Pisum sativum</i>	72
4.3.2 Sulfate and molybdate transporter genes in <i>Pisum sativum</i>	74
5 Out look	78
6 References	80
7 Appendixes	90
7.1 Sulfate and molybdate transporter genes sequence in <i>Pisum sativum</i>	90